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Effects of biosolids from a wastewater treatment plant receiving manufactured nanomaterials on *Medicago truncatula* and associated soil microbial communities at low nanomaterial concentrations

Chun Chen [a,*], Olga V. Tsyusko [b,*], Dave H. McNear Jr. [b], Jonathan Judy [c], Ricky W. Lewis [b], Jason M. Unrine [b,*]

[a] State Key Laboratory of Arid Region Crop Stress Biology, Northwestern Agriculture and Forestry University, Yangling, Shaanxi 712100, People's Republic of China
[b] Department of Plant and Soil Sciences, University of Kentucky, Lexington, KY 40546, United States
[c] Department of Soil and Water Science, University of Florida, Gainsville, FL 32611, United States

HIGHLIGHTS

- Little toxicity in *Medicago truncatula* from nanomaterial transformation products
- Tissue metal concentrations similar among all treatments
- Differences in microbial community composition between bulk and nano treatment
- Microbial community differences may be result of aging method artifacts.

GRAPHICAL ABSTRACT

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ABSTRACT

Concern has grown regarding engineered nanomaterials (ENMs) entering agricultural soils through the application of biosolids and their possible effects on agroecosystems, even though the ENMs are extensively transformed. The effects of exposure to biosolids containing transformation products of these ENMs at low concentrations remain largely unexplored. We examined the responses of *Medicago truncatula* and its symbiotic rhizobia *Sinorhizobium meliloti* exposed to soil amended with biosolids from WWTP containing low added concentrations of ENMs (ENM Low), bulk/dissolved metals (bulk/dissolved Low), or no metal additions (control). We targeted adding approximately 5 mg/kg of Ag and 50 mg/kg of Zn, and Ti. Measured endpoints included *M. truncatula* growth, nodulation, changes in the expression of stress response genes, uptake of metals (Ag, Zn and Ti) into shoots, and quantification of *S. meliloti* populations and soil microbial communities. After 30 days exposure, no effects on root or shoot biomass were observed in ENM Low and bulk/dissolved Low treatments, whereas both treatments had a larger average number of nodules (5.7 and 5.57, respectively) compared to controls (0.33). There were no significant differences in either total accumulated metal or metal concentrations in shoots among the treatments. Expression of five stress-related genes (metal tolerance protein (MTP), metal transporter (MTR), peroxidase (PEROX), NADPH oxidase (NADPH) and 1-aminocyclopropane-1-carboxylate oxidase-like protein (ACC_Oxidase)) was significantly down-regulated in both bulk/dissolved Low and ENM Low treatments. However, a change in soil microbial community composition and a significant increase in total microbial biomass were observed in ENM Low relative to control. The ENM Low treatment had increased abundance of Gram-negative and anaerobic bacteria and reduced abundance of eukaryotes compared to control. The study demonstrated that

* Corresponding authors at: Department of Plant and Soil Sciences, University of Kentucky, 1100 South Limestone Street, Lexington, KY 40546, United States.
E-mail addresses: chunchen@yahoo.com (C. Chen), olga.tsyusko@uky.edu (O.V. Tsyusko), jason.unrine@uky.edu (J.M. Unrine).

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although there were some subtle shifts in microbial community composition, plant health was minimally impacted by ENMs within the time frame and at the low exposure concentrations used in this study.

1. Introduction

As use of consumer products containing engineered nanomaterials (ENMs) increases, ENMs are likely entering wastewater treatment plants (WWTP) in increasing quantities. Within WWTP, nanoparticles are largely partitioned into sewage sludge (Mueller and Nowack, 2008). Treated sewage sludge is often applied to agricultural lands as biosolids in many parts of the world (Unrine et al., 2010). Recently, concerns have been raised about the potential for adverse impacts of ENMs in biosolids on agroecosystems (Judy and Bertsch, 2014). Current national regulations for amendment of soils with biosolids, such as 40 CFR part 503 in the United States, do not specifically address ENMs as a potential contaminant of concern (Hanson et al., 2011; USEPA).

Manufactured Ag, TiO_2 and ZnO ENMs are the most widely used types of metal-based ENMs in consumer products (Barton et al., 2015; Bondarenko et al., 2013). Research on the impacts of these ENMs introduced to terrestrial ecosystems have generally investigated the effects of these materials on single species such as plants or on soil microorganisms in isolation from each other. To date little is known on how ENMs influence plants and soil microbial community interactions. However, examining the effects of ENMs, with a focus on interactions among organisms in soil communities, provides more information on ecological consequences than single-species assessments (Colman et al., 2013; McKee and Filser, 2016). A handful of studies have been conducted with respect to the interactions between ENMs and plant-microbial systems. However, some of these experiments were still performed using as-manufactured ENMs introduced directly into hydroponic solution (Fan et al., 2014; Huang et al., 2014) or soil (Bandyopadhyay et al., 2015; Priester et al., 2012). More recent studies regarding the fate of ENMs in WWTP have demonstrated that, in biosolids or biosolids-amended soils, the majority of the as synthesized ENMs have undergone a range of physical and chemical transformations modifying their original properties (Impellitteri et al., 2013; Kaegi et al., 2011; Lombi et al., 2012; Ma et al., 2014; Pradas del Real et al., 2016). Thus, the toxicity predicted for as-manufactured Ag, TiO_2, and ZnO ENMs may not be reliable or valid for these transformed ENMs in the environment (Levard et al., 2012; Rathnayake et al., 2014; Starnes et al., 2016) and uncertainties exist how the accumulation of environmentally transformed ENMs in soils will interfere with plant-microbial systems.

In our previous studies, we reported adverse effects of soil amended with biosolids generated at a pilot WWTP receiving influent with a mixture of Ag, TiO_2 and ZnO ENMs on the *Medicago truncatula-Sinorhizobium meliloti* symbiosis and associated effects on soil microbial community composition. The biosolid-amended soils containing transformed ENMs led to a significant decrease in microbial biomass, plant biomass, and root nodulation as well as perturbation of the microbial community and an increase in Zn uptake and Zn concentrations in shoots compared to the bulk/dissolved metal treatment (Chen et al., 2015; Judy et al., 2015b). This difference was observed despite the fact that the speciation of the metals, as measured by X-ray absorption spectroscopy (XAS), was similar across treatments. Moreover, our transcriptomic analysis suggested that these differences in toxicity were due to enhanced bioavailability of Zn in the ENM treatment. These studies, which used exposures at the legal limit in the U.S. for long-term loading of Zn in biosolids, suggest that the current regulatory limits may not be protective when Zn is primarily introduced as ENMs.

Our previous works represented a worst-case exposure scenario based on the cumulative metals loading after 20 years of biosolids application assuming the majority of added metals are ENMs. Further work is necessary to determine possible adverse effects where input of ENMs relative to other forms of metals is comparatively small. This is the current exposure scenario based on recent estimates (Sun et al., 2016; Sun et al., 2014). In this study, we conducted a pot experiment with *M. truncatula* grown for 30 days in biosolids containing ENMs (Ag, TiO_2 and ZnO) or dissolved/bulk metals at low metal concentrations, or in a control treatment with no added metals. The added concentrations of metals in the low ENM and dissolved/bulk treatments were similar to recent predictions of concentrations of ENMs in biosolids (Gottschalk et al., 2015; Gottschalk et al., 2009; Keller and Lazareva, 2013; Sun et al., 2015), and are generally less than the background concentrations of these metals in the control biosolids.

The aims of this study were: 1) to characterize the physiological responses and metal bioaccumulation for *M. truncatula* grown in biosolid-amended soil at low added concentrations of ENMs (ENM Low) or bulk/dissolved metals (bulk/dissolved Low); (2) determine potential changes in soil *S. meliloti* populations or soil microbial community composition; (3) monitor mRNA levels of selected stress-response genes that responded to the ENM treatment in our previous study, including metal tolerance proteins (MTP), metal transporter (MTR), peroxidase (PEROX), NADPH oxidase (NADPH) and 1-aminocyclopropane-1-carboxylate oxidase-like protein (ACC_Oxidase).

2. Materials and methods

2.1. Biosolids-amended soil preparation

Three types of sewage sludge biosolids spiked with (i) TiO_2, ZnO and Ag ENMs mixture (ENM), (ii) bulk/ionic TiO_2, ZnO and $AgNO_3$ (bulk/dissolved metal), or (iii) no metal added (control) were generated at a pilot WWTP facility at Cranfield University. Details of the pilot scale WWTP process (e.g., the influent wastewater properties, the characterization and dosage of ENMS used, metal concentrations in the influent or effluent, sludge production and properties, and the biosolids processing, etc.) have been reported previously (Ma et al., 2014). The biosolids were mixed at a 1:1 mass ratio with Woburn soil (loamy sand) and aged in outdoor lysimeters for six months as described previously (Judy et al., 2015b). These soils/biosolids mixtures were stored at 4 °C prior to use. In this study, to achieve the relatively low metal concentrations, the 1:1 biosolids/soil mixtures from both ENM and dissolved/bulk treatments were diluted 20-fold with the control 1:1 biosolids/soil mixtures. We targeted adding approximately 5 mg/kg of Ag and 50 mg/kg of Zn, and Ti. While models make varying predictions of current concentrations of ENMs in sewage sludge, these concentrations are within the range of various model predictions (Gottschalk et al., 2015; Gottschalk et al., 2009; Keller and Lazareva, 2013; Sun et al., 2015). While a 1:1 ratio of soil to biosolids is high, we chose it because it is a worst case assumption used in the U.S. EPA risk assessment for land application of biosolids (USEPA, 1995). Further, in many cases biosolids may be broadcast onto the soil surface without tillage to mix them into a large volume of soil, such as in pasture or forest lands, or in no-till agriculture. The relevant ratio of soil to biosolids will likely vary widely depending on application method, agronomic practices and target species.

2.2. Exposures of Medicago truncatula

For each treatment, five pots were prepared and seeded with six pre-germinated seedlings per pot (Garcia et al., 2006), and randomly placed in a plant growth chamber with a 14 h light/10 h dark

photoperiod at 20 °C and 70% relative humidity, 135 ± 3 μmol photon m^{-2} s^{-1} illumination. Relative humidity was held constant throughout the experiment using a humidistat. Each plant was then inoculated with 1 mL of a washed suspension of *Sinorhizobium meliloti* Rm2011 in sterile DI water (OD_{600} = 0.8). Full details of the *M. truncatula* (wild-type A17) seed germination, *S. meliloti* culture preparation, seedling transplantation, and the pot experiment preparation were reported in our previous study (Judy et al., 2015b). Plants were grown for 30 days and subsequently harvested, and divided into shoots and roots. The pooled roots of six plants per pot were rinsed with deionized water and then scanned, then the number of nodules and root parameters (e.g., root average diameter and total surface area, etc.) were recorded by WinRhizo Pro (Regent Instruments, Quebec, Canada). Morphological parameters, such as shoot length, number of leaves, and the fresh mass of shoot and root tissues were also measured. Later, the roots with the nodules were dried with paper towels, flash frozen in liquid nitrogen, and stored at −80 °C prior to RNA extraction. Dry mass of shoots was recorded after drying the samples at 60 °C for 48 h. Metal (Ag, Zn and Ti) concentrations in dry shoot samples were measured after digestion using ICP-MS (Judy et al., 2015b). For quality control, we included standard reference materials (SRM 2781 - domestic sludge and SRM 2709 San Joaquin soil - National Institute of Standards and Technology, Gaithersburg, MD, USA), spike recovery samples, use of traceable calibration standards and reagent blanks. Primary calibration standards were verified by comparison to traceable standards from an independent lot number. Since there is was no available plant SRM with Ti and Ag, we analyzed *M. truncatula* tissue spiked with known masses of TiO_2, Ag and ZnO nanomaterials and measured recovery. Results were only accepted if spike recovery was 85–105% and the calibration verification was within 10% of the expected value. The mass of total accumulated metals in shoot tissues was also calculated. Metal concentrations in root tissues were not determined because the difficulty removing soil particles adhered to the roots surface, even with repeated washing, can lead to erroneous measurements (Watson et al., 2015).

2.3. Soil microbial community structure – phospholipid fatty acid (PLFA) analysis

After plants were harvested, soil from each pot was homogenized, divided into small aliquots and frozen at −80 °C. Soils were then lyophilized and thereafter stored at −20 °C until PLFA extraction. Fatty acids were extracted from samples and derivatized to form fatty acid methyl esters (FAME) following the high-throughput method described previously (Buyer and Sasser, 2012), and subsequently analyzed using gas chromatography-flame ionization detection (Microbial Identification System Inc., Newark, DE). The full details of the fatty acid extraction and analysis methods were described previously (see the Supporting Information) (Judy et al., 2015b). Biomarkers for major microbial groups were calculated by summing FAMEs as follows: Gram-positive bacteria (iso and anteiso branched), Gram-negative bacteria (monounsaturated), actinobacteria (10-methyl fatty acids), fungi (18:2 ω6c), protists (20:3 ω5c and 20:4 ω6c) and arbuscular mycorrhizae (16:1 ω5c), and total PLFA. Total microbial biomass was calculated by summing the mass of all of the PLFAs detected within each sample.

2.4. Quantification of Sinorhizobium meliloti

A modified real-time polymerase chain reaction (RT-PCR) method was used to quantify the amount of *S. meliloti* in soils (Judy et al., 2015b; Trabelsi et al., 2009). Standard calibration curves were generated for each treatment using cycle threshold (Ct) values for nodulation-specific gene *nod*C and corresponding log colony forming units (CFUs) g^{-1} dry soil. For this purpose, S. *meliloti* Rm2011 grown to logarithmic-phase (OD_{600} = 1.60) in TY medium was prepared as a stock solution for the sample spike. One hundred mg of soil samples from three treatments were spiked with the 100 μL of *S. meliloti* Rm2011 serial dilutions that ranged from 8.73×10^6 to 8.73×10^9 CFU mL^{-1}. Following the manufacturer's protocol, the MoBio PowerSoil DNA isolation kit was used to extract total DNA from samples, including the spiked, unspiked and post-experimental test soil samples. DNA concentrations and quality were measured using a Varian Cary 50 UV/Visible spectrophotometer (Agilent). Primers *nod*C-F (5′-GCCGCTATCTCAATCTACGC-3′) and *nod*C-R (5′-TTGAAGCTGGGGACGAT-AAC-3′) were used to amplify *nod*C gene according to the method of Trabelsi et al. (2009). The total amounts of *S. meliloti* in the post-experimental samples were measured in CFU g^{-1} units using the calibration curves.

2.5. RNA extraction and quantitative real-time PCR (qRT-PCR)

Five stress response genes, linked to response to metals and oxidative stress, including metal tolerance proteins (MTP), metal transporter (MTR), peroxidase (PEROX), NADPH oxidase (NADPH) and 1-aminocyclopropane-1-carboxylate oxidase-like protein (ACC_Oxidase) were selected from the top 10 up-regulated differentially expressed genes from our previous microarray data from *M. truncatula* grown in biosolids containing TiO_2, Ag and ZnO ENM transformation products (Chen et al., 2015). MTP and MTR genes are involved in metal binding, transport, and storage, especially for Zn homeostasis and tolerance to Zn excess in *M. truncatula* (Montanini et al., 2007; Ricachenevsky et al., 2013). The PEROX gene is known to respond to oxidative stress induced by metals and metals-based ENMs toxicity by metabolizing H_2O_2 (Kaveh et al., 2013). NADPH is known to prevent oxidative stress and is also involved in symbiotic nodule function (Marino et al., 2011). RNA extractions were conducted on the pooled roots of six plants per pot. All five replicate pots were selected from each treatment for the extraction. Total RNA was extracted, quantified and evaluated for purity following the protocol described previously (Chen et al., 2015). Eight hundred ng of total RNA was used for cDNA synthesis with high-capacity cDNA reverse transcription kit (Applied Biosystems). Primer sequences, probe sequences, amplicon sizes and amplification efficiencies have been presented previously (Chen et al., 2015). Actin 2 was selected as a reference gene because its expression showed stability in our previous study (Chen et al., 2015).

2.6. Statistical analysis

Biomass, shoot length, bioaccumulation, and PLFA concentration were analyzed by univariate analysis and pairwise comparisons using SAS. For normally distributed and homogenously varied data, one –way ANOVA was utilized with the Student-Newman-Keuls procedure being used for post hoc multiple comparisons. For non-normal data, a Kruskal-Wallis test and pairwise Mann-Whitney *U* tests were used. Statistical methods for multivariate analysis of PFLA data are found in the supplementary information.

3. Results and discussion

3.1. Soil characteristics

While Ti concentrations determined by ICP-MS were similar among treatments, total Ag and Zn concentrations were significantly ($p < 0.05$) higher in the bulk/dissolved Low or ENM Low versus control treatments (Table 1). Both ENM Low and bulk/dissolved Low treatments concentrations for all three metals were not significantly different. Recovery of Ti, Zn and Ag in SRM 2781 (domestic sludge) was 92.1% ± 0.4%, 100% ± 1.7% and 96.2% ± 0.2%, respectively. Recovery of Ti, Zn and Ag in SRM 2709 (San Joaquin Soil) was 91.3% ± 1.9%, 92.8% ± 1.7% and 61.0% ± 1.72%, respectively. Lower than expected recovery in SRM 2709 is due to the extremely low concentration of Ag in the SRM (0.4 mg/kg) which is close to the method detection limit. The major anion concentrations for control, bulk/dissolved Low, and ENM Low treatments were similar, except for NH_4^+ concentrations. The NH_4^+ concentrations in

Table 1
Characterization data of biosolid-amended soils. Concentrations expressed as mean ± one standard deviation from $n = 3$ for pH, NH_4^+ and anions, $n = 15$ for Zn, Ag and Ti. Values with the different lowercase letters are significantly different from each other ($p < 0.05$). Control = soil amended with control biosolids; Bulk/dissolved Low = soil amended with biosolids containing bulk/dissolved metal at low dose of metals; ENM Low = soil amended with biosolids containing engineered nanomaterials at low dose of metals; BDL, below method detection limit. We targeted adding approximately 5 mg/kg of Ag, 50 mg/kg of Zn, and Ti.

	Control	Bulk/dissolved Low	ENM Low
pH	6.5 ± 0.02[a]	6.7 ± 0.02[b]	6.7 ± 0.05[b]
F^- (mg/kg)	BDL	BDL	BDL
Cl^- (mg/kg)	34.7 ± 1.8[a]	36.3 ± 4.1[a]	29.9 ± 0.9[a]
SO_4^{2-} (mg/kg)	587.4 ± 63.2[a]	607.3 ± 46.0[a]	524.8 ± 11.7[a]
NO_3^- (mg/kg)	BDL	BDL	BDL
PO_4^{3-} (mg/kg)	99.5 ± 6.3[a]	101.8 ± 2.9[a]	100.4 ± 1.5[a]
NH_4^+ (mg/kg)	185.7 ± 2.6[a]	176.1 ± 0.2[b]	161.5 ± 0.9[c]
Zn (mg/kg)	335.2 ± 35.2[a]	389.8 ± 34.1[b]	375.4 ± 51.7[b]
Ag (mg/kg)	1.7 ± 0.3[a]	6.2 ± 0.8[b]	6.4 ± 1.0[b]
Ti (mg/kg)	1180.4 ± 32.7[a]	1194.4 ± 70.6[a]	1261.9 ± 124.3[a]

both the bulk/dissolved Low and ENM Low treatments were significantly less than the control, and the ENM low treatment was significantly less than the bulk/dissolved Low treatments. Similarly, soil pH was lower in the control soils compared to the bulk/dissolved Low and ENM Low treatments where the pH was similar. The speciation of the metals in the soil biosolids mixtures, according to X-ray absorption spectroscopy, does not differ by treatment. Zn existed primarily as ZnS, $Zn_3(PO_4)_2$, carboxyl coordinated Zn and Zn bound to silicates, while Ag existed entirely as Ag_2S (Judy et al., 2015b; Ma et al., 2014). It is likely that toxicity of these transformation products, which have low solubility, is lower than exposure to pristine nanomaterials (Starnes et al., 2016; Rathnayake et al., 2014).

3.2. Metal uptake and accumulation

Even though the ENM and Bulk/dissolved Low soils had significantly higher Zn and Ag concentrations than the control treatment, no significant differences in Ag, Zn and Ti concentrations in shoot tissues among the three treatments were detected in this study (Fig. 1). After 30 days of exposure, the Zn mean concentration in *M. truncatula* shoot tissues did not exceed 60 mg/kg dry weight in any of the treatments (Fig. 1), nor did they significantly differ among treatments. Recovery of Ti, Zn and Ag in our spiked plant tissue was 97.1% ± 6.4%, 114.4% ± 5.9% and 97.2% ± 2.7%, respectively. We previously observed that when plants were exposed to the biosolids at higher metal concentrations, plants in the ENM treatment had Zn concentrations that were approximately 30% higher than for the bulk/dissolved metal treatment, despite the fact that the XAS-determined coordination environment (speciation) of the metals was similar (Judy et al., 2015b).

3.3. Medicago truncatula and Sinorhizobium meliloti responses

There were no significant differences in plant biomass (fresh or dried shoot and root) or shoot length for the bulk/dissolved Low and ENM Low treatments relative to the control (Table 2). Fresh shoot biomass and shoot length in the bulk/dissolved Low were significantly greater than the ENM Low treatments as was root surface area. Notably, although not significant, the ENM Low treatment consistently had less, while the bulk/dissolved Low treatment had greater plant growth and developmental responses relative to the control (Table 2). We have previously observed phytotoxicity when where the shoot tissues from the ENM treatments contained 182 mg Zn kg^{-1} dry mass, but not in control or bulk/dissolved treatments which contained 63 mg/kg and 103 mg/kg, respectively. Also, the phytotoxicity threshold (PT10, the shoot metal concentration corresponding to a 10% plant biomass reduction) for alfalfa (*Medicago sativa*), a closely related species, has been previously determined to be 200 mg Zn kg^{-1} dry mass (Baran, 2013). Therefore, it is not surprising that we did not observe phytotoxicity given the tissue concentrations of Zn which were observed.

There was no significant difference in the number of nodules per plant between bulk/dissolved Low and ENM Low treatments, although there were significantly fewer nodules on control plants compared to the other two treatments (Table 2). We previously observed that high concentrations of added ENMs inhibit nodulation, while high added concentrations of bulk/dissolved metals did not (Judy et al., 2015a, 2015b). Several hydroponic studies of ZnO (Huang et al., 2014) and TiO_2 (Fan et al., 2014) show that high exposure concentrations affect early plant–rhizobia interactions, interfering with nodule development and subsequently delaying the onset of nitrogen fixation. This has also been observed in soybean (*Glycine* max) in soil not amended with biosolids (Priester et al., 2012). Lower nodule numbers in the control in the present study was not due to reduced abundance of *S. meliloti* in soil. On the contrary, the number of *S. meliloti* colony forming units (CFU) were similar between control and bulk/dissolved Low treatments, both of which were about two times greater than in ENM Low treatment; although the ENM low and bulk/dissolved Low treatments were not significantly different (Table 3). The significantly greater NH_4^+ concentrations in the control treatment compared to the bulk/dissolved and ENM Low treatments might partially explain the reduction in nodulation, as it appears nodulation may be attenuated by relatively high or low concentrations of NH_4^+ in *M. truncatula* (Fei and Vessey, 2009). Additionally, we also observed large shifts in microbial community composition and function that may explain the lack of nodulation.

3.4. Soil microbial community

The nonmetric multidimensional scaling (NMDS) ordination for microbial biomarker groups in the control, bulk/dissolved Low and ENM Low soils produced a two-dimensional solution with a final stress of 3.66 after 41 iterations (Fig. 2). The NMDS ordinations showed a clear separation in microbial communities in the control, bulk/dissolved Low and ENM Low soils along axis 1, which explained 93% of the variation. Multi-response permutation procedure (MRPP) analysis showed that the microbial community structure in ENM Low and bulk/dissolved Low treatments were different at a significance of $p = 0.051$ ($A = 0.149$), and both were significantly different from the control (ENM Low vs control, $A = 0.4273$, $p = 0.002$; bulk/dissolved Low vs control $A = 0.2327$, $p = 0.009$). The bioplot rays in Fig. 2 show which of the microbial biomarker groups best explains the separation between the treatments. For example, the fungus to bacteria ratio (F:B) was significantly greater in the control soils due to significantly lesser total bacterial biomass (TBB). Total microbial biomass in the ENM Low treatment was significantly greater than the control, whereas there was no significant difference between bulk/dissolved Low and control treatments. (Table 4). There was no significant difference in total microbial biomass between ENM Low and bulk/dissolved Low treatments, though ENM Low treatment had greater concentrations of Gram-negative (G^-) and anaerobic bacteria.

Previous reports have shown that some as-synthesized metal-based ENMs may change the soil microbial community (Frenk et al., 2013; Ge et al., 2011; Xu et al., 2015), however, only a few studies have examined the influence of transformed ENMs under realistic exposure scenarios. Colman et al. (2013) conducted a mesocosm experiment examining the ecosystem response to Ag ENMs by using biosolids spiked with Ag ENMs at environmentally relevant concentrations (0.14 mg Ag kg^{-1} soil). They found that at the end of the experiment (50 days) Ag ENMs were readily transformed and their sulfidized product significantly reduced microbial biomass relative to the control or $AgNO_3$ treatments. Changes in soil enzyme activities and increased emission of N_2O from the soil were also observed in the Ag ENMs treatment relative to control or $AgNO_3$. In addition, the Ag ENMs treatment resulted in a significant difference in microbial community composition compared to the control one-day post-dosing, but community composition of the Ag ENMs

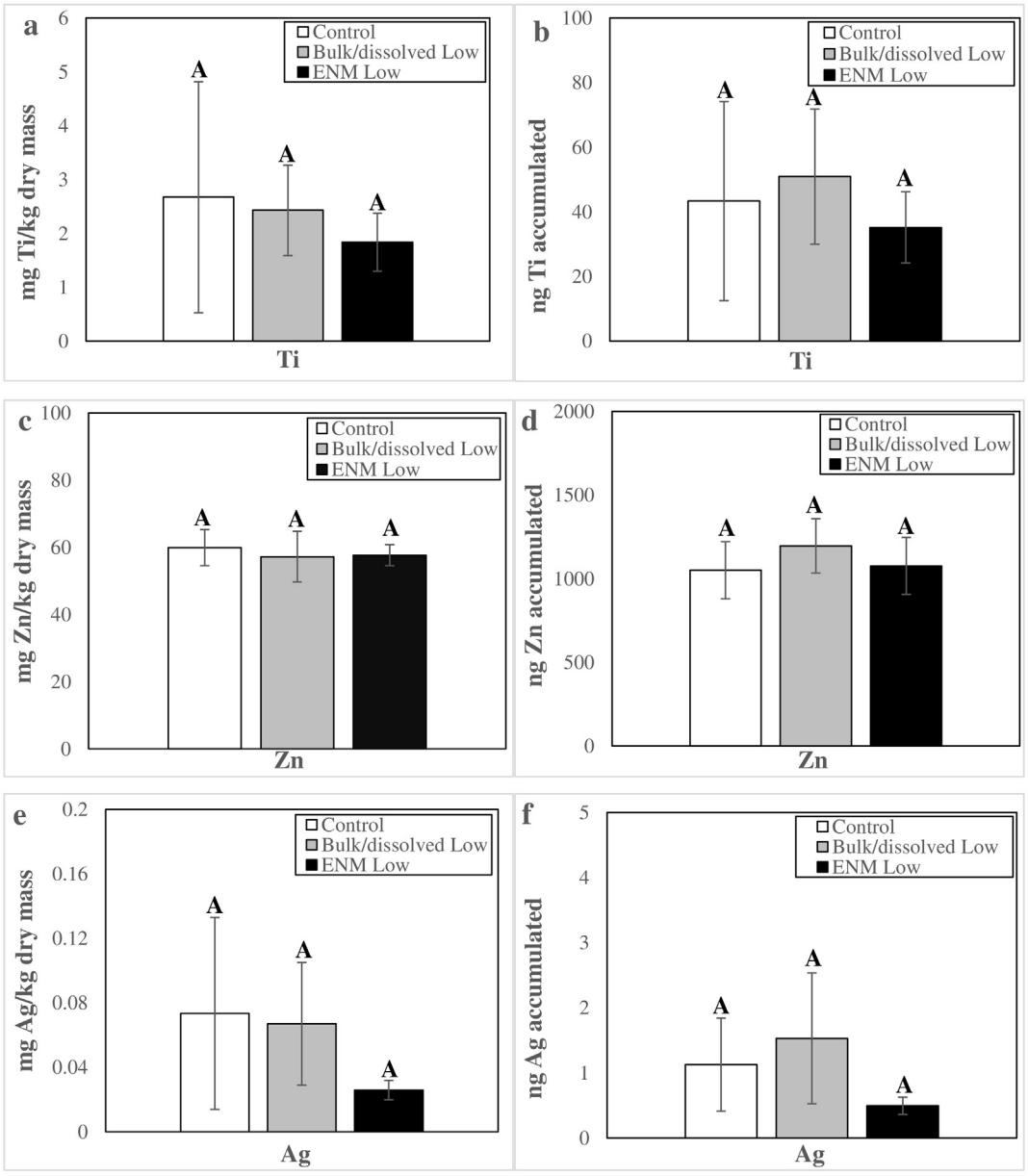

Fig. 1. Concentrations (panels, a, c, e) of Ti (a), Zn (c), and Ag (e) in *Medicago truncatula* shoot tissues and uptake of metals expressed as ng accumulated (panels, b, d, f) of Ti (b), Zn (d), and Ag (f). All error bars are standard error of the mean ($n = 10$), means with the same letter are not significantly different $p < 0.05$, Bulk/dissolved Low, soil amended with biosolids containing bulk/dissolved metal at low dose of metals; ENM Low, soil amended with biosolids containing engineered nanomaterials at low dose of metals.

treatment converged with the control after 50 days. A recent laboratory study showed Ag_2S ENMs significantly reduced the total microbial biomass at Ag concentrations of 1 and 100 mg/kg compared to control, whereas a significant change in the microbial community structure was only observed at 10 mg/kg (Judy et al., 2015a). The same soil-sludge/biosolids mixtures as in our previous study were recently examined by other authors from our research consortium, who observed a minimal difference in soil microbial community response in soils treated with sludge/biosolids enriched with either ENMs or dissolved/bulk metal salts (Durenkamp et al., 2016). Key differences between our studies and the Durenkamp et al. (2016) study was that our soil/biosolid mix was used as a plant growth medium and received *S. meliloti* inoculum,

Table 2
Growth and development responses of *Medicago truncatula* grown for 30 days. Each value represents mean ± standard deviation. Values with the different lowercase letters are significantly different from each other ($p < 0.05$).

	Control	Bulk/dissolved Low	ENM Low
Fresh shoot biomass (mg)	101.2 ± 34.6^{ab} ($n = 29$)	113.7 ± 30.8^{b} ($n = 29$)	96.2 ± 26.5^{a} ($n = 30$)
Dried shoot biomass (mg)	12.5 ± 5.0^{a} ($n = 14$)	14.0 ± 4.5^{a} ($n = 14$)	11.3 ± 3.8^{a} ($n = 15$)
Fresh root biomass (mg)	405.6 ± 88.4^{a} ($n = 5$)	475.9 ± 95.7^{a} ($n = 5$)	395.1 ± 73.7^{a} ($n = 5$)
Shoot length (cm)	7.5 ± 1.0^{ab} ($n = 14$)	7.8 ± 0.9^{b} ($n = 14$)	7.2 ± 1.1^{a} ($n = 15$)
Nodules per plant	0.3 ± 0.2^{a} ($n = 5$)	5.7 ± 0.8^{b} ($n = 5$)	5.6 ± 1.3^{b} ($n = 5$)
Root surface area (cm^2)	41.5 ± 7.9^{ab} ($n = 5$)	46.4 ± 8.5^{b} ($n = 5$)	35.7 ± 6.6^{a} ($n = 5$)
Average root diameter	0.27 ± 0.011^{a} ($n = 5$)	0.28 ± 0.007^{a} ($n = 5$)	0.29 ± 0.006^{a} ($n = 5$)

Table 3
Real time PCR estimates of *Sinorhizobium meliloti* quantity in biosolid-amended soils ($n = 4$). Values with the different lowercase letters are significantly different from each other ($p < 0.05$). Ct = threshold cycle; CFU = colony forming unit.

Samples	Ct values		CFUs·g^{-1} soil	
	Mean	STDEV	Mean	STDEV
Control	24.47	0.35	2.29E+08^{a}	5.25E+07
Bulk/dissolved Low	25.12	0.21	1.71E+08^{ab}	2.13E+07
ENM Low	23.54	0.52	1.00E+08^{b}	4.50E+07

rather than being analyzed directly from storage. Plant-microbial interactions may have altered the observed responses. In our previous study, we found a significant reduction in total microbial biomass and distinctive changes in microbial community structure in soils treated with biosolids containing high doses of Ag, TiO_2, and ZnO ENMs (Judy et al., 2015b).

A notable difference between our previous and current studies is the four-fold reduction in total microbial biomass in controls despite the fact that the same soil-biosolids mixture was used (SI Table S1). Soil chemical properties, including pH values and anions, were similar in the controls in both studies except for the total Zn concentration, which was lower in the control treatment in this study (SI Table S2). The most likely cause for this change in microbial community composition (SI Fig. S1) was storage of the high-dose biosolids at 4 °C for six months (from October 2013 to March 2014) prior to preparing the low dose treatments for this study. We did not examine microbial communities in the high-dose biosolid treatments after the storage prior to preparing the low dose treatments for this study, so we are uncertain how the microbial community might have changed prior to exposure. Storage of soil samples at 4 °C has been shown to affect the soil microbial community by shifting community composition and reducing microbial biomass (Haldeman et al., 1995; Klammer et al., 2005; Ross, 1991). While we did not determine

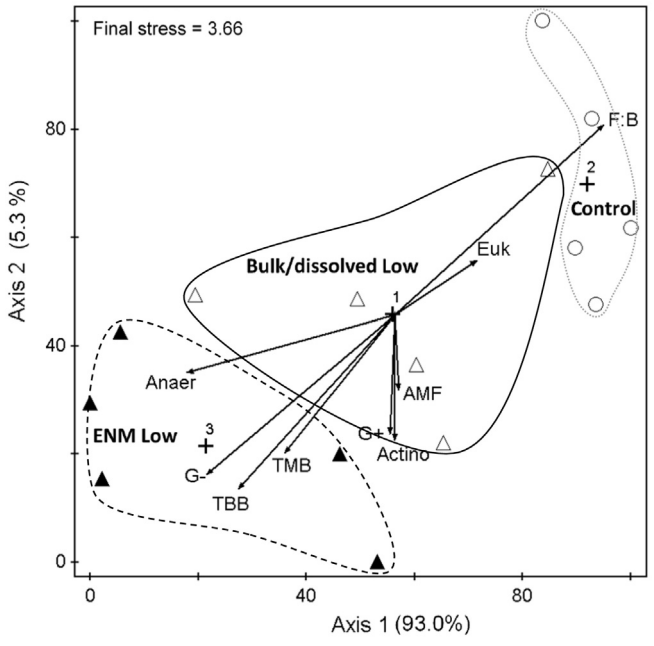

Fig. 2. PC-ORD non-metric multidimensional scaling (NMDS) ordination plot for the relationship between microbial communities in control, Bulk/dissolved Low and ENM Low treatments. Biplot correlation vectors of the concentration of fatty acids in diagnostic microbial biomarker groups with an $r^2 > 0.300$ were included. TBB = total microbial biomass; TFB = total fungal biomass; TBB = total bacterial biomass; F:B = fungus to bacteria ratio; Anaer = anaerobic bacteria; G^+ = Gram positive bacteria; G^- = Gram negative bacteria; AMF = Arbuscular Mycorrhizal Fungi; Actino = actinomycetes; Euk = eukaryotes. Final stress value reflects how well the ordination summarizes the observed distances among the samples.

Table 4
Concentrations (mean ± standard deviation; nmol g^{-1}, $n = 5$) of PLFA biomarker groups of the soil microbial community in control, bulk/dissolved Low and ENM Low treatments. Values with the different lowercase letters are significantly different from each other ($p < 0.05$). PLFA = phospholipid fatty acid.

Microbial group	Control	Bulk/dissolved Low	ENM Low
Gram positive bacteria	39.7 ± 4.7^{a}	39.3 ± 4.5^{a}	42.6 ± 3.1^{a}
Gram negative bacteria	116.0 ± 9.8^{a}	132.5 ± 13.9^{b}	152.0 ± 9.4^{c}
Anaerobic bacteria	3.6 ± 0.3^{a}	4.8 ± 0.8^{a}	8.5 ± 3.8^{b}
Actinomycetes	22.8 ± 1.4^{a}	24.2 ± 2.5^{a}	24.4 ± 1.7^{a}
Fungi	31.9 ± 3.0^{a}	31.0 ± 2.3^{a}	30.7 ± 2.3^{a}
AM-fungi	13.6 ± 1.7^{a}	14.0 ± 1.7^{a}	13.9 ± 1.0^{a}
Eukaryotes	29.2 ± 3.8^{a}	25.3 ± 1.8^{b}	24.6 ± 1.8^{b}
Total bacteria biomass	182.1 ± 15.7^{a}	200.8 ± 19.0^{a}	227.5 ± 13.7^{b}
Total fungus biomass	45.5 ± 3.9^{a}	45.0 ± 3.9^{a}	44.6 ± 22.9^{a}
Ratio of fungus to bacteria	0.25 ± 0.02^{a}	0.22 ± 0.01^{b}	0.20 ± 0.01^{c}
Total microbial biomass	256.8 ± 22.6^{a}	271.1 ± 21.8^{ab}	296.8 ± 17.2^{b}

microbial community structure until after the plants were grown and harvested, there were still treatment-specific differences even at the mixing rates used in this study to make the low-dose treatments (5% ENM or bulk/dissolved soil-biosolids to 95% control soil-biosolids) (Fig. 2 and Table 4). The near lack of nodules in the control treatment in this study may therefore be related to alterations in microbial community composition that occurred during storage leading to changes in community level interactions, and/or function. It could be that the metals present during storage of the high concentration biosolids may have resulted in a microbial community more favorable to nodulation. While we lack direct evidence for this hypothesis, our results do show that the control soils had less of G^- bacteria and total microbial biomass, and a greater fungus to bacteria ratio than the ENM Low and bulk/dissolved Low treatments (Fig. 2). However, further studies are necessary to confirm this hypothesis and to clarify the soil microbial community interactions that may have influenced nodulation, including those that might have influenced *S. meliloti* physiology. Regardless, presence of nodules on plants in the bulk/dissolved Low and ENM Low treatments indicates that low concentrations of ENMs containing biosolids do not inhibit nodulation.

3.5. Plant mRNA levels of stress response genes

Measurement of mRNA levels indicated that all five stress-response genes were significantly down-regulated in ENM Low and bulk/

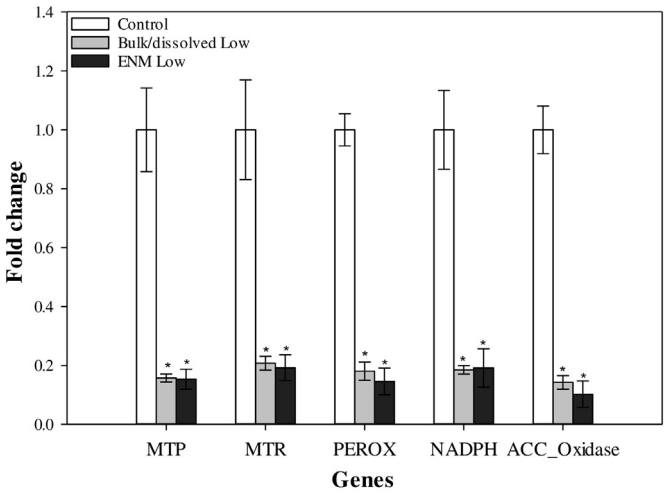

Fig. 3. Expression level of five representative genes in the root tissues from *Medicago truncatula* after 30 days of exposure. Data are presented as Mean ± standard error of $n = 5$ individual pots. *$p < 0.05$. MTP: metal tolerance protein; MTR: metal transporter; PEROX: peroxidase; NADPH: NADPH Oxidase; ACC_Oxidase: 1-aminocyclopropane-1-carboxylate oxidase-like protein.

dissolved Low exposed root tissues versus control (Fig. 3). One of these genes is 1-aminocyclopropane-1-carboxylic acid (ACC)-oxidase, involved in metabolizing ACC into ethylene, a hormone which may increase in response to various type of stress (Glick, 2006). In our previous study, where the plants were exposed to high concentrations of ENMs, this gene was up-regulated >60-fold (Chen et al., 2015). It is interesting that exposure to low concentrations of both bulk/dissolved metal and ENM resulted in down-regulation of this gene, suggesting that the plants were less stressed in in both bulk/dissolved Low and ENM Low treatments. Two other genes involved in Zn homeostasis and Zn excess in *M. truncatula* are MTP and MTR (Becher et al., 2004; Gaitan-Solis et al., 2015), which were highly up-regulated in the ENM treatment in our previous work (Chen et al., 2015). In contrast, again these two genes were significantly down-regulated in roots after exposure to ENM or bulk/dissolved Low treatments, which contained a relatively low concentration of Zn in shoots (about 60 mg/kg dry weight) lower than plants exposed to the previous high ENM treatment (182 mg/kg). The expression of the last two genes, PEROX and NADPH, encoding two proteins involved in oxidative stress and antioxidant defense, were down-regulated in the nodulated roots from ENM Low and bulk/dissolved Low treatments, whereas these two genes showed significant up-regulation in the non-nodulated roots from high ENM treatment in our previous work. It has been shown that nodule formation is accompanied by down-regulation of antioxidant proteins, included Mn-superoxide dismutase, peroxidase and others, perhaps as a part of a lowering of plant defenses to aid symbiosis (Brechenimacher et al., 2008; Kouchi et al., 2004; Lim et al., 2010), which may explain the different patterns of gene expression for PEROX and NADPH in our current and previous studies. Overall, the observed down-regulation in all five stress response genes further confirms that the plants exposed to bulk/dissolved Low or ENM Low treatments were less stressed than the controls. It is possible that mRNA levels of these stress-response genes are lower in the bulk/dissolved and ENM treated plants relative to control because they were nodulated and the control plants were not (El Yahyaoui et al., 2004).

4. Conclusions

The present study attempted to address whether biosolids containing aged ENMs at relatively low concentrations (ratio of nano-derived metals to background metals) would cause adverse effects on the legume-rhizobia symbiosis and soil microbial communities. Our previous research found that although transformed ENMs in biosolids likely have lower toxicity than corresponding pristine materials (Ma et al., 2014; Rathnayake et al., 2014; Starnes et al., 2016), biosolids from WWTPs receiving nanomaterials were still more toxic than those receiving bulk dissolve metals (Chen et al., 2015; Judy et al., 2015b). However, our previous research was conducted at high metal concentrations representing a worst-case exposure scenario. In the present study conducted at more environmentally realistic concentrations, based on current estimates of loading of ENMs to WWTPs, we found very little difference in the chemical soil properties, and in the apparent health of the plants (based on biomass production) among the treatments. However, the microbial community structure in the soils was significantly different between the control and each of the two metal treatments, which could have possibly been due to changes occurred during storage of biosolids/soil mixtures with high metal content prior to dilution with control biosolids/soil mixtures. The shift in microbial community structure coincided with a near absence of nodules in the control treatments and the presence of nodules in the ENM and bulk/dissolved treatments, where we observed significant down-regulation in all five stress-response related genes indicating the plants were less stressed than the controls. In the timeframe of this study, our results show that low concentrations of ENMs in biosolids have minimal adverse effects on plant health.

Conflicts of interest

The authors declare that they have no conflicts of interests with respect to this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.07.188.

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