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Aging reduces the toxicity of pristine but not sulphidised silver nanoparticles to soil bacteria

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ABSTRACT

In the environment engineered nanoparticles (ENPs) are subject to chemical and physical transformation processes. Thus, to understand their impact, it is important to consider how bioavailability and toxicity are influenced by these "aging" transformations with relation to environmental conditions and ENP properties. Here, two soil bacteria were exposed to Ag ENPs in ISO media (± fulvic acid) and soil pore water extracts with pH6 and pH8. The ENPs tested were 49 nm unfunctionalised, citrate stabilised (Ag-citr), 58 nm PVP-coated (Ag-PVP) and 36 nm sulphidised (Ag_2S-PVP); $AgNO_3$ was used as a positive control. Exposures were carried out using pristine (unaged) and 24h aged ENPs, and the 24h soluble fraction. Overall, toxicity was ranked $AgNO_3$ > Ag-PVP ≥ Ag-citr >> Ag_2S. Aging of $AgNO_3$, Ag-PVP and Ag-citr in the ISO medium caused little change from unaged exposures and growth inhibition was mainly caused by soluble silver. Added fulvic acid decreased silver toxicity after aging and reduced the contribution of dissolution; as was the case in the soil pore waters where toxicity could not be attributed to ionic silver. Ag_2S toxicity to *A. globiformis* in both ISO variants increased after aging, yet followed the same patterns as the metallic ENPs in the pore waters. For all ENPs pH effects were species dependent. Together this data showed that aging reduced toxicity in media with organic matter and despite soluble silver being the main driver of pristine ENP toxicity in the standard ISO medium, dissolution did not fully explain toxicity in the presence of organic matter.

INTRODUCTION

Nanomaterials have found widespread application in consumer products, medicine, technology etc[1]. Their growing use and production inevitably leads to an increased release into the environment and raises concern about their environmental fate and toxicity[2-4]. Silver in both its ionic and nanoparticulate form has strong antimicrobial properties, which is why it is commonly used as an antibacterial agent[5]. The bactericidal effects of Ag ENPs have been established for different aquatic and soil species[6-9] and were even shown to impact soil microbial community growth, activity and diversity[10-13]. The mechanisms for Ag ENP toxicity are most commonly attributed to the release of ionic silver through dissolution, with particle properties only having an indirect effect on toxicity by mediating dissolution kinetics[14]. A meta-analysis by Notter et al 2014 estimated that in as many as 93.8% of studies ionic silver was more toxic than nanosilver[15]. In *C. elegans* the toxicity of silver nanoparticles with different sizes and surface coatings was found to be directly linked to their dissolution and observed growth inhibition effects were rescued by Ag^+ chelating agents. However, a contribution of generated ROS to toxicity was observed for some of the tested ENPs[16]. Further studies have also found that toxicity of Ag ENPs could not be fully explained by dissolution alone. In daphnia organic matter altered Ag ENP toxicity without any changes in the concentration of dissolved silver in the media[17]. Soil enzyme activity was also unaffected at ionic silver concentrations matching those released from Ag ENPs[18] and in *Pseudomonas fluorescens* dissolved silver measurements did not reach required levels to cause the observed toxicity[8].

Once in the environment ENPs undergo transformation processes that alter their properties and ultimately how they are presented to, and taken up by, organisms. In the terrestrial environment various soil properties have been found to influence processes such as aggregation, dissolution or speciation changes. For example, the attachment of dissolved organic matter (DOM) to a particle surface can alter its stability and influence its mobility in the soil[19]. Additionally, ENP aggregation can also be affected by soil pH when it approaches the point of zero charge (PZC)[20]. Thus, establishing causation may not always be simple since such effects are often linked, e.g. the PZC is affected by surface absorbed DOM. DOM and pH in turn can influence ENP dissolution as a key driver of Ag ENP

toxicity[20, 21]. In fact, both mentioned soil properties were found to influence Ag accumulation and avoidance of the earthworm *Eisenia fetida*[22, 23]. However, when comparing the toxicity of Ag ENPs to microbial activity in five different soils pH and clay, rather than organic matter content, were the key drivers of observed effects under the given experimental conditions[24]. While it is important to gain a better understanding of the influence environmental properties have on fate and toxicity of "as produced" nanoparticles, studies investigating their environmental impact should also consider in which form the respective material is released. For silver nanoparticles the main route of entry into the environment is via sewage treatment plants where the majority will most likely be transformed into Ag_2S[25, 26]. This transformation of metallic to sulphidised nanosilver has been termed as the "natural antidote" to its toxicity[27] and has been demonstrated in duckweed and fish[27], nematodes[28] and bacteria[29, 30]. This decreased toxicity is attributed to lower reactivity and solubility of Ag_2S compared to metallic Ag ENPs. However, there is emerging evidence that Ag_2S transformed and exposed in sewage sludge can in fact cause toxicity to soil microbiota given longer exposure durations[31] or even become more toxic than ionic silver as was found in earthworms, *Medicago truncatula* and its symbiont *Sinorhizobium meliloti*[32, 33].

Given the degree to which nanoparticles can change in the environment, laboratory tests where organisms are exposed to ENPs immediately after their addition to even the most relevant conditions may not fully capture real effects. Diez et al. 2015 found that when Ag ENPs were aged in soils for a year before the earthworm *Eisenia fetida* was exposed it became more toxic than ionic silver (both freshly spiked and aged)[34]. On the other hand Ag ENP effects on microorganisms persisted over 100-140 days, with sewage sludge aged Ag ENPs remaining as toxic as unaged exposures[29]. Toxicity to daphnia of ENPs aged in media with and without natural organic matter for up to 48 hours also found nano ZnO toxicity unchanged across the tested media regardless of observed effects on ENP stability[17].

This study examines how antibacterial effects of Ag ENPs change in relation to the complexity of the exposure medium and the aging of metallic and sulphidised silver ENPs. Additionally, the extent to which dissolution contributes to the observed effects is considered. To test the hypothesis that aging

transformations decrease nanoparticle toxicity driven by soluble silver exposures were carried out in standard test media +/- organic matter and in pore water extracts from a natural soil adjusted to two different pH levels. Each of these parameters has been shown to affect ENP fate and behaviour thus increasing the understanding of aging effects on nanotoxicity and Ag^+ contribution under more environmentally relevant exposure conditions.

2. MATERIAL AND METHODS

2.1 Nanoparticles

Three spherical silver nanoparticles were tested: a 58.3 ± 12.9 nm Polyvinylpyrrolidone coated ENP (Ag-PVP) taken from the same batch used by Starnes et al 2015 and material characterisation contained therein[28], a 49.1 ± 6.3 nm unfunctionalised, citrate stabilised ENP (Ag-citr), and a 36.1 ± 9.7 nm Ag_2S ENP also PVP coated (Ag_2S). ENP stock characterisation and synthesis protocols can be found in the Supplementary Information (SI). Silver nitrate (purchased from Sigma Aldrich) was used as positive control.

2.2 Soil pore water

Soil properties and pH adjustment

The soil used in this study was previously collected from an acidic heathland site in Wareham forest, Dorset (Ordnance Survey Grid Reference: SU108058, Dorset, United Kingdom) and treated as described in Heggelund et al 2014[35]. All soils were homogenized, 2 mm sieved and air dried prior to use. A summary of the soil's initial properties can be found in the SI Table S1. The pH of the soil was adjusted to pH 4.8 and 7.2 by adding 2 and 8 g $CaCO_3$ per kg soil respectively.

Soil Pore Water Extraction

In order to prepare the soils for pore water extraction, after $CaCO_3$ was added, the soils were wet to 50% water holding capacity (WHC, 100%: 49.2 ml/100 g) and left for seven days at room temperature to allow for pH equilibration[35]. After seven days the soils were wet to the full 100% of their WHC for a

124 further 24 h. After 24 h the pore waters were extracted by centrifugation at 4000 rpm for 90 minutes
125 and collecting of the supernatant. They were then sterilised by syringe filtration (0.2 µm). Nutrients
126 were added at the same concentrations as the ISO 10 712 (1995) test medium (SI Table S3) to facilitate
127 bacterial growth. This addition changed the pore water pHs to pH 6.2 and 7.8 from the initial pH 4.8
128 and 7.2 respectively. Silver (ENP or $AgNO_3$) was added after extraction, filtration and nutrient addition.
129
130 **2.3 Bacterial toxicity assays**
131 *Test organisms*
132 Two soil bacteria were chosen as test organisms: the Gram-positive *Arthrobacter globiformis* (DSM
133 20124) and the Gram-negative *Pseudomonas putida* (DSM 50026). Strains were obtained from the
134 German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig Germany. Cultures
135 were maintained on LB agar (Merck Millipore) plates prepared according to the manufacturer's
136 instructions. They were grown at 25°C, subsequently stored at 3°C and transferred onto fresh agar
137 every six weeks.
138
139 *Test media*
140 Tests were carried out in four different types of exposure media: a) ISO 10712(1995) test medium
141 (composition see SI Table S3) with pH 7.0, b) ISO 10712(1995) test medium with added 50 mg/l fulvic
142 acid (FA) (Pahokee Peat Fulvic Acid Standard II 2S103F, purchased from the International Humic
143 Substance Society) with pH 6.8, c) pore water extracts from a field soil adjusted to pH 6.2 and d) pH
144 7.8.
145
146 *Toxicity assays*
147 The toxicity tests were carried out following a modified version of the ISO 10 712 (1995) guideline,
148 which is commonly used to assess the hazard of environmental pollutants like pharmaceuticals or
149 metals[7]. For this purpose bacteria were inoculated in the pre-culture medium (SI Table S3) 20 hours
150 prior to the test at 25°C, shaking at 150 rpm. Aliquots of the inoculum were subsequently transferred

into the test media to achieve an initial bacterial optical density (OD) of 0.05. Bacterial growth was measured as OD (absorbance at 420-580nm) in Honeycomb 100 well plates using a Bioscreen C MBR (Oy Growth Curves Ab Ltd, Finland). Assays were performed over 24 h at 25°C under constant shaking to prevent settling of the bacteria and formation of a biofilm. Exposures to a minimum of five concentrations were performed in triplicate with six untreated controls. Since Ag_2S did not cause significant growth inhibition at any tested concentration in any of the media, only data for the top concentrations will be shown.

2.4 Nanoparticle aging treatments

Three different treatments in each of the four media described above were used to establish the effect of aging on nanotoxicity to bacteria (Figure 1). Firstly, particles were added to each separate medium immediately before exposures were carried out, this is referred to as 'unaged' (UA). Secondly, particles were incubated in the respective exposure media in the absence of bacteria for 24 hours at 25°C on a shaker at 150 rpm this treatment is referred to as the 'aged total' (A). Aging was carried out over 24 h hours to mirror ENP changes under the test conditions and to gain an understanding whether the starting or final particle properties have a greater contribution to their toxicity. Finally, aliquots of the aged total were taken after the 24 h aging period and centrifuged for 3 hours at 20800 g following a method by Kroll et all 2016[36]. Centrifugation at this speed predicts settling of particles > 3 nm according to Stoke's law. The supernatant (top 1 ml) was taken and pooled for each concentration (3 replicates were centrifuged) and is referred to as 'soluble fraction' (S). This allowed for direct comparison of the toxicity of the exposure medium with NPs and ions present to the medium including only the soluble fraction (i.e. ions and ultra-small silver clusters). Where toxicity of the soluble fraction matched that of the aged total ionic silver was concluded to be the driver of the observed effects. All steps including centrifugation were also carried out with $AgNO_3$ treatments.

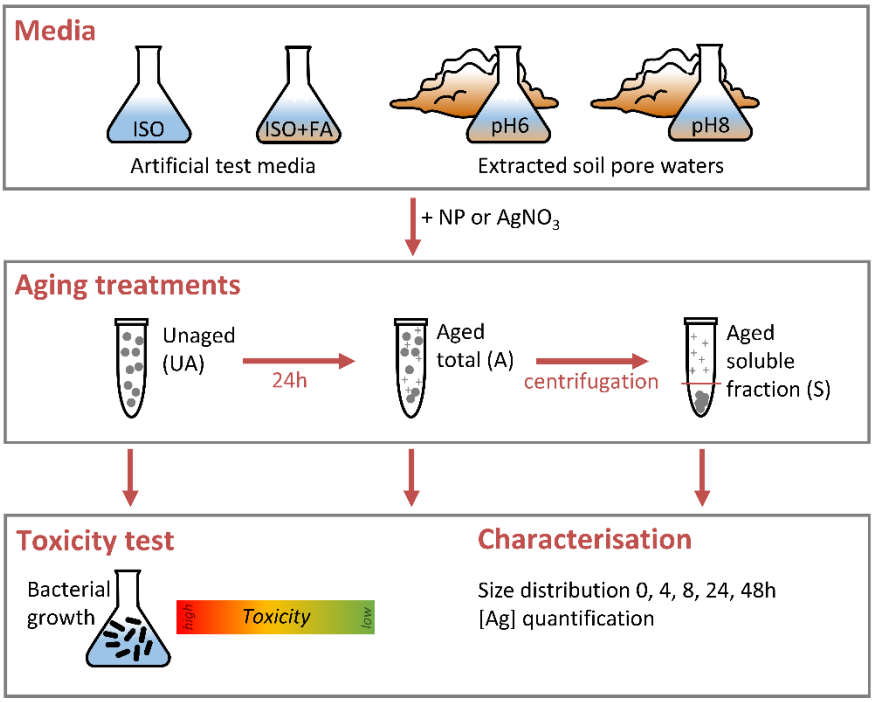

Figure 1: Experimental design and workflow. Four different media were prepared, the ionic or nano silver forms added, subjected to the three aging regimes and subsequently their bacterial toxicity and fate characterised.

2.5 Nanoparticle characterisation

Nanoparticle size (as hydrodynamic diameter) was measured over the test duration at t = 0 h, 4, 8, 24, and 48 h using a Nanosight NS500 instrument, fitted with a blue laser (405nm) (Malvern Instruments, UK). This allowed for monitoring of the aggregation dynamics and comparison of starting and final size distribution of both unaged (start t = 0 h, end t = 24 h) and aged exposures (start t = 24h, end t = 48 h) respectively. Due to interference from the soil particulate matter and bacterial cells these measurements could only conclusively be carried out in the ISO and ISO+FA media in absence of bacteria.

2.6 Concentration validation and dissolution measurements

Nominal exposure concentrations were validated in 10% of samples, randomly chosen from unaged and aged totals and all media. Samples were acidified using *aqua regia* comprised of a 3:1 ratio of 35% hydrochloric acid to 69% nitric acid (both Trace Select Ultra, Sigma-Aldrich). They were subsequently stored in the fridge, in the dark until analysis. The exposure concentrations were validated using flame atomic absorbance spectroscopy (Flame-AAS, Perkin Elmer AAnalyst 100). Samples to measure soluble

195 silver were generated in the same manner as for the toxicity assays 'soluble fraction' and also analysed
196 by Flame-AAS after acidification with *aqua regia*.
197
198 **2.7 Data Analysis**
199 Toxicity was expressed as percentage growth inhibition compared to control growth, Equation 1; with
200 a_0 and a_{24} as the absorbance in each well at the start and after 24h, and c_0 and c_{24} the average
201 absorbance in the control treatments at the start and after 24h. At higher concentrations ENPs were
202 found to increase absorbance, thus growth was determined as an increase in absorbance between
203 start and final measurement per well. Growth inhibition data was analysed for differences between
204 aging treatments, media and particle type using generalised linear models (GLM) with the fixed factors
205 "silver concentration" and "silver treatment" and their interaction term in Minitab 17. Differences
206 between treatments were further established by post hoc clustering using Tukey Pairwise Comparison.
207 Where silver concentration differed between treatment, e.g. when comparing Ag-PVP and Ag-citr,
208 results were analysed for concentration-response relationships in SigmaPlot 12.0 (Systat Software Inc,
209 USA) fitting a three-parameter logistic regression (Equation 2, y = reproduction, m = max reproduction,
210 x = exposure concentration, x_0 = EC_{50}, b = slope) and estimating upper asymptote, EC_{50} and slope
211 parameters for each of pore water media separately and differences between concentration-response
212 curves established using the F-test[37]. A p-value of <0.05 was considered to indicate a significant
213 difference. Full results of the statistical analysis can be found in the Supplementary Information.
214
215 $$I = (1 - \frac{a_{24} - a_0}{c_{24} - c_0}) x 100 \quad \text{(Equation 1)}$$
216 $$y = \frac{m}{1 + (\frac{x}{x0})^b} \quad \text{(Equation 2)}$$
217
218

3.1 Concentration validation and dissolution measurements

Exposure concentrations were found to be $AgNO_3$: 96.2 ± 11.8%, Ag-PVP: 65.7 ± 9.0%, Ag-citr: 79.3 ± 10.4%, Ag_2S: 76.0 ± 9.7% of the anticipated nominal concentrations. Where deviations were greater than 10%, i.e. for all ENP treatments, the exposure concentrations were recalculated to reflect actual concentrations and were reported as such.

Analysis of the soluble silver fraction in the aged exposures after centrifugation showed incomplete recovery (40-70%) of the added silver in $AgNO_3$ exposures (Figures 2 and 3). This could indicate precipitation of silver as particles; these phenomena were previously reported by Malysheva et al 2016[38] and Sharma et al 2015[39]. This type of precipitation likely also occurred in the totals lowering the amount of bioavailable silver in exposures derived from these treatments, thus resulting in similar effects in the ISO variants despite varying concentrations to the soluble fractions. The loss to container walls was considered negligible since Sekine et al 2015 demonstrated that dissolved silver shows very low binding to polypropylene tubes[40]. For both Ag ENP types, across all media, dissolution increased with increasing exposure concentration with Ag-PVP dissolving more than Ag-citr ENPs, with dissolution rates ranging between 0.25 and 7.4 %. The difference between the particles may have been linked to the higher stability of the Ag-PVP in the medium over the 24 h incubation period which could have increased their corrosion[41]. A comparison between media revealed lower levels of Ag-PVP and Ag-citr dissolution in the ISO test medium when FA was added (Figures 2 and 3). However, in the soil pore waters dissolution was increased compared to ISO+FA despite the natural organic matter content of the pore waters being much higher. This is consistent with findings that organic matter type and composition play a greater role in ENP dissolution than simply its concentration[42, 43]. Comparison of the two pore waters showed that a greater amount of soluble silver was present in the pH8 extract than in the pH6 pore water for both ENPs. Total Ag_2S dissolution after 24h was only determined in the top concentration and found to be much lower than that of the metallic ENPs: 0.144 ± 0.076 mg Ag/l (mean ± StDev) in the ISO medium, 0.033 ± 0.008 mg Ag/l in the ISO+FA, 0.030 ± 0.018 mg Ag/l in the pH6 soil pore water, and 0.022 ± 0.14 mg Ag/l at pH8.

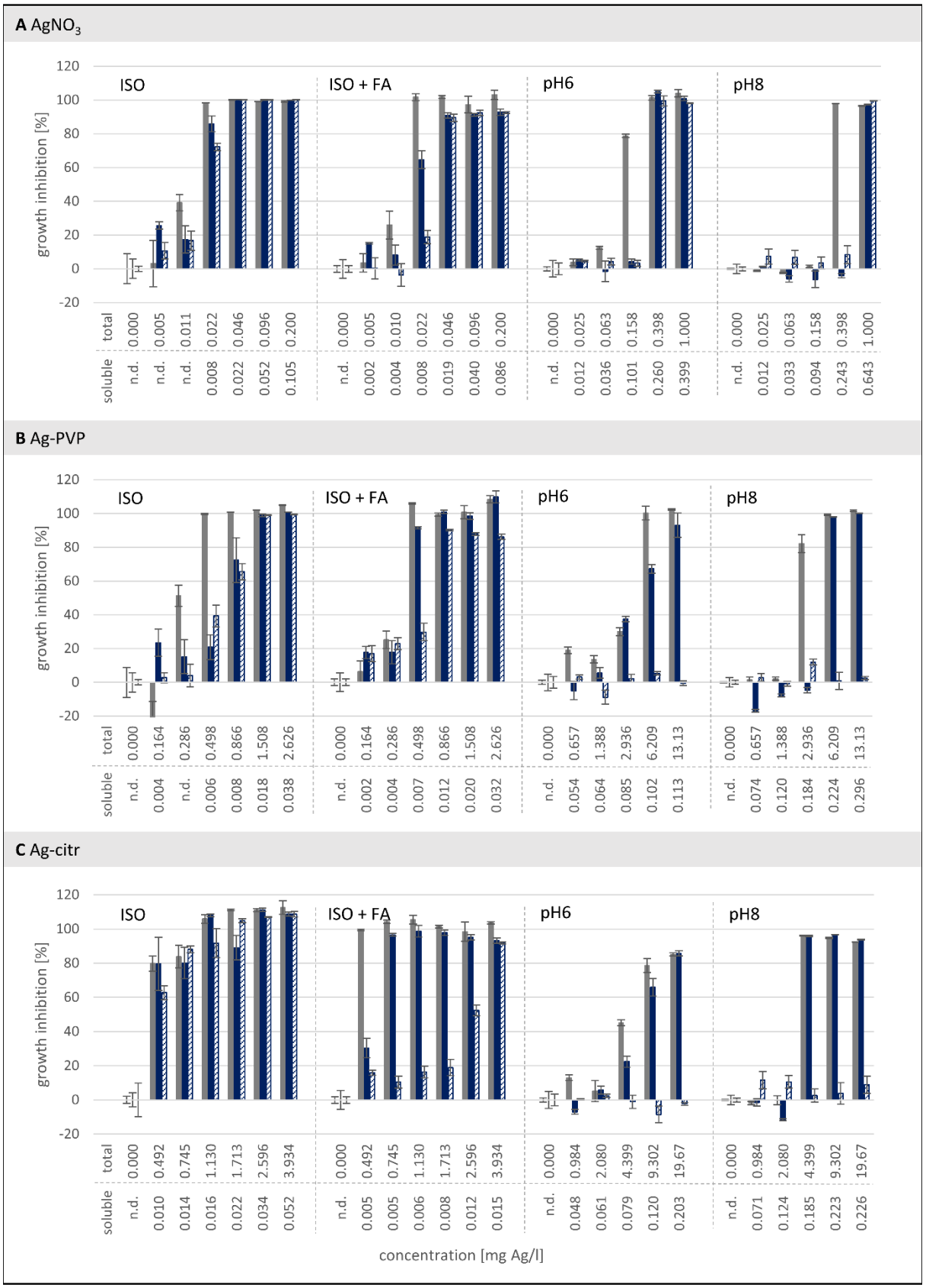

Figure 2: *A. globiformis* growth inhibition (averages ± SE) as percent inhibition in unexposed controls caused by exposure to A) $AgNO_3$, B) Ag-PVP and C) Ag-citr in ISO standard test medium, ISO medium with added fulvic acid (FA) and soil pore water extracts adjusted to pH6 and pH8. X-axis: Exposure concentrations [mg Ag/l] of totals (both unaged and aged and of the soluble fraction. Solid grey: unaged exposures, solid dark blue: aged total, dark blue striped: aged soluble fraction. n.d.: not detected.

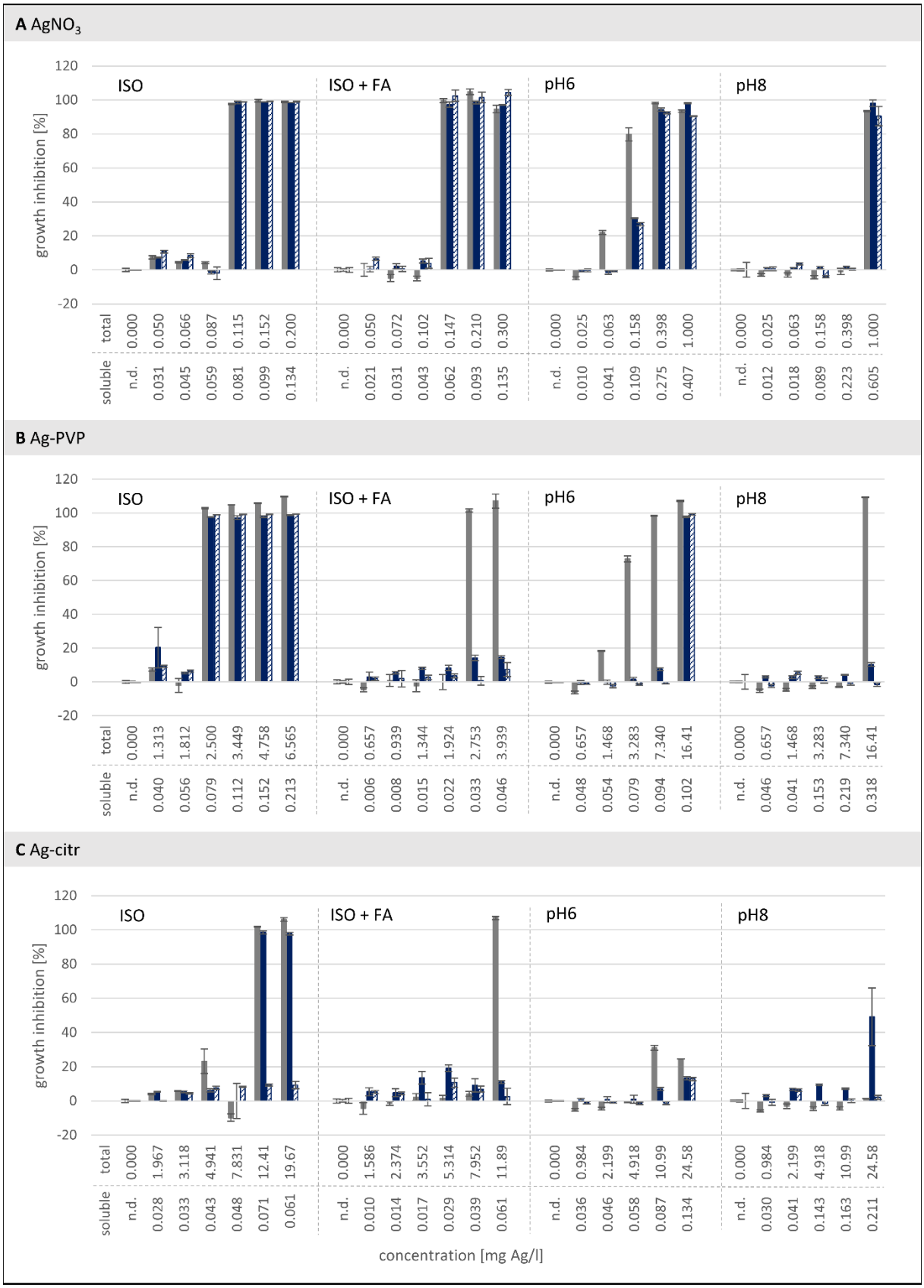

Figure 3: *P. putida* growth inhibition (averages ± SE) as percent inhibition in unexposed controls caused by exposure to A) $AgNO_3$, B) Ag-PVP and C) Ag-citr in ISO standard test medium, ISO medium with added fulvic acid (FA) and soil pore water extracts adjusted to pH6 and pH8. X-axis: Exposure concentrations [mg Ag/l] of totals (both unaged and aged and of the soluble fraction. Solid grey: unaged exposures, solid dark blue: aged total, dark blue striped: aged soluble fraction. n.d.: not detected.

3.2 Nanoparticle characterisation

Nanoparticle size characterisation using NTA was only reliably possible in the ISO media variants since soil particles present in the pore water extracts interfered with the measurements. However, no scattering from the fulvic acid molecules was detected in the ISO+FA medium. In the standard ISO medium Ag-PVP and Ag-citr both showed aggregation over the test duration, as can be seen by the increase in the 90th percentile hydrodynamic diameter) (D90), a well as the increased mode of Ag-PVP (Table 1). This meant that bacteria were exposed to particles with different properties at the start of the unaged versus the aged toxicity assays. The addition of FA to the medium stabilised both Ag-PVP and Ag-citr to remain close to the initial distribution at t = 0 h. Thus, in the bacterial toxicity tests exposure conditions with respect to the particle size distribution in the aged media matched those of the unaged ones, although, with the fulvic acid molecules already adsorbed to the particle surface. Ag_2S was, however, found to aggregate to a large degree regardless of presence of organic matter, although the FA did appear to decrease the aggregation. This difference to the Ag-PVP and Ag-citr may have been related to the greater affinity of organic matter to the metallic than the Ag_2S ENPs which would require a greater FA to ENP ratio to observe the same stabilising effects. The ENP stabilisation by organic matter is, like dissolution, dependent on the type of organic matter. The used Pahokee Peat Fulvic Acid has, in accordance with the findings presented here, previously been shown to stabilise Ag_2S ENPs in suspension[42]. As already stated nanoparticle size measurements in the soil pore waters were confounded by the presence of small soil particles that were not removed during pore water extractions and sterile filtering (prior to ENP addition). However, comparing size distributions of measurements with and without Ag-ENPs a distinct Ag-citr peak in pH6 remained over the test duration (SI Figure S2). Initially, this peak was also present at pH8, but was later indistinguishable from the pore water background, likely due to aggregation. This may have been an artefact of the way the soil pH was adjusted with $CaCO_3$, as divalent ions can increase aggregation[19].

Table 1: NTA characterisation hydrodynamic diameter (d.nm) mode ± SE. In brackets 10th (D10) and and 90th (D90) percentile sizes.

		0h	***4h***	***8h***	***24h***	***48h***
Ag-PVP	ISO	87.4 ± 3	87.8 ± 5.7	88.1 ± 6.6	80.9 ± 3.7	96.5 ± 13.9
		[67.8-128.4]	[70.9-127.6]	[67.6-130.3]	[73.1-141.1]	[78.8-218.0]
	ISO+fulvic	91.1 ± 2.9	79.8 ±.8	86.6 ± 4.1	81.1 ± 5.7	83.5 ± 5.9
		[67.1-129.8]	[67.9-135.8]	[67.7-135.0]	[70.1-133.8]	[62.9-142.6]
Ag-citr	ISO	54.1 ± 0.9	59.4 ± 2.9	56.9 ± 1.5	59.1 ± 3.1	53.5 ± 1.5
		[43.7-94.1]	[54.4-218.6]	[51.4-219.7]	[53.8-133.6]	[48.0-149.0]
	ISO+fulvic	52.3 ± 0.6	52.4 ± 1.1	52.3 ± 0.9	53.1 ± 0.5	52.6 ± 0.7
		[42.1-70.1]	[44.0-80.4]	[44.6-78.4]	[45.1-84.6]	[44.0-82.2]
Ag_2S	ISO	66.6 ± 4	85.1 ± 8.4	85.6 ± 5.6	59.1 ± 3.1	203.9 ± 77.2
		[51.0-166.2]	[66.4-162.0]	[60.4-146.7]	[70.5-244.6]	[79.2-256.1]
	ISO+fulvic	80.3 ± 15.4	68.8 ± 18.2	77.8 ± 2.3	78.7 ± 5.2	103.2 ± 14.8
		[61.3-149.6]	[53.9-134.7]	[69.7-158.6]	[59.9-151.1]	[68.0-263.3]

3.3 Bacterial toxicity assay

Comparing the two tested bacteria *A. globiformis* was found to be the more sensitive species (Figures 2 and 3). *P. putida* showed very steep concentration response curves, thus EC_{50} values could not always be reliably established. While differences between treatments were overall conserved across the two species there were some exposure conditions that induced opposite responses. At soil pore water pH6 *P. putida* growth inhibition caused by Ag ENP aged in the medium before exposure matched that of the soluble silver fraction, whereas for *A. globiformis* the latter did not explain the observed effects. The chosen species are Gram-positive and Gram-negative and since for bacteria the cell wall plays a key role in the interaction with ENPs this may have had an influence on the toxicity, especially in the absence of (or at low ratios) of organic matter. Further, the tested pHs were on either end of the optimal growth conditions for both bacteria (*A. globiformis* pH6-10, *P. putida* pH4-8). *A. globiformis* growth of controls was approximately half in the pH6 pore water compared to growth at pH8. However, growth of *P. putida* in the controls was unaffected by medium pH. The observed differences between the species may also be related to the generation of species specific exudates that could serve as protection from ENP toxicity[44]. These extracellular polymeric substances can alter ENP fate by forming an biomolecular corona on the particle surface that can cause contradictory effects depending on the test system[45] but which for Ag ENP have also been found to be pH dependent[46].

Effect of aging

Aging $AgNO_3$ in the ISO media prior to bacteria exposure had no significant influence on its toxicity to both *A. globiformis* ($F_{2,51}$=1.73, p=0.187, Figures 2A) and *P. putida* ($F_{2,50}$=1.92, p=0.157, Figures 3A), despite the already mentioned decrease in the measured silver concentration in the soluble fraction, i.e. after centrifugation after 24h. However, *A. globiformis* growth inhibition was significantly reduced (p<0.001) after $AgNO_3$ pre-incubation in all media containing organic matter. This may indicate silver binding to the organic matter that thus decreased its bioavailability.[47] Growth inhibition by the soluble fraction was the main driver of the observed aged silver effects in all but the ISO+FA medium ($F_{2,51}$=40.27, p<0.001). In *P. putida* aging also influenced toxicity in ISO+FA and pH6 pore water with the amount of soluble silver explaining observed responses ($F_{2,51}$=9.32, p<0.001 and $F_{2,45}$=281.88, p<0.001 respectively). Only in the pH8 pore water did the aging regime not influence $AgNO_3$ growth inhibition ($F_{2,45}$=2.79, p=0.072).

The pre-incubation of Ag-PVP in the media before exposure to the bacteria generally reduced toxicity to both bacterial species (GLM p<0.001), with the exception of *P. putida* in the standard ISO medium and *A. globiformis* in ISO+FA (Figures 2B and 3B). Further, there was no significant difference in growth inhibition of both species between total and soluble silver exposures after aging Ag-PVP ENPs in the ISO medium (SI Tables S4 and S6). This indicates sufficient dissolution of the ENP to cause the observed effects. Measured soluble silver concentrations were in the range of concentrations causing similar growth inhibition in the $AgNO_3$ exposures for this medium, further supporting this finding. The addition of FA to the medium decreased the effects of aging in *A. globiformis.* Unaged and aged total exposures were no longer significantly different. This may have been linked to the largely unchanged particle size distribution over the 24h pre-incubation duration shown in the NTA analysis that suggested comparable ENP exposure conditions between unaged and aged treatments. However, the proportion of the effect caused by the soluble fraction was found to be significantly lower in the presence of FA ($F_{2,51}$=35.92, p<0.001). On the other hand, *P. putida* responded to all three ISO+FA aging treatments differently, with the toxicity of UA > A > S ($F_{2,51}$=250.16, p<0.001). The attachment of FA to the particle surface and/or cell membrane may have altered the potential for interaction in a manner that

decreased toxicity for one bacterium but not the other, their distinct membrane structures possibly playing a role in this interaction. Gram-positive and Gram-negative soil bacteria have further been found to differ in their preferences for carbon sources, which may also have played a role in the observed differences[48]. In both soil pore waters, aging greatly decreased Ag-PVP toxicity to both bacteria. Again it is likely that the binding of the particles to the organic matter or clay particles still present in the pore water reduced their bioavailabilty[19]. No toxicity of their soluble fraction was observed in *A. globiformis*, indicating effects were related to the particle form rather than the chemical composition of the ENPs. This was also the case for *P. putida*, however, there was less inhibition of growth even in the aged total treatment. Additionally, for both bacteria measured soluble silver concentrations were lower than those needed in $AgNO_3$ exposures to cause corresponding effects.

Overall patterns of unaged/aged Ag-citr (Figures 2C and 3C) induced growth inhibition in *A. globiformis* matched those of Ag-PVP within the different media, with the exception of exposures carried out in the standard ISO medium where no significant differences were found between the three different aging regimes ($F_{2.51}$=2.44, p=0.098). Assays performed in ISO+FA media decreased Ag-citr toxicity from unaged to aged total, despite NTA analysis indicating almost identical particle size distributions for both unaged and aged exposures. This also suggested that the presence of FA decrease ENP bioavailability. Further, the significantly greater toxicity of the aged total than aged soluble exposures indicated that ionic silver could not be the sole driver of the observed effects. This was supported by the measured silver concentration in the soluble fraction, e.g. complete growth inhibition was reached at aged totals that released much lower concentrations of soluble silver than needed to cause the observed effects in $AgNO_3$ exposures. In the soil pore waters ENP toxicity was only reduced by aging at pH6, however at either pore water pH level little to no contribution of ionic silver to the toxicity was observed, despite corresponding silver concentrations causing effects in the $AgNO_3$ treatments having been reached. *P. putida* was overall less affected by Ag-citr exposure at the tested concentrations and only in the ISO variants could full inhibition be observed. Here effects in the standard ISO medium of unaged and aged totals matched while growth inhibition caused by the soluble fraction was significantly lower. As for *A. globiformis* the addition of FA reduced toxicity of aged ENPs. In the soil

pore water extracts such differences could not conclusively be discerned due to the limited impact of Ag-citr on bacterial growth at any of the tested concentrations.

Ag_2S caused little (maximum 22%) or no growth inhibition at the highest tested concentration (26.6 mg/l).

Effect of media

Comparing silver effects between the two ISO variants showed that the addition of FA to the standard medium significantly altered toxicity to *A. globiformis* in aged exposures (GLM $p<0.01$, Table S5), while remaining unaffected by medium organic matter content in unaged ones (GLM $p>0.05$). This may have been related to the changed affinity of "naked" ENPs surfaces to bacterial cells compared to FA conjugated ones and the antioxidant effect of FA. Toxicity of aged $AgNO_3$ and Ag-citr was reduced while Ag-PVP caused more growth inhibition after 24 h pre-incubation in the presence of FA. Similar trends were also visible in *P. putida* exposures to Ag-PVP, however, for Ag-citr growth inhibition was not sufficient to draw reliable conclusions. Likely the attachment of the organic molecules reached an equilibrium during the incubation prior to the toxicity assay that changed their behaviour and toxicity compared to unaged exposures where such attachment reactions took place while the bacteria were already present. Absence of a difference between the unaged ENPs toxicities suggested that a large proportion of the toxic effect occurred before the equilibrium was reached thus negating the potential protective effects of FA against $AgNO_3$ and Ag-citr toxicity or indeed the increased toxic effects in the case of Ag-PVP. Assessing the bacterial growth over time (data not shown) revealed that toxicity was largely related to an increase in the lag phase, i.e. a delay in bacterial growth, therefore toxicity in fact being related to effects at the start of the assays. Using Fourier Transform Infrared (FTIR) analysis humic acid sorption reactions have been shown to occur quickly (<1 min) for Cu, Mn, Al, SiO_2 ENPs[49]. However, sorption kinetics of proteins to Ag ENPs have been found to be dependent on their affinity and chemistry[50]. Thus, employing FTIR or surface enhance RAMAN spectroscopy could shed light on the sorption kinetics occurring here. Without FA both particles rapidly agglomerated in the medium,

as discussed above. Agglomeration has been shown to decrease toxicity of ENPs to bacteria thus the aging dependent increase in Ag-PVP growth inhibition could have been linked to the more stable dispersion in the presence of FA[26]. Further, growth inhibition by soluble silver was decreased, despite similar levels of measured silver concentrations of unaged and aged ENPs, suggesting that FA reduced Ag^+ bioavailability[51].

Soil properties which can vary vastly have the potential to influence the toxicity of ENPs depending on the terrestrial environment they enter[19] and soil pH has been showed to influence ENP availability and toxicity[24, 35]. To reflect this we included pore water extracts from soil at two distinct pH levels as exposure media. The pH of the tested pore waters significantly altered the toxicity of unaged and aged silver exposure to both bacteria species (GLM $p<0.05$, SI Tables S5 and S7). Increasing pore water pH reduced toxicity of all silver treatments to *P. putida*. While it is suggested that at lower pH ENP dissolution increases thus increasing toxicity[19], here the measured soluble silver concentrations suggest that the opposite was the case with Ag-PVP dissolution being 2-3 times higher at pH8 than at pH6. Additionally, no toxicity of the soluble fraction was found, further excluding ionic silver as driver of toxicity. In the $AgNO_3$ exposures a protective effect of higher pH was also observed for *A. globiformis*. Yet, unlike for *P. putida*, ENP induced growth inhibition in *A. globiformis* increased with increasing pore water pH. Such opposite trends suggest media effects on toxicity to be linked not only to ENP fate but also to be organism specific, such as the species dependent production of extrapolymeric substances as already mentioned above[45].

Effect of particle properties

Both metallic nanoparticles showed similar toxicity to *A. globiformis* as well as *P. putida* when compared to $AgNO_3$ and Ag_2S. However, Ag-PVP generally induced growth inhibition at lower concentrations and was found to be more soluble than Ag-citr, which was also reflected in the contribution of the soluble fraction to ENP toxicity. Only *A. globiformis* exposed in ISO+FA and pH8

showed the same concentration response patterns for both particles when freshly spiked. However, aging in ISO+FA did change responses in a particle dependent manner making their toxic effects significantly different (*F*- and *p*-values in Table S8). Although, individual comparisons between Ag-PVP and Ag-citr were significantly different (e.g. both aged in ISO medium), trends of the effects of aging and contribution of soluble silver to the observed toxicity, as described earlier, were consistent between the two particle types. This is in broad agreement with Whitley et al 2013[52] who found aging Ag-PVP and Ag-citrate in soil had different effects on their fate, but not once having been pre-incubated in sewage sludge. In *P. putida* aging decrease toxicity in all media containing natural organic matter to levels where no meaningful comparisons could be drawn.

Sulphidised silver ENPs

There is a general acknowledgement in the literature that the environmentally relevant release of Ag ENP from sewage treatment plants will largely be in form of sulphidised nanosilver, thus Ag_2S ENPs were included in this study. Strikingly, even at the highest tested concentration (26.6 mg Ag/l) there was only a maximum of 22% deviation from control growth in any of the exposure media (Figure 4A, B). Consequently, only limited conclusions could be drawn about bacterial responses to the aging treatments and various media. For *A. globiformis* in the ISO media variants aging significantly increased toxicity (ISO: $F_{2,6}$=18.42, p=0.003, ISO+FA: $F_{2,6}$=8.05, p=0.02). This effect was more pronounced in the absence of FA. However, overall growth inhibition was greater in the presence of FA, despite the lower amount of soluble silver released (ISO: 0.144 mg Ag/l, ISO+FA: 0.033 mg Ag/l). Collin et al 2016 also showed that Ag_2S ENP toxicity to *C. elegans* was unaffected by the presence of organic matter regardless of its influence on ENP solubility[42]. Further, here the measured concentrations of Ag_2S derived soluble silver did cause greater toxicity to *A. globiformis* at equal silver concentrations in the in $AgNO_3$ exposures in ISO and ISO+FA media. In the soil pore waters at both pHs ENP toxicity was reduced by aging. As for the metallic ENPs this may have been influenced by the type of organic matter present that showed a different binding affinity than the FA, thus changing the ENP bioavailabilty[21]. In

P. putida observed differences were even smaller than for *A. globiformis* and while Ag_2S did induce growth inhibition these were too small to be reliably interpreted.

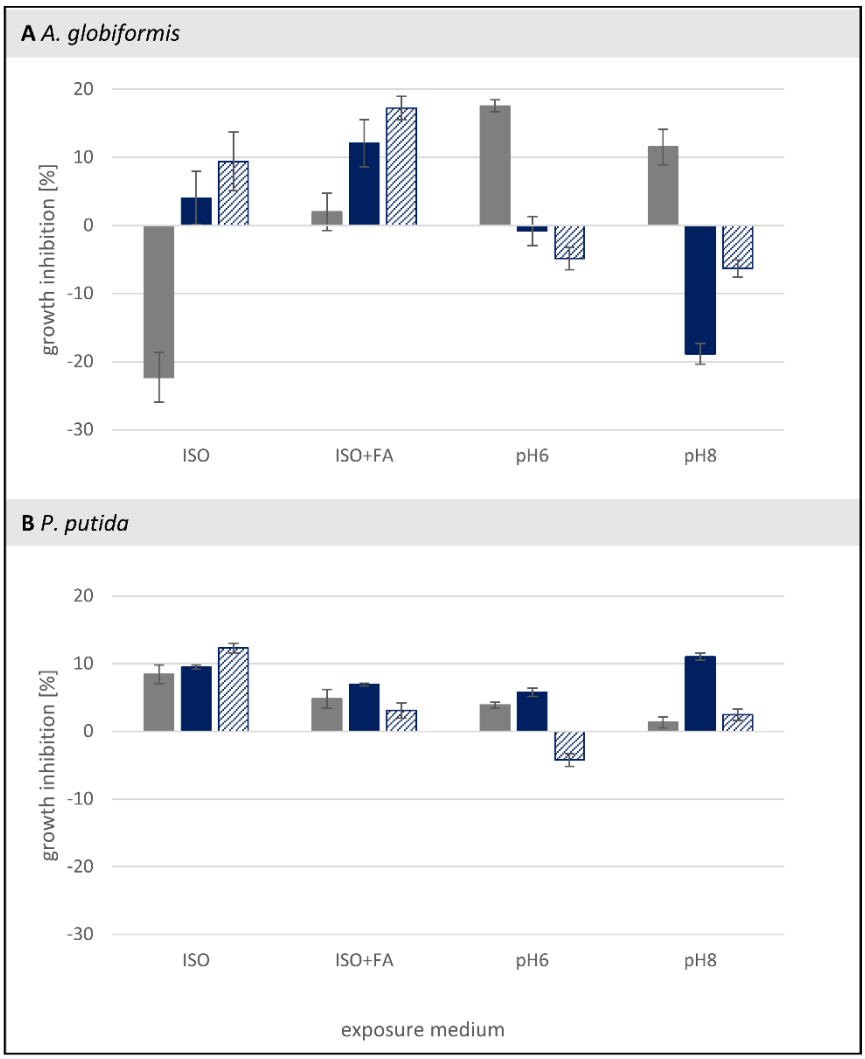

Figure 4: Growth inhibition (average ± SE) caused by Ag_2S exposure to A) *A. globiformis* and B) *P. putida* as percent inhibition in unexposed controls in ISO standard testmedium, ISO medium with added fulvic acid (FA) and soil pore water extracts adjusted to pH6 and pH8; Solid grey: unaged exposures, solid dark blue: aged total, dark blue striped: aged soluble fraction.

4. CONCLUSION

This study examined how aging silver nanoparticles and ionic silver in the exposure media prior to toxicity assays alters their effect to bacteria in relation to the media and nanoparticle properties. An overview of the results of the toxicity assays is presented in Table 2. In 80% of the tested Ag^+ and Ag ENP exposure scenarios aging significantly reduced toxicity. The majority of cases where aging had no effect had been carried out in the standard ISO test medium. Once organic matter was present in the

exposure media, in form of a FA supplement to the standard medium or in the extracts of natural soil pore waters, aging reduced ENP toxicity regardless of any other media property. Strikingly, while soluble silver largely explained Ag-PVP and Ag-citr toxicity in the standard ISO medium, the observed toxic effects in the media containing natural organic matter cannot be solely attributed to the soluble fraction and thus to ionic silver. This was additionally supported by the silver concentration measurements in the soluble fraction. The amount of soluble silver present in aged totals compared to concentrations of $AgNO_3$ needed to cause similar effects was reached in the ISO medium, yet was much lower in the other media. Thus toxicity beyond that of ionic silver was revealed, likely caused by different species of silver compounds in solution and particle specific effects unrelated to the core material, such as the attachment to the cell surface can alter membrane functions, interaction with respiratory chain, penetrating into the cell and interacting with proteins and DNA[53]. When considering Ag_2S as relevant sewage treatment plant release form of Ag ENPs, both aging and media composition influenced fate and toxicity to *A. globiformis*. Together this demonstrated that under the most environmentally relevant combination of here tested exposure scenarios, i.e. Ag_2S in soil pore water, aging reduced ENP toxicity. In the context of nanotoxicology research the presented finding stress the importance of environmentally relevant exposure designs. The use of relevant release forms (here Ag_2S) can greatly influence conclusions drawn from toxicity tests. However, even the very simple addition of standard fulvic acid to the test medium already altered the effects of ENP aging and contribution of ionic silver to toxicity. Further, aging/pre-incubation of materials in the exposure medium, as is likely to occur in the environment, significantly impacted test results and is easily achievable in standard test systems. Thus, even small adjustments to test designs can help to draw more environmentally meaningful conclusions.

Table 2: Overview of effects of aging treatments and media on silver toxicity to *A. globiformis* and *P. putida*. Treatments: unaged UA, aged total A, aged soluble fraction S; Aging effects: ↔ unchanged, ↑ increased or ↓ decreased toxicity with comparisons made in relation to unaged exposures; in brackets toxicity of aged total compared to soluble fraction. Media effects: changes in toxicity after addition of FA to ISO media or effect of increased pH between the two soil pore waters; Ø denotes no observed effects of soluble fraction, n.d. = not determinable.

	AGING effects				**MEDIA effects**	
	ISO	**ISO+FA**	**pH6**	**pH8**	**FA addition**	**pH increase**
A. globiformis						
$AgNO_3$	↔ (A=S)	↓ (A>S)	↓ (A=S)	↓ (A=S)	UA↔ A↓ S↓	UA↔ A↓ S↓
Ag-PVP	↓ (A=S)	↔ (A>S)	↓ (A>S)	↓ (A>S)	UA↔ A↑S↓	UA↔ A↑S↓
Ag-citr	↔ (A=S)	↓ (A>S)	↓ (A>S)	↔ (A>S)	UA↔ A↓S↓	UA↔ A↓S↓
Ag_2S	↑ (A≤S)	↑ (A≤S)	↓ (A≥S)	↓ (A<S)	UA↑A↑ S↑	UA↑A↑ S↑
P. putida						
$AgNO_3$	↔ (A=S)	↓ (A=S)	↓ (A=S)	↔ (A=S)	UA↔ A↓ S↔	UA↓ A↓ S↔
Ag-PVP	↔ (A=S)	↓ (A>S)	↓ (A>S)	↓ (A>S)	UA↔ A↑ S↔	UA↓ A↓ SØ
Ag-citr	↔ (A>S)	↓ (A>S)	n.d.	n.d.	n.d.	UA↓ A↓ SØ
Ag_2S	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

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CONFLICT OF INTEREST

There are no conflicts of interest to declare.

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Aging reduces the toxicity of pristine but not sulphidised silver nanoparticles to soil bacteria

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SUPPLEMENTARY INFORMATION

12 pages, 2 figures, 2 tables

Soil and media properties

Nanoparticle synthesis and characterisation

Results statistical data analysis

References

Soil properties

Table S1: Soil properties: Classification, origin, soil texture, 100% water holding capacity (WHC) in mL per 100 g soil (dry weight), soil pH measured in 0.01 M CaCl2 and pore water (PW) pH, organic matter content (OM), and cation exchange capacity (CEC). Data taken from Heggelund et al 2014 [1].

Origin	Classification	Sand %	Silt %	Clay %	100% WHC [mL]	Soil pH_{CaCl2}	PW pH_{H2O}	OM %	CEC [mval/100g]
Acidic Heath	Sandy	91.7	4.7	3.5	49.2	3.1	4.2	8.00	5.4

Nanoparticle synthesis

Synthesis details can be found for Ag-PVP in Starnes et al 2015[2] and for Ag-citr in Cobaleda-Siles et al 2017[3].

Ag_2S synthesis (manuscript in prep):

Briefly, a 1L aqueous solution of $Na_2S.9H_2O$ and Polyvinylpyrrolidone (PVP) 55kDa was heated to a specific temperature under vigorous stirring. Then a concentrated solution of the $AgNO_3$ precursor was injected at a defined $[AgNO_3]$/[PVP] ratio for desired nanoparticle size. To ensure complete reaction of the precursors the solution was stirred at the synthesis temperature for 15 min. To remove excess S^{2-} the synthesised Ag_2S nanoparticles were purified by centrifugation and resuspended in Milli-Q-water with 55kDa PVP (1 mg/mL).

Nanomaterial characterisation

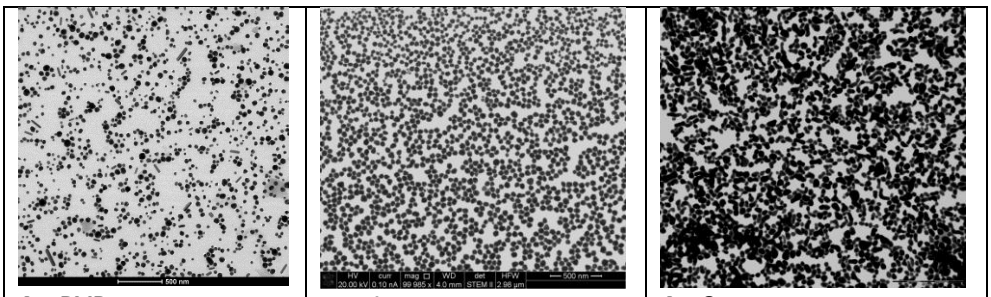

Figure S1: TEM images of tested nanoparticles. Ag-PVP image provided by Dr Stella Marinakos from Duke University. Scale bars are 500 nm.

Table S2: Nanoparticle stock characterisation. Asterices denote information provided by the suppliers

Nano-material	Coating/stabiliser	TEM Size [nm]	NTA mean size [nm]	Zeta potential [mV]	Reference

Ag-citr	5 mM sodium citrate	49.1 ± 6.3	60.5 ± 0.6	-50.0 ± 2.3	
Ag-PVP	Polyvinylpyrrolidone	58.3 ± 12.9[2]*	88.2 ± 1.1	-11.6 ± 0.3	Starnes et al 2015[2]
Ag_2S	Polyvinylpyrrolidone	36.1 ± 9.7	84.8 ± 1.1	-25.7 ± 1.7	

ISO 10712 medium

Table S3: Nutrient concentrations in the ISO 10 721 (1995) media

Nutrients	Preculture Solution (mg/L)	Test Nutrient Solution (mg/L)
$NaNO_3$	500	500
$K_2HPO_4 \times 3H_2O$	120	120
KH_2PO_4	60	60
yeast extract	50	-
$C_6H_{12}O_6$	2000	2000
$MgSO_4 \times 7H_2O$	200	200
iron(III) citrate	0.5	0.5

Nanoparticle NTA characterisation

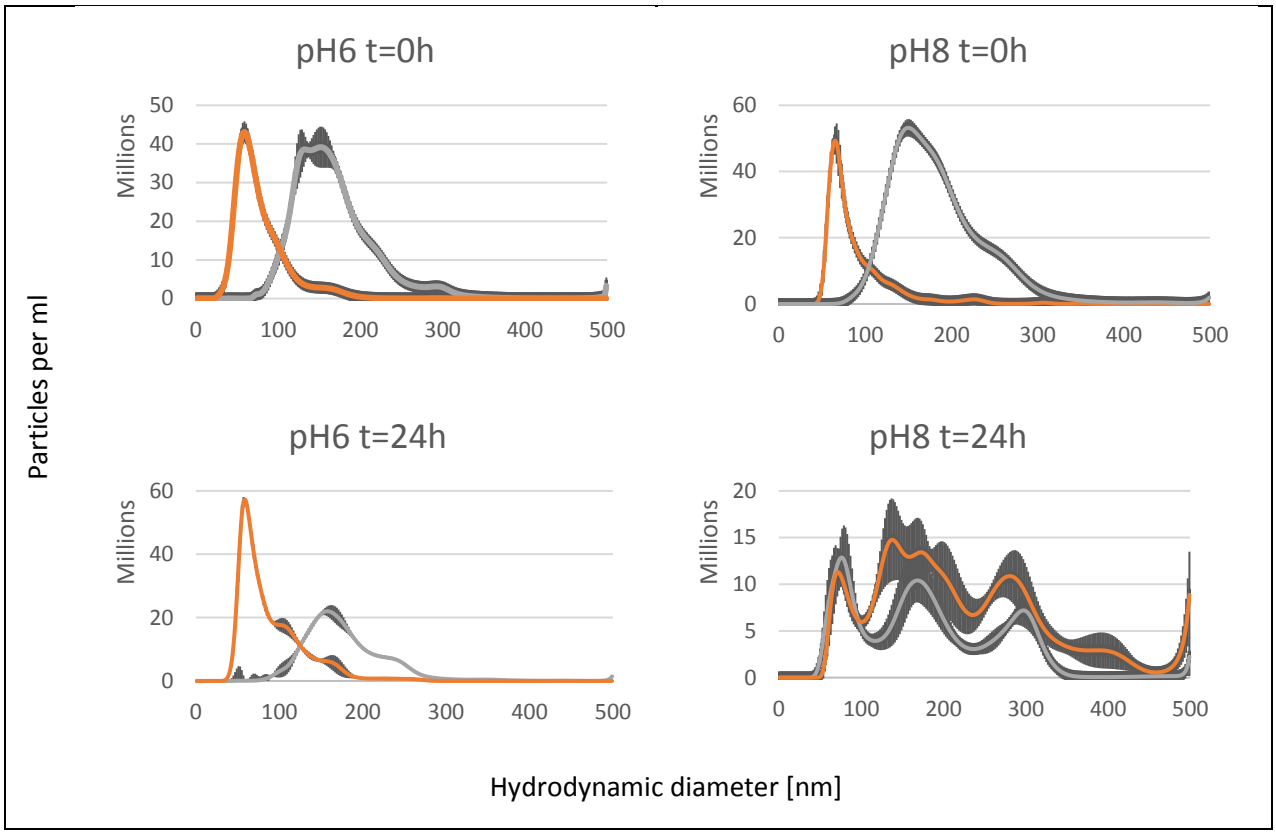

Figure S2: NTA hydrodynamic diameter [nm] ± standard deviation of Ag-citr in soil pore water extracts with different pHs (pH6 and pH8) at t=0h and t=24h, orange: particles in pore water, grey: pore water without particles.

Results statistical data analysis

Table S4: Results of GLM analysis and post hoc Tukey pairwise comparison comparing growth inhibition of *A. globiformis* by different aging treatments in ISO standard testmedium, ISO medium with added fulvic acid (FA) and soil pore water extracts adjusted to pH6 and pH8. Different letters denote significant differences between treatments (p>0.05).

	Comparison	R^2	df	*F*-value	*p*-value	Tukey	
$AgNO_3$							
ISO	concentration	96.25	6	214.08	0.000	unaged	A
	treatment		2	1.73	0.187	aged	A
	interaction		12	1.88	0.059	dissolved	A
ISO+FA	concentration	97.93	6	367.01	0.000	unaged	C
	treatment		2	40.27	0.000	aged	B
	interaction		12	12.34	0.000	dissolved	A
pH6	concentration	98.73	5	651.14	0.000	unaged	B
	treatment		2	39.11	0.000	aged	A
	interaction		10	25.69	0.000	dissolved	A
pH8	concentration	99.01	5	718.25	0.000	unaged	C
	treatment		2	81.35	0.000	aged	A
	interaction		10	80.07	0.000	dissolved	B
Ag-PVP							
ISO	concentration	95.23	6	147.39	0.000	unaged	B
	treatment		2	15.42	0.000	aged	A
	interaction		12	9.09	0.000	dissolved	A
ISO+FA	concentration	98.28	6	445.12	0.000	unaged	B
	treatment		2	35.92	0.000	aged	B
	interaction		12	14.79	0.000	dissolved	A
pH6	concentration	97.31	5	177.99	0.000	unaged	C
	treatment		2	215.85	0.000	aged	B
	interaction		10	44.71	0.000	dissolved	A
pH8	concentration	99.36	5	737	0.000	unaged	C
	treatment		2	640.35	0.000	aged	B
	interaction		10	234.71	0.000	dissolved	A
Ag-citr							
ISO	concentration	93.46	6	117.62	0.000	unaged	A
	treatment		2	2.44	0.098	aged	A
	interaction		12	1.61	0.118	dissolved	A
ISO+FA	concentration	98.6	6	349.67	0.000	unaged	C
	treatment		2	513.32	0.000	aged	B
	interaction		12	54.94	0.000	dissolved	A
pH6	concentration	96.66	5	127.75	0.000	unaged	C
	treatment		2	178.73	0.000	aged	B
	interaction		10	42.03	0.000	dissolved	A
pH8	concentration	99.06	5	536.4	0.000	unaged	B
	treatment		2	437.84	0.000	aged	B
	interaction		10	138.95	0.000	dissolved	A

Table S5: Results of GLM analysis and post hoc Tukey pairwise comparison comparing growth inhibition of *A. globiformis* in different media treatments under unaged (UA) and aged (A) conditions in ISO standard testmedium, ISO medium with added fulvic acid (FA) and soil pore water extracts adjusted to pH6 and pH8.

	Comparison	R2	df	F-value	p-value
$AgNO_3$					
ISO v ISO+FA	concentration	95.81	6	129.03	0.000
UA	treatment		1	0.06	0.813
	interaction		6	0.38	0.889
ISO v ISO+FA	concentration	96.81	6	169.65	0.000
A	treatment		1	12.18	0.001
	interaction		6	0.94	0.482
pH6 v pH8	concentration	96.13	5	135.03	0.000
UA	treatment		1	13.79	0.001
	interaction		5	6.60	0.000
pH6 v pH8	concentration	88.8	5	39.25	0.000
A	treatment		1	9.89	0.004
	interaction		5	5.13	0.002
Ag-PVP					
ISO v ISO+FA	concentration	96.93	6	175.61	0.000
UA	treatment		1	0.11	0.741
	interaction		6	3.29	0.012
ISO v ISO+FA	concentration	95.11	6	99.54	0.000
A	treatment		1	18.18	0.000
	interaction		6	8.25	0.000
pH6 v pH8	concentration	99.49	5	1108.73	0.000
UA	treatment		1	8.93	0.006
	interaction		5	66.66	0.000
pH6 v pH8	concentration	97.92	5	265.18	0.000
A	treatment		1	4.43	0.044
	interaction		5	16.83	0.000
Ag-citr					
ISO v ISO+FA	concentration	98.96	6	531.23	0.000
UA	treatment		1	0.52	0.477
	interaction		6	9.59	0.000
ISO v ISO+FA	concentration	95.7	6	119.1	0.000
A	treatment		1	8.29	0.007
	interaction		6	6.02	0.000
pH6 v pH8	concentration	99.35	5	850.37	0.000
UA	treatment		1	50.64	0.000
	interaction		5	52.19	0.000
pH6 v pH8	concentration	98.37	5	314.03	0.000
A	treatment		1	65.07	0.000
	interaction		5	37.53	0.000

Table S6: Results of GLM analysis and post hoc Tukey pairwise comparison comparing growth inhibition of *P. putida* by different aging treatments in ISO standard testmedium, ISO medium with added fulvic acid (FA) and soil pore water extracts adjusted to pH6 and pH8. Different letters denote significant differences between treatments (p>0.05).

	Comparison	R2	df	F-value	p-value	Tukey	
$AgNO_3$							
ISO	concentration	99.91	6	8720.21	0.000	unaged	A
	treatment		2	1.92	0.157	aged	A
	interaction		12	2.68	0.007	dissolved	A
ISO+FA	concentration	99.66	6	2447.53	0.000	unaged	B
	treatment		2	9.32	0.000	aged	A
	interaction		12	3.13	0.002	dissolved	A
pH6	concentration	99.88	5	6634.1	0.000	unaged	B
	treatment		2	281.88	0.000	aged	A
	interaction		10	297.33	0.000	dissolved	A
pH8	concentration	98.77	5	721.6	0.000	unaged	A
	treatment		2	2.79	0.072	aged	A
	interaction		10	0.84	0.592	dissolved	A
Ag-PVP							
ISO	concentration	99.41	6	1429.16	0.000	unaged	A
	treatment		2	1.62	0.207	aged	A
	interaction		12	3.57	0.001	dissolved	A
ISO+FA	concentration	98.47	6	198.82	0.000	unaged	A
	treatment		2	250.16	0.000	aged	B
	interaction		12	140.39	0.000	dissolved	C
pH6	concentration	99.86	5	1805.55	0.000	unaged	A
	treatment		2	5490.81	0.000	aged	B
	interaction		10	1381.66	0.000	dissolved	C
pH8	concentration	98.18	5	168.73	0.000	unaged	C
	treatment		2	88.65	0.000	aged	B
	interaction		10	145.01	0.000	dissolved	A
Ag-citr							
ISO	concentration	98.91	6	491.83	0.000	unaged	B
	treatment		2	248.42	0.000	aged	B
	interaction		12	105.50	0.000	dissolved	A
ISO+FA	concentration	97.75	6	124.46	0.000	unaged	C
	treatment		2	47.57	0.000	aged	B
	interaction		12	112.01	0.000	dissolved	A
pH6	concentration	99.59	5	658.68	0.000	unaged	C
	treatment		2	1195.66	0.000	aged	B
	interaction		10	460.46	0.000	dissolved	A
pH8	concentration	76.27	5	8.7	0.000	unaged	A
	treatment		2	23.73	0.000	aged	B
	interaction		10	6.39	0.000	dissolved	A

Table S7: Results of F-test for ISO variants and GLM analysis and post hoc Tukey pairwise comparison for soil pore waters comparing growth inhibition of *P. putida* in different media treatments under unaged (UA) and aged (A) conditions in ISO standard testmedium, ISO medium with added fulvic acid (FA) and soil pore water extracts adjusted to pH6 and pH8. n.d.: not determined due to F-test constraints.

		R2	df	F-value	p-value
$AgNO_3$					
ISO v ISO+FA UA				4.217	0.018
ISO v ISO+FA A				1.852	0.169
pH6 v pH8 UA	concentration	99.78	5	1851.92	0.000
	treatment		1	1529.03	0.000
	interaction		5	617.36	0.000
pH6 v pH8 A	concentration	99.91	5	5325.97	0.000
	treatment		1	1016.57	0.000
	interaction		5	1122.80	0.000
Ag-PVP					
ISO v ISO+FA UA				n.d.	n.d.
ISO v ISO+FA A				n.d.	n.d.
pH6 v pH8 UA	concentration	99.92	5	5896.52	0.000
	treatment		1	3259.02	0.000
	interaction		5	1549.55	0.000
pH6 v pH8 A	concentration	93.72	5	80.93	0.000
	treatment		1	3.58	0.068
	interaction		5	7.97	0.000
Ag-citr					
ISO v ISO+FA UA				n.d.	n.d.
ISO v ISO+FA A				n.d.	n.d.
pH6 v pH8 UA	concentration	99.68	5	935.97	0.000
	treatment		1	951.58	0.000
	interaction		5	765.67	0.000
pH6 v pH8 A	concentration	78.55	5	14.77	0.000
	treatment		1	14.8	0.001
	interaction		5	4.87	0.002

Table S8: Results of F-test comparing growth inhibition of Ag-PVP and Ag-citr in the same media under unaged (UA) and aged (A) conditions in ISO standard testmedium, ISO medium with added fulvic acid (FA) and soil pore water extracts adjusted to pH6 and pH8. n.d.: not determined due to F-test constraints.

		A. globiformis		*P. putida*	
		F-value	**p-value**	**F-value**	**p-value**
ISO	UA	3.901	0.023	309.32	0.000
	A	8.686	0.001	317.12	0.000
ISO+FA	UA	0.139	0.935	766.83	0.000
	A	21.17	0.000	n.d.	n.d.
pH6	UA	14.35	0.000	1071.87	0.000
	A	1.529	0.241	n.d.	n.d.
pH8	UA	1.529	0.468	n.d.	n.d.
	A	0.241	0.708	n.d.	n.d.

Table S9: Results of GLM analysis and post hoc Tukey pairwise comparison comparing growth inhibition of *A. globiformis* and *P. putida* by different aging treatments in ISO standard testmedium, ISO medium with added fulvic acid (FA) and soil pore water extracts adjusted to pH6 and pH8. Different letters denote significant differences between treatments (p>0.05).

	R2	df	F-value	p-value	Tukey	
A. globiformis						
ISO	85.99	2	18.42	0.003	unaged	B
					aged	A
					dissolved	A
ISO+FA	72.84	2	8.05	0.020	unaged	B
					aged	AB
					dissolved	A
pH6	89.97	2	40.35	0.000	unaged	A
					aged	B
					dissolved	B
pH8	95.63	2	65.7	0.000	unaged	A
					aged	C
					dissolved	B
P. putida						
ISO	61.62	2	4.82	0.057	unaged	A
					aged	A
					dissolved	A
ISO+FA	53.17	2	3.41	0.103	unaged	A
					aged	A
					dissolved	A
pH6	95.15	2	58.8	0.000	unaged	A
					aged	A
					dissolved	B
pH8	94.4	2	50.56	0.000	unaged	B
					aged	A
					dissolved	B

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