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Effects of silver sulfide nanomaterials on mycorrhizal colonization of tomato plants and soil microbial communities in biosolid-amended soil

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ABSTRACT

We investigated effects of Ag_2S engineered nanomaterials (ENMs), polyvinylpyrrolidone (PVP) coated Ag ENMs (PVP-Ag), and Ag^+ on arbuscular mycorrhizal fungi (AMF), their colonization of tomato (S*olanum lycopersicum)*, and overall microbial community structure in biosolids-amended soil. Concentration-dependent uptake was measured in all treatments. Plants exposed to 100 mg kg^{-1} PVP-Ag ENMs and 100 mg kg^{-1} Ag^+ exhibited reduced biomass and greatly reduced mycorrhizal colonization. Bacteria, actinomycetes and fungi were inhibited by all treatment classes, with the largest reductions measured in 100 mg kg^{-1} PVP-Ag ENMs and 100 mg kg^{-1} Ag^+. Overall, Ag_2S ENMs were less toxic to plants, less disruptive to plant-mycorrhizal symbiosis, and less inhibitory to the soil microbial community than PVP-Ag ENMs or Ag^+. However, significant effects were observed at 1 mg kg^{-1} Ag_2S ENMs, suggesting that the potential exists for microbial communities and the ecosystem services they provide to be disrupted by environmentally relevant concentrations of Ag_2S ENMs.

KEYWORDS: Nanotoxicology, nanoparticles, nanotechnology,

CAPSULE: Although Ag_2S ENMs are less toxic to soil microorganisms than pristine nanomaterials or ions, some effects are observed on soil microbial communities at relevant concentrations.

27 **INTRODUCTION**

28 Over the past 10-15 years, it has become clear that nano-scale materials have useful
29 properties (e.g. physicochemical, optical, and electrical) that can be harnessed to produce
30 beneficial technologies. Consequently, a steadily increasing number of consumer products
31 employing nanotechnology has become available for purchase (WWCIS 2014). Silver (Ag)
32 engineered nanomaterials (ENMs) have anti-microbial properties and as a result, are among
33 the most widely used ENMs, being employed in products as ubiquitous as clothing,
34 toothpaste, and toys (Sondi 2004, Benn 2008, Benn 2010). However, Ag from the ENMs
35 within these consumer products can be released as either intact ENMs or as dissolved Ag^+
36 into wastewater streams during use and will be subsequently incorporated into sludge during
37 wastewater treatment (Benn 2008). In many parts of the world sludge from wastewater
38 treatment is processed into biosolids and applied to crop land as fertilizer. In these areas, a
39 potential exists for ENMs to enter and accumulate in agroecosystems with unknown
40 consequences for terrestrial biota.

41 Prior to discharge into agroecosystems, as-manufactured, pristine ENMs (or their
42 dissolution products) will be transformed during wastewater treatment into fundamentally
43 different end products (Levard 2011, Rathnayake 2014). These aged ENMs (a-ENMs) will
44 have altered chemical and physical properties and will likely induce different biological
45 responses compared to pristine ENMs. In the case of Ag ENMs, recent studies have shown
46 that they will be transformed to Ag_2S a-ENMs during the wastewater treatment process prior
47 to release into agroecosystems (Levard 2011, Wiesner 2011, Doolette 2013, Ma 2013).

48 How the accumulation of a-ENMs such as Ag_2S a-ENMs in agroecosystems will affect
49 crop productivity, soil microbial communities, and soil health remains unclear. Soil bacteria
50 and fungi are involved in many critical ecosystem processes, including C and nutrient cycling
51 (Judy 2014). Although there have been studies over the past few years examining the effects

52 of ENMs on beneficial bacteria (Bandyopadhyay 2012, Calder 2012), fungi, and whole soil
53 microbial communities (Colman 2013), these studies have largely examined impacts as a
54 result of exposure to pristine ENMs and little information exists with which to evaluate the
55 risk posed by a-ENMs to soil bacteria and fungi and the ecosystem services they deliver.
56 There has been a particularly large amount of research conducted examining the effects of
57 pristine ENMs on microbial communities in municipal waste, activated sludge and compost
58 (Doolette 2013, Gitipour 2013, Sun 2013, Ma 2015). However, soil microbial communities
59 contain a different assemblage of organisms than is present in municipal waste and therefore,
60 the above studies provide little information regarding how a-ENMs will affect soil microbial
61 communities and the ecosystem services (plant growth promotion, nitrogen fixation, etc.) soil
62 microorganisms participate in.

63 Dozens of studies have been published examining the phytotoxicity and potential for plant
64 uptake of ENMs (Lin 2007, Judy 2012, Judy 2014). The results of these studies, mostly
65 conducted using pristine ENMs in hydroponic exposure media and employing different
66 exposure methods and plant species, have produced contradictory results (Judy 2014).
67 Although the magnitude of the impact to crop productivity as a result of plant uptake or direct
68 phytotoxicity remains unclear, there is sufficient evidence that ENMs can be bioavailable and
69 toxic to crop plants under some circumstances (Judy 2012).

70 In addition to direct acute toxicity, a-ENMs could can potentially negatively affect crop
71 productivity indirectly by influencing beneficial plant-microbial relationships (Gambino
72 2015), although few studies have focused on this indirect toxicity. For example, the impact
73 of a-ENMs on beneficial soil fungi and plant-fungi symbioses is largely unstudied (Dubchak
74 2010). The majority of terrestrial plant species form mutualistic symbioses with arbuscular-
75 mycorrhizal fungi (AMF), in which the plant provides the AMF with carbohydrates while the

76 AMF facilitates the uptake of inorganic nutrients, including N, P, and Zn into the plant
77 (Rengel 1999, Judy 2014).

78 Very little research to date has examined the effects of ENMs on mycorrhizal colonization
79 of plant roots (Dubchak 2010, Watts-Williams 2014). One such study examined how
80 mycorrhizal colonization of sunflower (*Helianthus annuus*) responded to the presence of
81 pristine Ag ENMs.(Dubchak 2010) In this study, the authors reported that Ag ENMs
82 inhibited mycorrhizal colonization of *Helianthus annuus* at a soil concentration of
83 approximately 150 mg kg^{-1} (Dubchak 2010). Another more recent study by Feng et al. (2013)
84 which examined how pristine FeO and Ag ENMs affected AMF colonization of clover roots
85 in perlite/sand mix reported significant biomass reduction as a result of exposure to 3.2 mg
86 kg^{-1} FeO ENMs (Feng 2013).

87 However, as previously mentioned, the overwhelming evidence suggests that pristine Ag
88 ENMs such as those used in the two above studies will be unlikely to accumulate in the
89 environment. As a result, it remains unclear if the presence of a-ENMs in soil or the
90 adsorption a-ENMs on the plant root surface will interfere with effective plant-AMF
91 symbiosis. In this study, we have investigated the potential for polyvinylpyrrolidone (PVP)
92 coated Ag (PVP-Ag) ENMs, Ag_2S a-ENMs, and Ag^+ to affect mycorrhizal colonization of
93 tomato (S*olanum lycopersicum*) roots, plant growth, and overall soil microbial communities
94 in biosolid-amended soil.

95 MATERIALS AND METHODS

96 **Soil exposure media.** Exposure media consisted of 95% (wt/wt) sandy loam collected from
97 Blackpoint, South Australia and 5% sludge collected from a wastewater treatment plant
98 (WWTP) located in the Barossa Valley, South Australia (Table 1). The Blackpoint soil was
99 collected from the top 10 cm of the soil profile, air-dried, and sieved to < 2mm. The pH,

100 Table 1. Soil media characterization data. For the mix, *denotes a value calculated from
101 measurements of the Blackpoint soil and Barossa Valley biosolids. WHC= water holding
102 capacity. TOC= total organic carbon. CEC= cation exchange capacity.
103

Analyte	Biosolids-soil mixture (mean ± SD)
HCO_3-ext.P (mg kg^{-1})	3.7*
WHC (mL/100g)	28.0 ± 3.5
S (mg kg^{-1})	884*
pH	6.8 ± 0.1
Total C %	1.8 ± 0.1
% Sand	61.2 ± 0.6
% Silt	12.6 ± 0.4
% Clay	23.0 ± 0.7
CEC (cmol kg^{-1})	18.2 ± 1.4

104
105 cation exchange capacity (CEC), water holding capacity (WHC), and other selected physical
106 and chemical properties of the soil-sludge mix were assessed (Table 1). CEC was
107 determined using NH_4Cl and pH was measured using 5 g of soil-sludge mix and 10 mL 1 M
108 KCl (Sparks 1996). The WHC of the soil media was measured using a gravity filtration
109 method (Ma 2013). Recent studies have estimated that the concentrations of Ag ENMs in
110 biosolids can approach 1 mg kg^{-1} (Gottschalk 2009), thus we examined the effects of
111 exposure to soil concentrations of 1, 10, or 100 mg Ag kg^{-1} as PVP-Ag ENMs and Ag_2S
112 ENMs to simulate a range of concentrations that might be expected following long-term
113 applications of biosolids to soil and compared these to dissolved Ag^+ (as $AgNO_3$) and a blank
114 control.
115 At the end of the experiment, readily soluble or labile Ag concentrations in the soil-sludge
116 mix were estimated by extraction with 1 M NH_4NO_3 (Hall 1998). For these extractions,
117 approximately 10 g of media were weighed into 50 ml centrifuge tubes into which 25 ml
118 NH_4NO_3 were added. The resulting suspensions were shaken end-over-end for 2 h then
119 allowed to settle, after which an aliquot of supernatant was collected and filtered through 0.45

120 μm cellulose acetate filters. Total Ag concentration in filtrates was determined by
121 inductively coupled plasma-mass spectrometry (ICP-MS, Agilent 7700).

122 Total Ag concentrations in soil media treatments (actual exposure concentrations) were
123 determined using a modified US-EPA 3051A strong acid extraction procedure (USEPA
124 2007). For this procedure, approximately 0.25 g soil media was weighed into 50 ml digestion
125 vessels into which 7.5 ml of nitric acid (HNO_3) and 2.5 ml hydrochloric (HCl) acid were
126 added. The soils were open-vessel digested at room temperature for 12 h, then sealed and
127 heated for 45 min at 175ºC (after a 10 min ramp period) in a microwave oven at 1600 W
128 (Mars Express, CEM). Ag concentrations in the digestions were determined by ICP-MS and
129 soil Ag was calculated on a dry weight basis. Standard reference materials (SRMs) Montana
130 Soil 2711a (NIST, Gaithersburg, MD, USA) and marine sediment PACS-2 (National
131 Research Council of Canada (NRC), Ottawa, ON, Canada) were included to assess the
132 accuracy of the digestion and analysis procedures (n=7), resulting in recoveries of 105 ± 13%
133 and 101 ± 11%, respectively. Analytical runs contained internal calibration verification
134 samples and duplicate dilutions.

135 **Nanomaterial synthesis and characterization**. Ag_2S and PVP-Ag ENMs were prepared
136 according to established protocols (Kim 2006, Levard 2011). PVP-Ag ENMs were
137 synthesized by dissolving PVP into ethylene glycol, heating to 120° C, adding $AgNO_3$ and
138 allowing to heat for 2 h. After adding acetone to remove ethylene glycol, PVP-Ag ENMs
139 were washed five times with ultrapure deionized water (DI, Milli-Q, Millipore). For Ag_2S
140 ENMs, PVP-Ag ENMs were sulfidized by adding Na_2S to a 2:1 S:Ag molar ratio and
141 aerating at room temperature for 48 h (Levard 2011). Finally, Ag_2S ENMs were washed with
142 DI four times.

143 Purified ENMs were characterized using TEM, XRD, and dynamic light scattering (DLS;
144 Table 2). Electrophoretic mobility measurements were collected via phase angle light
145 scattering (PALS, Table 2) and were converted to zeta potentials using the Hückel

146 Table 2. Nanomaterial characterization data. PDI= polydispersity index.

147

Nanomaterial	Zeta potential (Mean ± SD; mV)	Z-average diameter (Mean ± SD; nm)	PDI	TEM diameter (Mean ± SD; nm)	Background Ag^+ (%)
Ag_2S	-54.1 ± 1.0	90.1 ± 0.8	0.2	30.1 ± 11.5	BDL
PVP Ag	-7.9 ± 0.1	59.7 ± 1.6	0.3	20.6 ± 6.9	0.19 ± 0.02

148
149

150 approximation. DLS and PALS measurements were collected using a Nano-ZS Zetasizer
151 (Malvern Instruments Ltd, Worcestershire, UK). X-ray diffractograms were collected from
152 2-3 drops of suspension evaporated onto a Si wafer and analyzed using a X'Pert Pro multi-
153 purpose diffractometer (PANalytical, Almelo, Netherlands) using Fe-filtered Co-Kα radiation
154 and equipped with an automatic divergence slit, a 2° anti-scatter slit, and a fast X'Celerator Si
155 strip detector. Diffractograms were recorded from 3 to 80° 2-theta in steps of 0.017° with a
156 0.5 second counting time per step. TEM micrographs of the ENM treatments were collected
157 using a Tecnai G2 Spirit (FEI, Hillsboro, Oregon, USA) TEM. TEM particle diameter was
158 quantified using ImageJ software and was based upon at least 100 particles from at least 3
159 different micrographs. The amount of soluble Ag present in the background of ENM stock
160 suspensions was quantified by filtering samples of each suspension through 3 kDa
161 regenerated cellulose membranes (Amicon Ultra, Millipore, Billerica, MA, USA). Ag
162 concentrations in filtrates were determined by ICP-MS.

163 **Plant growth experiment.** Commercially available tomato (S*olanum lycopersicum*)
164 seeds ("Gross lisse" variety; Mr. Fothergill's, South Windsor, NSW, Australia) were shaken
165 in dilute bleach for 10 min and then rinsed with DI five times. Based upon manufacturer
166 recommended application rates, 6 g of commercial mycorrhizal inoculum (3000 propagules g^-

^{1}; MycoApply Start Up Super, Microbe Smart Pty, Ltd, Melrose Park, SA, Australia) were mixed into 1 kg of media prior to potting. Biosolid-soil mixtures were brought to 80% WHC prior to seeding. Plants were grown in a climate controlled growth room at 65% relative humidity with a 12h light (23 °C)/12h dark (15 °C) light cycle. Tomato was selected for this study as it is an agriculturally important crop species as well as an USEPA recommended test plant for plant uptake and translocation of contaminants (USEPA 1996). Three seeds were introduced into 1 L closed pots. After 2 weeks, the number of seedlings were thinned to one per pot. The WHC of soil media was maintained at 80% by watering pots to weight three times weekly. Four weeks after planting, a modified 10% Long-Ashton (no chloride salts or P added) nutrient solution was added in equal volumes to each pot once per week (Hewitt 1966, Cavagnaro 2001). All plants were harvested 8 weeks after planting.

Tomato shoot collection and analysis. Shoots were collected by cutting them immediately above the soil surface and dried in an oven at 60 °C to determine dry shoot biomass. The dry biomass was then digested in 3 mL nitric acid for 4 h at 115°C using an Environmental Express SC189 HotblockPro (Environmental Express, Charleston, SC, USA) and subsequently analyzed for Ag concentrations via ICP-MS. SRMs Citrus Leaves NCS ZC73018 (China National Analysis Center for Iron and Steel, Beijing, China) and Tomato Leaves 1573a (National Institute of Standards and Technology (NIST), Gaithersburg, MD, USA) were digested and analyzed along with the tissue samples. The mean and standard deviation of the recovery of the Ag content of these SRMs were 101 ± 5 % and 101 ± 2%, respectively (n=3). Analytical runs contained internal calibration verification samples, duplicate dilutions, and spike recovery samples. Considering the need to use root biomass for AMF colonization assessment as well as the difficulty in distinguishing root-adsorbed ENMs from root-accumulated ENMs, root Ag content was not assessed.

191 **Mycorrhiza colonization of tomato roots**. Roots were collected, rinsed with DI and
192 cleared of cellular contents by soaking in 10% KOH for 4 d at room temperature. Roots were
193 then rinsed with DI and fungal tissue associated with the roots was stained by soaking root
194 tissue in 5% black ink (Sheaffer Pen, Shelton, CT, USA)/95% white vinegar for 5 min at
195 approximately 85°C (Vierheiling 1998). Roots were de-stained in 5% white vinegar
196 overnight, rinsed, and subsequently stored in 50% (wt/wt) glycerol until counting.
197 Mycorrhizal colonization of roots was quantified using the grid-line intersect method
198 (Giovannetti 1980).

199 **Neutral and phospholipid fatty acid analysis**. Neutral (NLFAs) and phospholipids
200 (PLFAs) were extracted from 5 g of freeze-dried soils following the method of White et al.
201 (1979) as modified by Frostegård et al. (1991) using a solution of chloroform, methanol, and
202 citrate buffer (1:2:0.8 v:v:v, 0.15 M, pH 4.0) (White 1979, Frostegård 1991). Additional
203 citrate buffer and chloroform were added to split the extracts into two phases and NLFAs,
204 glycolipids, and PLFAs were eluted from Supelclean™ silica columns (Supelco, Bellefonte,
205 Pennsylvania, USA) using chloroform, acetone, and methanol, respectively. The NLFAs and
206 PLFAs were converted into fatty acid methyl esters (FAME) by alkaline methanolysis and
207 quantified relative to an internal standard (methyl decanoate) and two FAME mixtures (a 27
208 component bacterial acid methyl esters (BAME) mix (Sigma-Aldrich, St. Louis, MO, USA)
209 and a 37 component FAME mix (Supelco, Bellefonte, Pennsylvania, USA)) on an Agilent
210 7890B GC and 5977A MS using a DB-5 (5% diphenyl, 95% dimethyl polysiloxane) column.

211 Microbial PLFAs were divided into putative diagnostic groups as follows: i15, a15, i16,
212 i17 and a17 as gram-positive PLFAs, 16:1ω7c, 18:1ω7c, cy17 and cy19 as gram-negative
213 PLFAs, 10Me16, 10Me17, and 10Me18 as actinomycetes, 18:2ω69c as ectomycorrhizal and
214 saprotrophic fungi, and 16:1ω5c as AMF (Olsson 1995, Zelles 1999, Ruess 2010). The
215 NLFA 16:1ω5c was also used as a marker for AM fungi (Olsson 1995). Although the PLFA

216 16:1ω5c is more strongly correlated with AMF biomass than the NLFA 16:1ω5c, the PLFA
217 is found in bacteria as well as AM fungi, while the NLFA is more specific to AMF fungi
218 (Olsson 1999). Therefore, both the NLFA and PLFA 16:1ω5c are reported here to provide a
219 more accurate representation of the treatment effects of AMF fungal biomass. Total
220 microbial PLFA biomass was estimated by summing the PLFAs for each diagnostic group.

221 **Statistical analysis.** Differences between treatments were analyzed using SAS 9.3. Data
222 sets were checked for normality and homoscedasticity using Shapiro-Wilke's test and
223 Bartlett's test, respectively. Normal and homogenously varied data were analyzed via
224 analysis of variance (ANOVA; PROC ANOVA) and differences between treatment means
225 determined post-hoc using the Student-Neuman-Keuls procedure. If data could not be made
226 normal via a log transformation, data were analyzed via a Kruskal-Wallis test and differences
227 between treatment means determined using pair wise Mann-Whitney U-tests.

228 Proportional distributions of PLFAs were calculated by dividing yields of individual
229 PLFAs by total PLFA yields (nmol PLFA g^{-1} soil). Microbial community structure was
230 assessed using these proportional mol% distributions via principle component analysis (PCA)
231 and analysis of similarity (ANOSIM) in PRIMER (v6.1.16).

232 **RESULTS AND DISCUSSION**

233 **Characterization of Ag ENMs in stock suspensions.** The stock suspensions (PVP-Ag
234 and Ag_2S) were relatively monodisperse, contained roughly spherical ENMs that had primary
235 particle sizes, as determined by TEM, of approximately 20-30 nm (Table 2; Figure S1, see
236 supporting information). Electrophoretic mobility measurements revealed negative mean
237 zeta potentials of -8 and -54 mV for the PVP Ag and Ag_2S ENMs, respectively. Previous
238 reports using PVP-Ag and Ag_2S ENMs synthesized using methods similar to those employed
239 here have reported slightly different zeta potentials than those measured in this
240 study,(Reinsch 2012, Levard 2013) although these methods consistently generate stable

241 suspensions of negatively charged PVP-Ag and Ag_2S ENMs. XRD confirmed that the PVP-
242 Ag ENMs were Ag^0 and that the Ag_2S ENMs were Ag_2S (acanthite; Figure S1, see
243 supporting information). Background soluble Ag concentrations were < 0.2% of the total Ag
244 concentrations in ENM stock suspensions (Table 2).

245 **Total and ammonium nitrate extractable Ag concentrations in soil media.**
246 Measurement of the total Ag concentrations of the exposure media confirmed concentrations
247 approximately equal to the expected nominal concentrations of the treatments (Table 3).
248 Ammonium nitrate extractable Ag concentrations were <0.15% of the total Ag concentrations
249 in all added Ag treatments (Table 3). Extractable Ag in the Ag_2S treatments was not
250 significantly different from control, even at 100 mg kg^{-1}. Extractable Ag concentrations were
251 significantly higher in the 100 mg kg^{-1} PVP-Ag ENMs and in the 10 and 100 mg kg^{-1} soluble
252 Ag treatments, with mean extractable Ag concentrations of 4.2, 3.2, and 11.4 µg kg^{-1},
253 respectively.

254 Table 3. Metals analysis of media, post exposure. 1 M NH_4NO_3 extractable Ag method
255 detection limit=0.15 µg kg^{-1}. BDL= below detection limit. For statistical analysis of BDL
256 data, ½ the instrument detection limit (4.6 ng L^{-1}) was substituted for each BDL data point.
257 Data presented as mean ± one standard deviation. Treatments with the same superscripts are
258 not significantly different at α=0.05.

259

Treatment/Concentration (mg kg^{-1})	Total Ag (mg kg^{-1})	1 M NH_4NO_3 extractable Ag (µg kg^{-1})
Control	0.04 ± 0.01	BDL[a]
PVP Ag 1	1.2 ± 0.1	BDL[ab]
PVP Ag 10	10.7 ± 2.5	0.4 ± 0.3[b]
PVP Ag 100	84.3 ± 20.5	4.2 ± 1.2[d]
Ag_2S 1	1.4 ± 0.3	BDL[ab]
Ag_2S 10	11.0 ± 1.2	BDL[ab]
Ag_2S 100	88.6 ± 16.9	0.2 ± 0.2[ab]
Ag^+ 1	1.4 ± 0.4	BDL[ab]
Ag^+ 10	12.1 ± 1.8	3.3 ± 1.2[c]
Ag^+ 100	102.2 ± 5.6	11.7 ± 2.7[d]

260 **Shoot biomass.** Dry shoot biomass was significantly reduced for the 100 mg kg^{-1} PVP-Ag
261 ENM and 100 mg kg^{-1} Ag^+ treatments (Table 4). Dry shoot biomass was not significantly
262 affected at any of the Ag_2S ENM concentrations tested. Surprisingly, plants exposed to 10
263 mg kg^{-1} Ag^+ generated significantly more shoot biomass than the control (Table 4). The
264 toxicity observed in the 100 mg kg^{-1} PVP-Ag treatment could not be unequivocally linked to
265 extractable Ag pools, as 1 M NH_4NO_3 extractable Ag in the 100 mg kg^{-1} PVP Ag treatment
266 was only slightly higher than that measured in the 10 mg kg^{-1} Ag^+ treatment in which positive
267 effects were observed.
268
269 Table 4. Dry shoot biomass, shoot accumulation and shoot uptake. Data presented as mean
270 ± one standard deviation. Treatments with the same superscripts are not significantly
271 different at α=0.05.
272

Treatment/Concentration (mg kg^{-1})	Dry shoot biomass (mg)	Shoot Ag Concentration (µg kg^{-1})	Ag uptake (ng)
Control	24.9 ± 7.1^{bc}	66.3 ± 49.5^{a}	1.6 ± 1.0^{a}
PVP Ag 1	22.9 ± 6.2^{bc}	63.9 ± 25.3^{a}	1.5 ± 0.7^{a}
PVP Ag 10	32.8 ± 11.1^{b}	284.0 ± 162.6^{bc}	8.6 ± 5.1^{cd}
PVP Ag 100	14.3 ± 4.5^{a}	1536.3 ± 455.9^{d}	22.4 ± 9.5^{de}
Ag_2S 1	26.3 ± 9.5^{bc}	137.0 ± 113.3^{ab}	6.5 ± 6.5^{ab}
Ag_2S 10	21.6 ± 5.3^{bc}	214.8 ± 114.3^{b}	4.4 ± 2.2^{bc}
Ag_2S 100	29.6 ± 6.7^{b}	734.3 ± 984.2^{c}	23.2 ± 35.3^{de}
Ag^+ 1	15.7 ± 5.1^{ac}	134.8 ± 59.9^{b}	2.0 ± 0.8^{ab}
Ag^+ 10	50.4 ± 19.6^{d}	212.9 ± 77.2^{b}	11.0 ± 6.5^{cd}
Ag^+ 100	13.0 ± 4.0^{a}	2354.7 ± 863.5^{d}	29.7 ± 11.9^{e}

273
274
275 The mechanism for the observed significant increase in tomato growth in the presence of
276 10 mg kg^{-1} soluble Ag is unknown. However, hormetic growth stimulation has been
277 observed in plants exposed to low concentrations of toxic metals in soils such as Cd and Pb
278 (Poschenrieder 2013). Poschenrieder et al. (2013) suggested three main modes of action for
279 toxic metal ions that can lead to hormetic plant growth responses: 1) substrate interactions

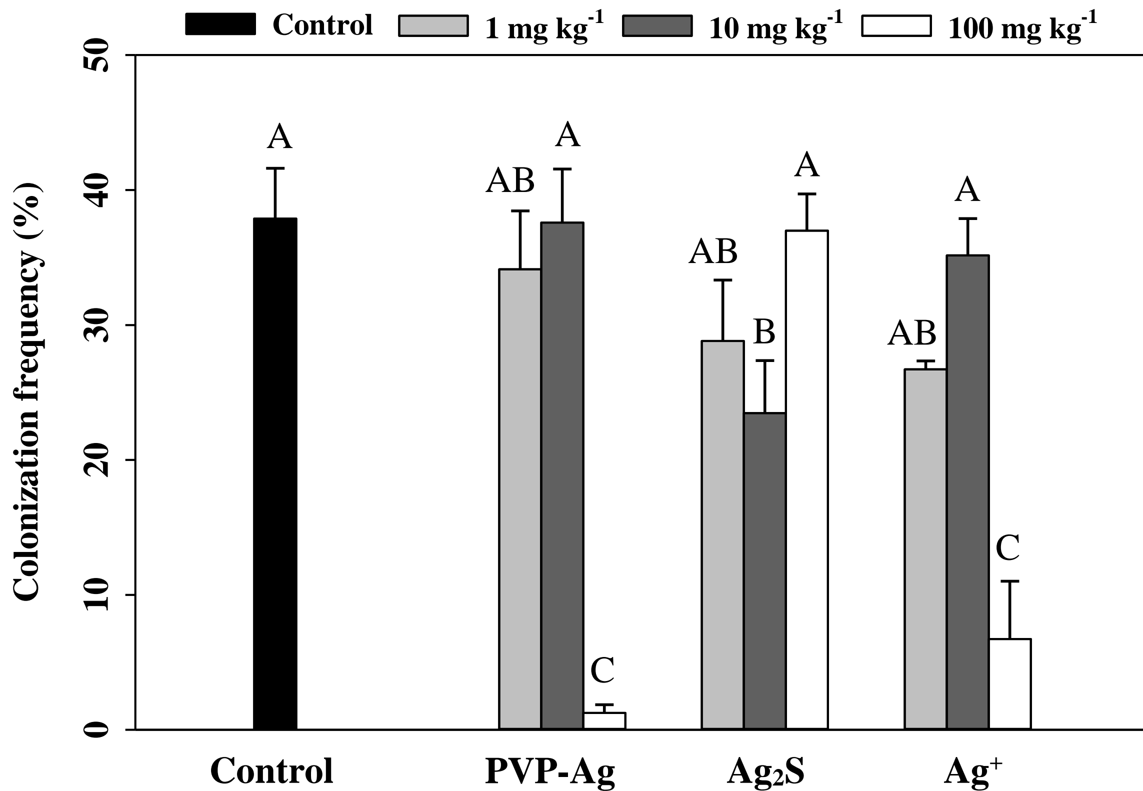
280 consistent with amelioration (e.g. root membrane and rhizosphere), 2) metal induced
281 activation of specific defense reactions in plants (e.g. activation of metal tolerance genes),
282 and 3) metal induced general defense reactions that can lead to protection against another
283 stressor such as antioxidant defenses. In addition, a recent study reported that sub-lethal
284 concentrations of Ag^+ in soils resulted in upregulation of nitrifying genes (amoA1 and
285 amoC2) in the nitrifying bacterium, *Nitrosomonas europaea* (Yang 2013).

286 **Shoot tissue Ag concentrations and uptake .** The concentration of Ag in shoots from
287 plants grown in the 1 mg kg^{-1} PVP-Ag ENMs and in the 1 mg kg^{-1} Ag_2S ENMs treatments
288 were not significantly different from the Ag concentration in control tissue samples (Table 4).
289 Tissue concentrations in all three 10 mg kg^{-1} treatments were significantly higher than
290 controls, though not significantly different from each other. The mean Ag tissue
291 concentration in the 100 mg kg^{-1} Ag_2S treatment was significantly higher than all the 1 and
292 10 mg kg^{-1} treatments, but significantly lower than was measured in plant tissues from the
293 100 mg kg^{-1} PVP-Ag and Ag^+ treatments. Re-examining these data as total Ag uptake
294 revealed different trends. Uptake appeared to be concentration-dependent, with no
295 significant differences between treatments at each of the three concentrations (Table 4). This
296 finding suggests that translocation of Ag into shoot tissue was not related to the reduction in
297 biomass observed in the 100 mg kg^{-1} PVP-Ag and Ag^+ treatments.

298 Whether or not shoot-accumulated Ag was taken up as dissolved Ag^+ or as intact Ag
299 ENMs is unclear, especially in the case of the PVP-Ag treatment. The Ag_2S ENMs are
300 highly insoluble and contain very little background Ag^+ (Table 2), suggesting that the uptake
301 measured in plants exposed to this treatment was primarily the result of the uptake of intact
302 Ag_2S ENMs.

303 **Mycorrhiza colonization.** Mycorrhizal colonization of tomato roots for all Ag treatments
304 of 1 mg kg^{-1} Ag treatment exposure concentration was not significantly different compared to

305 the control (Figure 1). In contrast, mycorrhizal colonization was significantly reduced
306 compared to the control at 100 mg kg^{-1} in the Ag-PVP ENMs and Ag^+ treatments (Figure S2;
307 see supporting information). Colonization was also significantly reduced in the 10 mg kg^{-1}
308 Ag_2S treatment but not at the higher 100 mg kg^{-1} exposure concentration.
309



310
311

312 Figure 1. Mycorrhizal colonization frequency of tomato roots. Treatments with the same
313 letter are not significantly different at α=0.05 as determined by pair-wise Mann-Whitney U-
314 tests. Error bars=standard error.

315 Why colonization was affected in a seemingly concentration independent manner is
316 unclear. However, this result is similar to a recent report in which *Trifolium repens* was
317 exposed to pristine FeO and Ag ENMs in an artificial medium (Feng 2013). In this study,
318 mycorrhizal colonization was affected at 0.01 mg kg^{-1} Ag ENMs, but not at 0.1 mg kg^{-1} or 1
319 mg kg^{-1} Ag ENMs. These authors speculated that higher concentrations of Ag generated a

320 stress response that was not induced at 0.01 mg kg^{-1} Ag ENMs which allowed the plants to
321 better tolerate the presence of Ag.

322 **Total microbial biomass**. Control biosolids-soil contained significantly higher total
323 microbial PLFA biomass relative to all treatments except 1 mg kg^{-1} PVP-Ag and 10 mg kg^{-1}
324 Ag_2S ENMs (Table 5). Biomass was lower than in the control in the 1 and 100 mg kg^{-1}
325 Ag_2S ENM treatments, although biomass at 10 mg kg^{-1} Ag_2S ENMs was not significantly
326 different than control. Again, the reason for the lack of concentration dependance in
327 response to the Ag_2S ENMs is unclear. In this instance, there was no significant difference in
328 biomass between the three concentrations of Ag_2S and the difference between the 10 mg kg^{-1}
329 Ag_2S ENMs and the control treatment, though significant, is small. In contrast, biomass
330 measured in the PVP-Ag and Ag^+ treatments clearly trended downwards as concentration
331 increased, although this concentration dependence was only significant when comparing 1
332 and 100 mg kg^{-1} treatments.

333 **AM fungal biomarker concentration.** PLFA 16:1ω5c decreased in response to all three
334 treatments (Table 5). Ag_2S ENMs reduced 16:1ω5c concentration at 1 and 100 mg kg^{-1},
335 although, as with total biomass, there was not significant reduction compared to the control in
336 the 10 mg kg^{-1} treatment. PLFA 16:1ω5c reduction was largest at 100 mg kg^{-1} Ag^+ and PVP-
337 Ag. However, these reductions were not significantly different than those those measured in
338 other treatments in which root colonization was not heavily affected (e.g. Ag_2S and Ag^+ 1 mg
339 kg^{-1}; Figure 1), suggesting that the reductions in colonization observed in the 100 mg kg^{-1}
340 Ag^+ and PVP-Ag treatments did not result solely by inhibition of soil AMF populations.

341 NLFA 16:1ω5c was not significantly affected by any treatment (Table 5). Considering
342 previous work suggesting that NLFA 16:1ω5c is more selective for AMF than PLFA
343 16:1ω5c, this result may indicate that the differences detected in measured PLFA 16:1ω5c
344 concentrations are the result of reductions in microbial groups other than AMF that contain

345 PLFA 16:1ω5c, such as gram-negative bacteria (Olsson 1999). Alternatively, NLFA
346 16:1ω5c is thought to be associated with AMF spores whereas PLFA 16:1ω5c is associated
347 with AMF hyphae (Olsson 1995, Olsson 1999). The differences observed in measured
348 NLFA and PLFA 16:1ω5c could indicate that AMF hyphal growth was inhibited by the
349 treatments that impacted PLFA 16:1ω5c.

350

351 Table 5. Total microbial biomass, NLFA 16:1ω5c, and PLFA 16:1ω5c concentrations (nmol
352 g^{-1}). Data presented as mean ± one standard deviation. Treatments with the same
353 superscripts are not significantly different at α=0.05.

354
355

Treatment/Concentration (mg kg^{-1})	Total biomass	NLFA 16:1ω5c	PLFA 16:1ω5c
Control	23.6 ± 2.0^{a}	2.2 ± 0.5^{a}	0.7 ± 0.1^{a}
PVP Ag 1	22.0 ± 1.6^{ab}	2.1 ± 0.6^{a}	0.6 ± 0.1^{ab}
PVP Ag 10	19.8 ± 3.0^{bc}	2.0 ± 0.8^{a}	0.6 ± 0.1^{ab}
PVP Ag 100	17.4 ± 2.0^{c}	1.5 ± 0.7^{a}	0.5 ± 0.1^{cd}
Ag_2S 1	19.3 ± 2.5^{bc}	1.6 ± 0.4^{a}	0.6 ± 0.1^{bc}
Ag_2S 10	22.3 ± 2.8^{ab}	2.2 ± 0.6^{a}	0.7 ± 0.1^{ab}
Ag_2S 100	20.2 ± 3.1^{bc}	1.8 ± 0.5^{a}	0.6 ± 0.1^{bc}
Ag^{+} 1	19.4 ± 1.1^{bc}	1.7 ± 0.6^{a}	0.5 ± 0.0^{bc}
Ag^{+} 10	18.3 ± 2.0^{c}	2.1 ± 0.5^{a}	0.6 ± 0.1^{bc}
Ag^{+} 100	16.9 ± 3.3^{c}	1.3 ± 0.7^{a}	0.4 ± 0.1^{cd}

356

357 **Soil concentrations of microbial classes**. Gram-negative bacteria were significantly
358 reduced relative to the control in the 10 and 100 mg kg^{-1} PVP-Ag, the 1 and 100 Ag_2S ENM,
359 and at all concentrations of Ag^{+} (Table 6). The gram-positive bacteria population was
360 significantly decreased at 100 mg kg^{-1} PVP-Ag and Ag_2S ENMs and at both 10 and 100 mg
361 kg^{-1} Ag^{+}. Actinomycetes were significantly reduced in every treatment. In each of these
362 three microbial classes, inhibition was the largest in the 100 mg kg^{-1} PVP-Ag and Ag^{+}.
363 Fungi were impacted to a lesser degree, but significant decreases in fungal PLFAs were
364 measured in the 100 mg kg^{-1} PVP-Ag and the 1 mg kg^{-1} Ag_2S ENMs.

365

366 Table 6. Concentrations (nmol g^{-1}) of PLFAs indicative of gram-negative bacteria, gram-
367 positive bacteria, actinomycetes and fungi. Data presented as mean ± one standard deviation.
368 Treatments with the same superscripts are not significantly different at α=0.05.

369
370

Treatment/Concentration (mg kg^{-1})	Gram-negative bacteria	Gram-positive bacteria	Actinomycetes	Fungi
Control	5.2 ± 0.5^{a}	5.7 ± 0.5^{a}	1.1 ± 0.1^{a}	0.6 ± 0.1^{ad}
PVP Ag 1	4.7 ± 0.4^{ab}	5.5 ± 0.4^{a}	1.0 ± 0.1^{b}	0.6 ± 0.1^{de}
PVP Ag 10	4.1 ± 0.6^{bc}	4.9 ± 0.8^{ab}	0.8 ± 0.2^{bcde}	0.6 ± 0.1^{cde}
PVP Ag 100	3.8 ± 0.5^{c}	4.3 ± 0.5^{b}	0.7 ± 0.1^{de}	0.5 ± 0.1^{e}
Ag_2S 1	4.3 ± 0.6^{bc}	4.8 ± 0.6^{ab}	0.9 ± 0.1^{bcd}	0.5 ± 0.1^{bce}
Ag_2S 10	4.9 ± 0.8^{ab}	5.5 ± 0.6^{a}	0.9 ± 0.1^{bc}	0.6 ± 0.1^{cde}
Ag_2S 100	4.3 ± 0.6^{bc}	5.0 ± 0.8^{b}	0.8 ± 0.1^{bcde}	0.6 ± 0.2^{cde}
Ag^{+} 1	4.2 ± 0.3^{bc}	4.8 ± 0.3^{ab}	0.8 ± 0.1^{bcde}	0.5 ± 0.1^{bce}
Ag^{+} 10	3.9 ± 0.4^{c}	4.5 ± 0.5^{b}	0.8 ± 0.1^{cde}	0.6 ± 0.1^{cde}
Ag^{+} 100	3.6 ± 0.8^{c}	4.1 ± 0.8^{b}	0.7 ± 0.1^{e}	0.5 ± 0.1^{bc}

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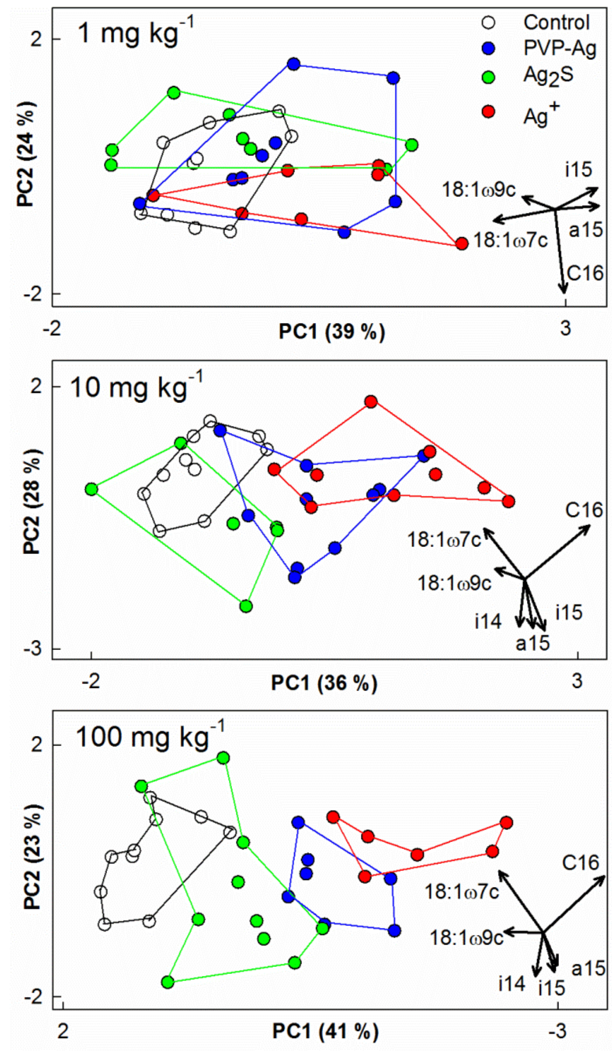
373 **Proportional distribution of microbial classes**. Both PCA and ANOSIM analysis
374 indicated that the microbial community structure was significantly affected by both treatment
375 and exposure concentration (Figure 2; see supporting information Table S1 for details of
376 PCA eigenvectors). For Ag^{+} and PVP-Ag, even the lowest exposure concentration of 1 mg
377 kg^{-1} significantly changed microbial community structure ($R = 0.332$ $p = 0.005$ and $R =$
378 0.213, $p = 0.011$, respectively) whereas for Ag_2S ENMs a significant change in microbial
379 community structure was only detected at 10 mg kg^{-1} ($R = 0.431$, $p = 0.009$). Differences in
380 the microbial communities measured in the treatments relative to the control were largely
381 driven by relative decreases in gram-negative bacteria (18:1ω7c, 18:1ω9c), relative increases
382 in gram-positive (i15, i15, a15) bacteria populations, and relative increases in the general
383 microbial marker, C16.

384



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387 Figure 2. Principle component analysis of the proportional distribution of phospholipid fatty

388 acids (PLFAs) detected within the (top) 1 mg kg^{-1}, (middle) 10 mg kg^{-1} and (bottom) 100 mg

389 kg^{-1} treatments. Vectors with correlations > 0.30 are shown in the lower right-hand corner of

390 each panel.

391

392 Gram-positive bacteria and fungi are typically associated with the rhizosphere and

393 turnover of fresh, plant-derived material, while gram-positive bacteria and actinomycetes are

394 typically associated with the turnover of soil organic (Treonis A. M. 2004, Kramer 2006).

395 The observed change in microbial community structure, with proportional decreases in gram-

396 negative and increases in gram-positive bacteria, potentially suggests a decrease in
397 rhizosphere development or plant inputs with the ENM treatments.

398 **Implications for agroecosystems**. Although little research has examined how a-ENMs
399 will affect beneficial plant-microbe relationahips, this is not the first study examine how
400 sulfidation impacts the toxicity of Ag ENMs. Reinsch et al. (2012) observed that Ag ENM
401 sulfidation dramatically reduced *Escherichia coli* growth inhibition induced by pristine Ag
402 ENMs (Reinsch 2012). Levard et al. (2013) reported that sulfidation of Ag ENMs reduced
403 toxicity to *Danio rerio*, *Fundulus heteroclitus*, *Caenorhabditis elegans,* and *Lemna minuta*.
404 Another recent study examined the effects of Ag_2S ENMs to *Caenorhabditis elegans*
405 reproduction, which is often a more sensitive endpoint than growth or mortality, in an
406 exposure solution (Starnes 2015). The authors found the EC_{10} for reproduction of Ag_2S
407 ENMs to be 802.4 $\mu g\ L^{-1}$ and that the observed toxicity could not be completely explained
408 by the presence of Ag ions alone. However, it is likely that the EC_{10} for *C. elegans*
409 reproduction for Ag_2S ENMs would be much higher in soil.

410 The results reported here add clarification to a recent study by Judy et al. (2015) which
411 reported that PLFA biomarkers associated with AMF were significantly reduced and soil
412 microbial communities significantly affected in biosolids-amended soils spiked with ENMs
413 relative to biosolids-amended soils treated with bulk/ionic metal (Judy 2015). In that study,
414 wastewater treatment plant influent was spiked with either Ag, TiO_2, and ZnO ENMs, spiked
415 with Ag, TiO_2, and ZnO bulk/ionic metal, or unamended with metals (control). PLFA-based
416 soil microbial community analysis indicated that the AMF, gram-negative bacteria, gram-
417 positive bacteria, and fungi populations in the ENM treatment were significantly lower than
418 were present in the bulk metal or control treatments. The authors were unable to deconvolve
419 which ENM or combination of ENMs within the ENM treatment was responsible for this
420 effect, although toxicogenomic analysis suggested that Zn ions were largely responsible

421 (Chen 2015) . The present study provides evidence that Ag_2S ENMs may have contributed to
422 the alteration of the soil microbial community reported in this earlier work.

423 **CONCLUSIONS**

424 This study presents some of the first data elucidating how sulfidation of Ag ENMs affects
425 agronomically beneficial plant-microbial interactions and soil microbial communities. We
426 observed that exposure to Ag_2S a-ENMs, material analogous to that will be discharged into
427 the soil environment, resulted in a greatly different biological response compared to pristine
428 PVP-Ag ENMs, with no phytotoxicity observed as a result of exposure to the Ag_2S a-ENMs.
429 The PVP-Ag and Ag^+ treatments induced a strongly concentration-dependent response in the
430 plants and soil microorganisms, whereas the Ag_2S ENM did not. However, although the
431 Ag_2S a-ENMs were less phytotoxic and had a smaller effect on soil microorganisms than the
432 PVP-Ag and Ag^+, toxic effects were observed in the soil microbial community in response to
433 1 mg kg^{-1} Ag_2S a-ENMs, suggesting that unintended discharge of Ag ENMs into terrestrial
434 ecosystems may pose a risk for soil microbial communities. The difference between the
435 results observed in the PVP-Ag ENM treatment and the Ag_2S ENM treatment highlights the
436 importance of considering ENM life cycle and the conditions presented within various
437 discharge pathways when evaluating the potential environmental risk of ENMs. Critically,
438 the lack of inhibition to mycorrhizal colonization observed at 100 mg kg^{-1} Ag_2S ENMs
439 suggests that the risk posed by Ag_2S ENMs to plant-mycorrhiza symbiosis from unintentional
440 discharge of Ag ENMs into agoecosystems may be minimal. Considering Ag^+ caused greater
441 effects than either of the Ag ENMs, risk assessment of Ag ENM release in biosolids based on
442 experiments examining the toxicity of the soluble Ag^+ ion would be conservative.

443 **Supporting information available.** TEM and XRD analysis of ENMs, light micrographs
444 of stained plant roots, eigenvectors from PLFA analysis.

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