



# Nanoparticles within WWTP sludges have minimal impact on leachate quality and soil microbial community structure and function<sup>☆</sup>



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## ABSTRACT

One of the main pathways by which engineered nanoparticles (ENPs) enter the environment is through land application of waste water treatment plant (WWTP) sewage sludges. WWTP sludges, enriched with Ag and ZnO ENPs or their corresponding soluble metal salts during anaerobic digestion and subsequently mixed with soil (targeting a final concentration of 1400 and 140 mg/kg for Zn and Ag, respectively), were subjected to 6 months of ageing and leaching in lysimeter columns outdoors. Amounts of Zn and Ag leached were very low, accounting for <0.3% and <1.4% of the total Zn and Ag, respectively. No differences in total leaching rates were observed between treatments of Zn or Ag originally input to WWTP as ENP or salt forms. Phospholipid fatty acid profiling indicated a reduction in the fungal component of the soil microbial community upon metal exposure. However, overall, the leachate composition and response of the soil microbial community following addition of sewage sludge enriched either with ENPs or metal salts was very similar.

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## 1. Introduction

One of the main pathways for consumer product-derived engineered nanoparticles (ENPs) in the environment is via sewage systems entering waste water treatment plants (WWTPs) (Batley et al., 2013), where ENPs tend to accumulate in sludge solids, rather than the watery effluent of WWTPs (Wang et al., 2012). In the EU and US, the majority of these sludges are applied to agricultural soils (Gottschalk et al., 2009), thereby forming the primary pathway for ENPs to enter the human food chain. Legal guidelines on environmental concentrations are available for many metallic ions; however, there is uncertainty whether current guidelines are appropriate when these metals originally enter the system as ENPs (Batley et al., 2013). Pristine ENPs are rapidly transformed in WWTPs (including in this study; Ma et al., 2014), and laboratory

and soil studies both show sulfidation of silver (Ag) ENPs (Kim et al., 2010; Ma et al., 2014) and sulfidation and phosphatidation of zinc (Zn) ENPs (Lombi et al., 2012; Ma et al., 2014). These reactions are likely to alter the reactivity and toxicity of ENPs. Dissolution and aggregation are additional major factors influencing the environmental impact of ENPs and these processes need to be elucidated in natural systems (Gottschalk et al., 2013). Dissolution is often a large factor in ENP toxicity, acting generally to increase toxicity, but specific ENP effects have also been observed (Ma et al., 2013). A complicating factor is the fact that the toxic range for a particular ENP to individual species can be large, depending on the specific properties of the material and environmental conditions (Gottschalk et al., 2013). Aggregation increases the effective diameter of nanomaterials, diminishing size-related ENP toxicity effects. Transformations of pristine ENPs in soil have been studied previously (mainly for Ag) and depend on soil type, pH, organic matter content, availability of humic and fulvic acids, etc. (Cornelis et al., 2013; Sagee et al., 2012). The observed differences in ENP retention and leaching between natural soils and simple porous media such as quartz and glass beads (Sagee et al., 2012) emphasizes the need to use natural conditions to obtain representative results. ENP-enriched sludge, mixed with soil, is the most realistic scenario

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to study leaching after the various transformations have occurred during WWTP.

The agricultural use of WWTP sludges can also have variable effects on greenhouse gas (GHG) emissions. Although carbon dioxide (CO<sub>2</sub>) emissions are increased in sewage sludge amended soil, the net impact may be positive due to increased carbon sequestration in soil. On the other hand, emissions of nitrous oxide (N<sub>2</sub>O) and methane (CH<sub>4</sub>) are generally increased as well (Brown et al., 2010). ENPs and/or associated metal ions may have detrimental effects on soil bacteria, thereby influencing GHG emissions. Colman et al. (2013) reported increased emission of N<sub>2</sub>O in Ag ENP enriched sewage sludges compared to an AgNO<sub>3</sub> control. Methanogenesis was decreased by the addition of Ag ENPs (Yang et al., 2013a, 2012), and ZnO ENPs decreased methanogenic activity during WWTP sludge production (Gonzalez-Estrella et al., 2013). Agriculture is one of the main contributors (approx. 20%) of GHG emissions (Lemke et al., 2007) and any changes in these emissions due to ENP exposure would influence their environmental impact.

Many studies have observed effects of ENPs on mixed microbial species *in vitro* (Auffan et al., 2009; Lee et al., 2008) or on single culturable species (Calder et al., 2012). Studies of microbial community-level responses to ENP exposure in a soil context are limited. Effects are likely to be context dependent, as ENP bioavailability varies with soil properties such as texture, organic matter and humic acid contents, surface charge changes and precipitation with other ions (Calder et al., 2012; Pawlett et al., 2013; Tong et al., 2007). In addition, toxicological effects of ENPs to microorganisms are likely to be specific to microbial species or even single strains. Many microorganisms excrete complex mixtures of extracellular polymeric substances which can act as a barrier, protecting against ENP toxicity by chelating metal ions and delaying cellular damage caused by reactive oxygen species (Joshi et al., 2012). Community-level studies often do not include fungi and are limited to effects on soil bacterial communities (Collins et al., 2012). Although organismal studies can provide valuable insights, from an ecological perspective it is the community as a whole that provides key soil functions. Few studies have encompassed the whole microbial community (Kumar et al., 2011; Pawlett et al., 2013), and there remains little information as to how soil microbial community exposure to ENPs affects soil function. It is also becoming evident that effects of ENPs on microorganisms are specific to the ENPs selected and their size, shape and speciation (Domingos et al., 2009; Sotiriou and Pratsinis, 2010). Current evidence using pristine ENPs demonstrates a minimal impact of fullerenes (C<sub>60</sub>) (Tong et al., 2007), but clear toxicity effects have been reported for TiO<sub>2</sub> and ZnO (Ge et al., 2011) and Ag on soil microbial communities (Kumar et al., 2011). Conversely, minimal (Cullen et al., 2011), or context dependent effects (Pawlett et al., 2013) have been reported for zero-valent iron. Microbial community structure may be affected by metal salts addition, as well as ENPs, and nitrification rates were shown to be dependent on soil Ag (Langdon et al., 2014) and Zn (Cela and Sumner, 2002) concentrations. ENPs may inhibit specific groups of bacteria involved in the nitrogen (N) cycle, with nitrification (Yang et al., 2013b) and N<sub>2</sub>-fixation (Ge et al., 2012) apparently being especially susceptible to ENP toxicity.

Our objective was to study natural ageing and leaching of WWTP sludges enriched during anaerobic digestion with Ag and ZnO ENPs or corresponding metal salts, upon mixing with soil. Ag and ZnO were chosen as they are some of the ENPs with the highest concentration in WWTP sludge (Sun et al., 2014). However, during the production of these sludges, we showed that Ag was transformed to Ag<sub>2</sub>S and Zn to ZnS, Zn<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> or Zn associated Fe oxy/hydroxides, regardless of the form of Ag or Zn added (Ma et al., 2014). Dry sludges were mixed with soil and aged for 6 months in lysimeters under ambient UK conditions. We hypothesised that

leaching of ENPs and their ionic counterparts would be minimal as they are known to be transformed during sludge production and retained within sludge solids (Ma et al., 2014). Furthermore, GHG emissions and microbial community structure were expected to be affected in the ENP and metal salts enriched sludges because Ag and Zn(O) are known to inhibit several microbial processes. To our knowledge this is the first study that combines sludges enriched in ENPs with soil incubation after transformation of ENPs and assesses their effects on microbial community structure and GHG emissions.

## 2. Materials and methods

### 2.1. Sludge production and preparation and mixing with soil

Sludges were produced with ZnO and Ag ENPs, or their metal salts, added to both WWTP primary sludge and the activated sludge basin, followed by anaerobic digestion of primary clarifier solids and waste activated sludge, as described previously for this study (Ma et al., 2014). This resulted in three sludges: Control (SC) to which no metals were added, Metal salts (M) to which Zn(NO<sub>3</sub>)<sub>2</sub> and AgNO<sub>3</sub> were added, and Nanoparticle (NP) to which ZnO and Ag ENPs were added. Synthesis and characterization of ZnO and Ag ENPs added in the WWTP have been described (Ma et al., 2014). Briefly, the original Ag ENPs were 52 nm in diameter and stabilized with polyvinylpyrrolidone (55 kDa), resulting in a hydrodynamic diameter of 80 nm, with a zeta potential of -6.1 mV at pH 7. The ZnO ENPs used had a primary particle size of 30 nm, but a hydrodynamic diameter in DI water of 253 nm. Zeta potential was +15 mV at pH 7. The resulting wet sludges (approx. 25% dry weight) were air-dried and milled <2 mm. Soil was collected to a depth of 25 cm from a site in Woburn, Bedfordshire, UK, previously described by McGrath et al. (2012). The sandy soil (Arenosol, FAO) was sieved <2 mm and air-dried. Soil and sludges were mixed for 10 min using a Hobart bench mixer, leaving three bulk soil/sludge mixtures: SC, M and NP. Soil and sludge were mixed at a ratio of 58%:42%, targeting a total Zn concentration of 1400 mg kg<sup>-1</sup> in both M and NP mixtures. The experiment was designed as a worst case scenario, based on the current U.S. EPA part 503 cumulative pollutant loading limit for Zn in soils amended with biosolids, combined with the guideline for soil/sewage sludge ratios of 1:1 in the top 15 cm of the soil and known concentrations in U.S. sewage sludge (EPA, 1995). Total Ag concentrations were approximately 140 mg kg<sup>-1</sup>, also based on known concentrations, although Ag is not regulated in the U.S. Baseline concentrations of Zn and Ag in the SC mixtures were approximately 400 and 3 mg kg<sup>-1</sup>, respectively. Mixtures were wetted with locally collected rainwater to 50% of their water holding capacity before the start of the experiment.

### 2.2. Lysimeter set-up, leachate sampling and end-sampling

Lysimeters consisted of PVC tubes (11 cm diameter, 23 cm length) with a funnel attached to the bottom and separated from the lysimeter body by a nylon mesh. Twenty four lysimeters (8 replicates per treatment) were packed with 900 g dry weight equivalent soil/sludge mixture and incubated outdoors in a 300 × 55 cm wooden box, filled with sand to promote soil-like temperatures. The experiment was started on 3 January 2013 and finished on 26 June 2013. Total rainfall was 285 mm over the course of the experiment (determined on site), occasionally including snow fall in the first two months. Minimum and maximum air temperatures were -9.8 and 13.0 °C in January and 1.8 and 24.4 °C in June, respectively. From May 15 onwards, additional pre-collected rainwater was added with a pressure sprayer (approximately 0.2 L per lysimeter every few days when it did not rain), since precipitation was low in that period. Leachates were collected

in 0.5 L brown flasks and analysed at days 25, 42, 77, 140, 151 and 165. At the end of the experiment, soil/sewage sludges were removed from the lysimeters, homogenized and subsamples taken for determination of dry weight, nitrate ( $\text{NO}_3^-$ ) and ammonium ( $\text{NH}_4^+$ ) content (approx. 70 g fresh from 4 lysimeters per treatment), and for microbial community analysis (approx. 110 g fresh from all 8 lysimeters per treatment). The remainder of the soil/sludges was bulked per treatment and air dried for further analysis.

### 2.3. Microbial phenotypic community structure and functional profiling

Phospholipid fatty acid (PLFA) profiles were determined using an adaptation (Pawlett et al., 2013) of the method of Frostegard et al. (1993). The proportion of the microbial community attributed to fungi was estimated using the fatty acid indicator 18:2 $\omega$ 6 (Frostegard and Baath, 1996), and the bacterial community by the sum of *i*15:0, *ai*15:0, 15:0, 16:1, *i*16:0, 16:1 $\omega$ 9, 16:1 $\omega$ 7 t, *i*17:0, *ai*17:0, *cyc*-17:0, 17:0 and *cyc*-19:0 (Frostegard and Baath, 1996). The fungal:bacterial ratio was calculated using the fungal biomarker 18:2 $\omega$ 6 divided by summed mol. % of bacterial fatty acids (Frostegard and Baath, 1996). Soil community function was assessed using the multiple substrate induced respiration (MSIR) method of Campbell et al. (2003). The following seven carbon substrates were selected: *D*-glucose,  $\gamma$ -aminobutyric acid, *L*-arginine, citric acid,  $\alpha$ -ketoglutaric acid, *L*-malic acid, alanine (four replicates), and compared with water only as a measure of basal metabolic rate. Substrate was added at 30 mg g<sup>-1</sup> soil water, of which 25  $\mu$ L was dispensed into each soil (400  $\mu$ L soil volume).

### 2.4. Emissions of N<sub>2</sub>O, CH<sub>4</sub> and CO<sub>2</sub>

Emissions of N<sub>2</sub>O, CH<sub>4</sub> and CO<sub>2</sub> were determined 24 h after a simulated rain event, resulting in almost 100% soil saturation and consequently high emission levels. Gas emissions were determined on 18 June 2013 between 1100 and 1200 h BST (18 °C air temperature) by placing an inverted, airtight conical plastic propagator flask on top of 4 out of 8 lysimeter tubes per treatment. The total headspace amounted to 1.72 L and a 30 mL sample was taken after 0, 20, 40 and 60 min. All collected gas samples were analysed for N<sub>2</sub>O, CO<sub>2</sub> and CH<sub>4</sub> using a gas chromatograph. See supplementary data for further details.

### 2.5. Chemical analyses

For determination of Zn and Ag, 5 mL leachate was digested with 5 mL conc. nitric acid/perchloric acid (87:13 v/v; analytical grade) (Zhao et al., 1994). Digest solutions were analysed by ICP-OES (Perkin Elmer Optima 7500DV) for Zn and by ICP-MS (Perkin Elmer NexION 300 $\times$ ) for Ag.

Leachates were centrifuged for 10 min at 16,000 g and the supernatant was analysed on a Skalar Continuous Flow Analyser (Skalar SAN<sup>PLUS</sup>; Skalar (UK) Ltd, Whel Drake, UK) to determine nitrate ( $\text{NO}_3^-$ ) and ammonium ( $\text{NH}_4^+$ ) concentrations.

For determination of Zn and Ag, soil/sewage sludges were milled in a Retsch PM400 ball mill for 10 min at 250 rpm. 0.25 g samples were digested in 5 mL aqua regia (4 mL hydrochloric acid and 1 mL conc. nitric acid), based on the method of McGrath and Cunliffe (1985). Digest solutions were analysed by ICP-OES. For determination of Ag, digests were re-dissolved in 5 mL 10% HCl:10% HNO<sub>3</sub> and further diluted to 1% HCl:1% HNO<sub>3</sub> before analysis by ICP-MS. A certified reference material (BCR 143) was included for quality assurance.

For the determination of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  contents in soil/sewage sludge mixtures, 50 g fresh soil/sewage sludge (24–26 g dry

weight equivalent) was extracted with 80 mL 2 M potassium chloride, shaken for 2 h at 120 rpm and centrifuged at 3000 rpm for 10 min. The resulting supernatant was filtered through Whatman 1 filter papers and stored at 4 °C until analysis.  $\text{NO}_3^-$  and  $\text{NH}_4^+$  in these extracts were analysed by Skalar.

The pH of soil/sewage sludge mixtures was determined in deionized boiled water (10 g soil/sewage sludge: 25 mL H<sub>2</sub>O).

### 2.6. Statistical analyses

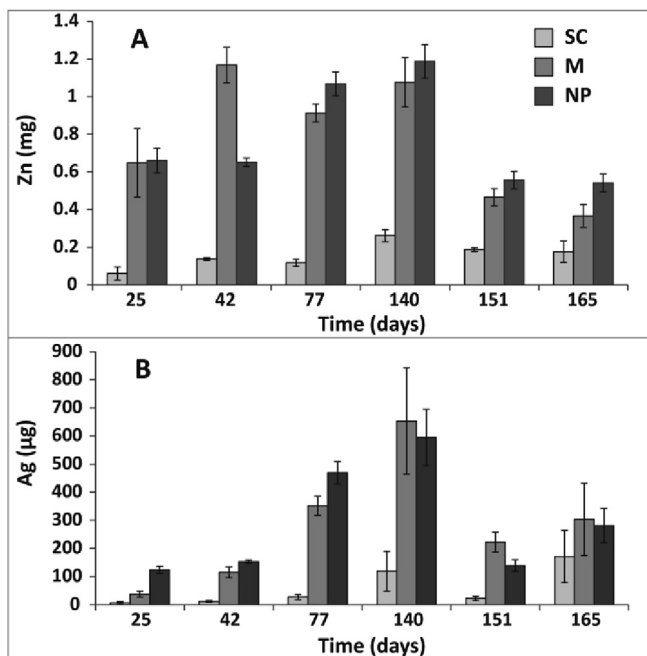
All data were analysed using analysis of variance (ANOVA). Soil/sewage sludge Zn and Ag concentrations and total leachate amounts were square root transformed before analysis to establish homogeneity of variance. In cases where homogeneity could not be achieved by transformation, the non-parametric Mann–Whitney U-test was used (Genstat, VSN International, Hemel Hempstead, UK). PLFA and MSIR data were analysed using principal component analysis (PCA) followed by ANOVA of the factor scores to determine whether there were any significant effects of the experimental design on the PCA factor scores. PLFA and MSIR statistics were performed using Statsoft, Inc. (2012) STATISTICA version 11, stipulating an alpha value of 0.05.

## 3. Results and discussion

### 3.1. Zn and Ag leaching

Total leachate volumes from the lysimeters over the duration of the experiment were very similar in Control (SC, Sludge Control), Metal salts (M) and Nanoparticle (NP) treatments, although less leachate was collected from the SC treatment in the first month (Table S1). Leachates were often brown in colour, mainly due to organic matter derived from the sewage sludge in the mixtures. Extra watering with on-site collected rainwater mainly affected the last two leachate collections (at 151 and 165 days), often leading to a decrease in elemental concentrations due to dilution. Total amounts of Zn leached over the 6 month period were 1.0, 5.2 and 5.2  $\mu$ g g<sup>-1</sup> soil for SC, M and NP treatments, respectively (calculated from Fig. 1A). This amounted to only 0.2, 0.3 and 0.3% of total Zn in the soil/sewage sludges, indicating that concentrations in soil/sludge mixtures did not change markedly over the course of the experiment. Although total Zn leached over the 6-month period was the same in the M and NP treatments, the amount of Zn leached at day 42 was higher in the M treatment, whereas it was slightly lower afterwards (Fig. 1A, Table S2). This may indicate that Zn from the M treatment was more mobile at the start of the experiment. In contrast to almost negligible leaching amounts, there was a small but significant decrease in M and NP soil/sewage sludge Zn concentrations between the start and end of the experiment (Tables 1, S2), which may have been due to sampling and analytical error.

Total amounts of Ag leached over the 6-month period were 0.40, 1.87 and 1.96  $\mu$ g g<sup>-1</sup> soil for SC, M and NP treatments, respectively, amounting to 14.5, 1.2 and 1.4% of total Ag available (calculated from Fig. 1B). M and NP were not significantly different from each other. As these leaching amounts are low, they do not explain the apparent decrease in Ag concentration between the start and the end of the experiment in M and NP treatments (Tables 1, S2). Although the total amounts of Ag leached over the 6-month period were the same, Ag leached was higher in the NP compared to M treatment at days 25, 42 and 77, but more Ag was leached from the M treatment at day 151 (Fig. 1B, Table S2). Concentrations and total amounts leached peaked at day 140 in M and NP treatments. It appears that Ag was initially more mobile in the NP treatment, whereas Zn was initially more mobile in the M treatment, even



**Fig. 1.** Total amounts of Zn (A; mg) and Ag (B; µg) in leachates from control (SC), metal salts (M) and nanoparticle (NP) soil/sewage sludge lysimeters at each sampling. Data represent the mean of 8 replicate measurements ( $\pm$ standard error). See Table S2 for ANOVA results.

though Ma et al. (2014) reported that these ENPs and their metal salts appeared very similar upon transformation in these WWTP sewage sludges.

Organic matter (Coutiris et al., 2012) and natural colloids (Cornelis et al., 2013, 2011) generally retain metals in the soil, although the addition of humic acid often leads to increased leaching (Sagee et al., 2012). Aggregation and transformations during WWTP sewage sludge production (Ma et al., 2014) and/or incorporation into soil may result in a reduced mobility in the leaching columns (Ben-Moshe et al., 2010), although Schlich et al. (2013) reported that Ag ENPs in soil, incorporated via sewage sludge, still exerted toxic effects on soil microbes comparable to pristine ENPs. In studies where pristine ENPs were directly added to soil rather than incorporated into WWTP sewage sludges, considerably higher leaching rates have been observed for both Ag and ZnO (Collins et al., 2012; Sagee et al., 2012). This is a salient difference between our study and many previous studies. Cornelis et al. (2011) added CeO<sub>2</sub> NPs to soils together with WWTP sewage sludges and did not observe an impact of sewage sludges on ENP retention in the soil. However, in this case, ENPs were not added to the sewage sludges during its production and the initial transformations observed by Ma et al. (2014) could not take place.

The pH of soil/sewage sludge mixtures reflected both starting materials. The pH of original Woburn soil, sewage sludges and soil/sewage sludge mixtures was 6.6, 7.5 and 7.2, respectively, and no differences were observed between treatments (data not shown).

Ageing and leaching with rainwater for 6 months did not affect the pH of the soil/sewage sludges. Waalewijn-Kool et al. (2013) observed higher dissolution rates of ZnO NPs into pore water at lower pH. The pH in our sewage sludges may partly explain the lower leaching rates observed, as less Zn would be dissolved and enter into the soil pore water.

### 3.2. Nitrate and ammonium

Nitrate (NO<sub>3</sub><sup>-</sup>) and ammonium (NH<sub>4</sub><sup>+</sup>) ion concentrations at the start of the experiment were approximately 0.2 and 0.6 mg g<sup>-1</sup> soil dry matter (DM), respectively. No significant differences in total inorganic N were observed between treatments; however, wetting air dried soil/sewage sludges by adding rainwater could lead to changes in the ratio between NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup>, irrespective of the treatments (data not shown). Inorganic N in the soil/sewage sludges at the end of the experiment was 5- to 8-fold lower compared to starting concentrations. Whereas the majority was present in the form of NH<sub>4</sub><sup>+</sup> at the start of the experiment, NO<sub>3</sub><sup>-</sup> predominated (up to 90%) at the end, especially in the M and NP treatments (Fig. S1). By the end of the experiment, NO<sub>3</sub><sup>-</sup> concentrations were not significantly different between treatments (Table S2), whereas NH<sub>4</sub><sup>+</sup> concentrations were lower in M and NP treatments compared to SC (Mann–Whitney U test;  $p = 0.03$ ). The increased contribution of NO<sub>3</sub><sup>-</sup> at the end of the experiment was reflected in the leaching of inorganic N from the lysimeters. During the course of the experiment 0.37, 0.64 and 0.55 mg-N g<sup>-1</sup> soil DM was leached from SC, M and NP treatments, respectively (calculated from Table S1 and Fig. S2). This was mainly as NH<sub>4</sub><sup>+</sup> (0.36, 0.57 and 0.40 mg-N g<sup>-1</sup> soil DM, respectively). Over the last 3 time points, NO<sub>3</sub><sup>-</sup> was also leached. Significant differences were observed between treatments, with the lowest amount of N leached from the SC treatment (Fig. S2, Table S2). Significantly more NH<sub>4</sub><sup>+</sup> was leached from the NP treatment during the first 3 time points compared to M, but this was reversed in the last 3 time points (Fig. S2, Table S2). NO<sub>3</sub><sup>-</sup> leached was consistently highest for the NP treatment (0.16 mg-N g<sup>-1</sup> soil DM), with intermediate amounts leached from M (0.07 mg-N g<sup>-1</sup> soil DM), and very low amounts from the SC soil/sewage sludges (0.01 mg-N g<sup>-1</sup> soil DM). The absence of NO<sub>3</sub><sup>-</sup> in leachates for the first 3 time points (Fig. S2) and its increased contribution in soil/sewage sludges at the end of the experiment (Fig. S1) suggests an inhibition or suppression of nitrification. This may have been caused by the lower temperatures at the start of the experiment, with increasing temperatures and biological activity in the later stages. Ag can inhibit nitrification in soils as well, with high pH and/or organic matter content mitigating these effects (Langdon et al., 2014). Half maximal effective concentration (EC<sub>50</sub>) values derived from salt additions were 50–100 mg-Ag kg<sup>-1</sup> for most soils (Langdon et al., 2014), similar to concentrations used in our soil/sewage sludges. Pristine Ag ENPs have also been shown to inhibit nitrification (Yang et al., 2013b), possibly through dissolution into ionic Ag. However, after transformations during sludge formation, Ag was likely less bio-available in our experiment, based on the low leaching rates (Fig. 1). Furthermore, the high organic matter content of the sewage sludges will have increased Ag binding and possibly

**Table 1**  
Total Zn and Ag concentrations in control (SC), metal salts (M) and nanoparticle (NP) soil/sewage sludge mixtures at the beginning of the experiment and following a 6-month leaching period. Data represent the mean of 4 replicate measurements ( $\pm$ standard error). See Table S2 for ANOVA results.

Treatment	Zn at start (mg g <sup>-1</sup> soil)	Zn at end (mg g <sup>-1</sup> soil)	Ag at start (mg g <sup>-1</sup> soil)	Ag at end (mg g <sup>-1</sup> soil)
SC	0.41 $\pm$ 0.03	0.41 $\pm$ 0.002	0.003 $\pm$ 0.001	0.003 $\pm$ 0.001
M	1.83 $\pm$ 0.06	1.72 $\pm$ 0.03	0.152 $\pm$ 0.003	0.123 $\pm$ 0.002
NP	1.87 $\pm$ 0.03	1.69 $\pm$ 0.04	0.137 $\pm$ 0.005	0.112 $\pm$ 0.001

transformation, reducing its bio-availability.

### 3.3. GHG emissions and microbial community structure

N<sub>2</sub>O, CO<sub>2</sub> and CH<sub>4</sub> emission rates from soil/sewage sludge mixtures are shown in Table 2. There were no significant treatment effects (Table S2) for N<sub>2</sub>O and CO<sub>2</sub> with average effective emission rates of 1.2 and 634 kg ha<sup>-1</sup> day<sup>-1</sup>, respectively. Bacteria involved in N cycling have been shown to be sensitive to ENPs (Ge et al., 2012; Throckmole et al., 2007; Yang et al., 2013b). Although some changes in soluble N were observed during the course of the experiment, N<sub>2</sub>O emissions near the end were not affected. Copper (Cu) ENPs decreased N<sub>2</sub>O generation during the WWTP activated sludge process through NP dissolution (Chen et al., 2012). The ENPs used in our study were either less toxic than Cu, or were transformed during sewage sludge production, mixing with soil and ageing for 6 months, diminishing their effect on N<sub>2</sub>O generation. Also, the presence of more NO<sub>3</sub><sup>-</sup> and less NH<sub>4</sub><sup>+</sup> in the NP treatment (Fig. S2, 5th sampling) did not affect N<sub>2</sub>O emission at that time. Colman et al. (2013) observed a 4.5-fold higher N<sub>2</sub>O flux after 8 days exposure from ENP spiked sewage sludges in soil compared to sewage sludges without ENPs. However, this difference was negligible after 50 days, more comparable to our incubation of nearly 5 months. Furthermore, Colman et al. (2013) added ENPs to finished sewage sludges: in our more realistic experiment ENPs were added during sewage sludge production and were already transformed before their addition to soil (Ma et al., 2014). Transformations may render ENPs less active and less labile, diminishing their bioavailability and environmental impact.

The CH<sub>4</sub> emission rate was 3.0 kg ha<sup>-1</sup> day<sup>-1</sup> for the SC treatment. This value is 1- to 2-orders of magnitude higher than typical rates found in the literature (Colman et al., 2013; Diaz-Rojas et al., 2014), which may be explained by the favourable conditions for emissions of these gases, i.e. moist soil and high substrate availability in the sewage sludges (Le Mer and Roger, 2001). In comparison, CH<sub>4</sub> emission could not be detected in M and NP treatments. CH<sub>4</sub> emission occurs under anoxic conditions (as does denitrification) in soil due to activity of methanogens and may be mitigated by the mainly aerobic CH<sub>4</sub> consuming methanotrophs (Le Mer and Roger, 2001). However, all lysimeters received the same amount of rainwater. The differences in CH<sub>4</sub> emission between the SC and the M and NP treatments suggest an effect of metal toxicity on CH<sub>4</sub> production. It is known that Zn and Ag can have a detrimental effect on microbial communities, mainly in ionic form, however, these metals were not readily available in pore water of our soil/sewage sludge mixtures, as indicated by the low amounts leached (Fig. 1). Ge et al. (2012) observed a specific decrease in Methylobacteriaceae, responsible for methane oxidation, whereas our results may suggest a detrimental impact on CH<sub>4</sub> producing methanogens.

Together, principal component (PC) 1 and PC2 of the microbial community's phenotypic (PLFA) PCA profile accounted for 53% of the variation (Fig. 2A). M and NP treatments had significantly ( $p < 0.001$ ) altered profiles compared to the SC treatment on both PC1 and PC2 (Fig. 2A). However, for PC1 there was no significant

difference between the PLFA profiles of M and NP. PC2 did distinguish between the PLFA profiles of M and NP communities. Fatty acids contributing to the loadings (range, -0.8 to +0.8) on PC1 were 15:0i, cy17:0, 18:2 $\omega$ 6,9 and 20:4 (6, 10, 14, 18). Fatty acids contributing to the loadings (range, -0.8 to +0.8) on PC2 included 16:1 $\omega$ 11c, 16:0i and 18:1 $\omega$ 10or11. Indicator fatty acids demonstrate that application of both the M and NP treatments significantly ( $p < 0.001$ ) reduced the fungal:bacterial ratios compared to the SC treatment, but with no significant difference between the M or NP treatments (Fig. 3). This reduction was through a reduced ( $p < 0.001$ ) fungal proportion determined from the reduced relative mol. %, as there was no significant effect on the bacterial portion; a possible well-documented metal-dependant fungicidal effect. Although there is generally more evidence supporting a detrimental effect of ENPs on (soil) bacteria (Ge et al., 2011, 2012), impacts on fungi have also been observed (Lipovsky et al., 2011). Evidence of differences between M and NP treatments is provided by their distinction on PC2 of the PLFA profile. This secondary effect

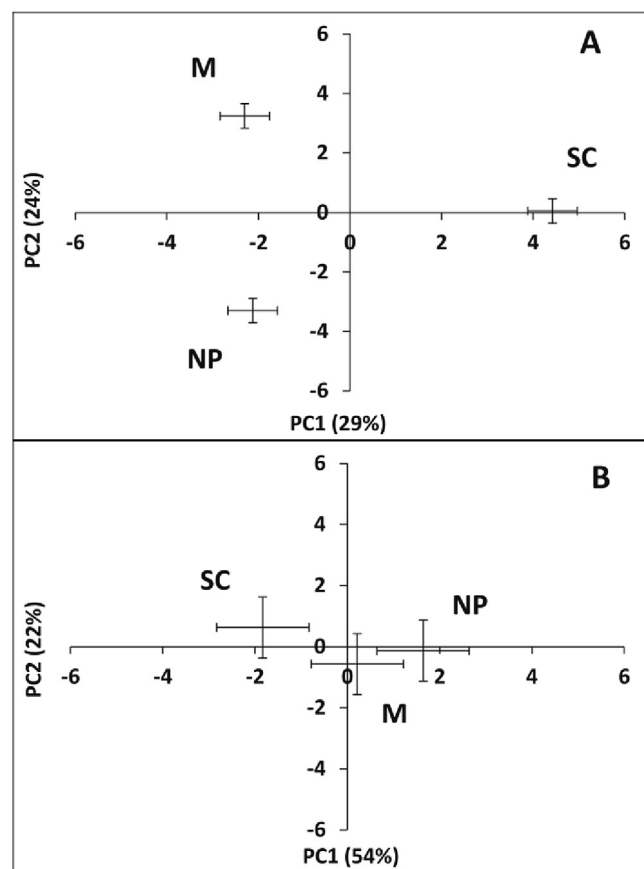


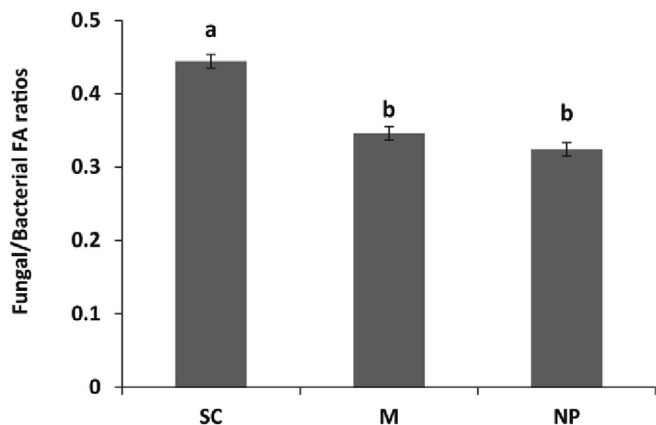
Fig. 2. PCA score plot of ordination means comparing the effect of control (SC), metal salts (M) and nanoparticle (NP) soil/sewage sludges at the end of the experiment on the PLFA phenotypic profile (A) and the MSIR functional profile (B). Data represent the mean of 8 replicate measurements ( $\pm$ pooled standard error).

Table 2

N<sub>2</sub>O, CO<sub>2</sub> and CH<sub>4</sub> emission rates from wet control (SC), metal salts (M) and nanoparticle (NP) soil/sewage sludge mixtures in outside lysimeters. Data represent the mean of 4 replicate measurements ( $\pm$ standard error). See Table S2 for ANOVA results.

Treatment	N <sub>2</sub> O (kg N <sub>2</sub> O ha <sup>-1</sup> day <sup>-1</sup> )	CO <sub>2</sub> (kg CO <sub>2</sub> ha <sup>-1</sup> day <sup>-1</sup> )	CH <sub>4</sub> (kg CH <sub>4</sub> ha <sup>-1</sup> day <sup>-1</sup> )
SC	1.47 $\pm$ 0.29	603 $\pm$ 104	3.03 $\pm$ 1.19
M	0.91 $\pm$ 0.15	707 $\pm$ 47	<0.01 <sup>a</sup>
NP	1.15 $\pm$ 0.41	592 $\pm$ 18	<0.01 <sup>a</sup>

<sup>a</sup> Detection limit.



**Fig. 3.** Fungal:bacterial fatty acid (FA) ratios in control (SC), metal salts (M) and nanoparticle (NP) soil/sewage sludges at the end of the experiment. Data represent the mean of 8 replicate measurements ( $\pm$  pooled standard error) and letters above the bars represent homogenous groupings.

was attributed to three fatty acids: 16:1 $\omega$ 11c, 16:0i and 18:1 $\omega$ 10 or 11. Further studies are warranted to establish whether these are specific for addition of ENP enriched sewage sludge to soil, and could be used as possible ENP biomarkers. In the functional (MSIR) profiles, PC1 and PC2 of the PCA accounted for 76% of the total variation (Fig. 2B). Significant ( $p < 0.001$ ) effects were limited to PC1 which differentiated between M and NP treatments compared to the SC treatment, however, there was no significant difference between the profiles of the M and NP treatments.

A study by Colman et al. (2013), using Ag ENPs and AgNO<sub>3</sub> added to soil together with sewage sludges, detected differences in microbial community structure after only 1 day. However, differences were not detected after 50 days, suggesting that a period of weathering/aging of ENPs in either sewage sludge and/or soils renders the potential toxic effects to be minimal. Microbial communities may have either adapted to metal exposure in the longer term, or recovered from an initial toxic stress as transformation of ENPs in soil and/or sewage sludge reduced metal toxicity and environmental impact over long time periods. This could also explain the minimal effects in our experiments. Other studies on the impact of ENPs on soil microbial community structure (Kumar et al., 2011; Pawlett et al., 2013; Tong et al., 2007) are less relevant, as the ENPs were generally added directly to soil, with the possibility of large initial effects due to the reactivity of pristine ENPs.

#### 3.4. Environmental implications

Metal ENP toxicity can be divided into direct ENP-specific effects, and indirect effects resulting from dissolution into ions, the latter often being predominant. It was already observed in previous work with these sewage sludges that Ag and Zn were transformed to Ag<sub>2</sub>S and ZnS, Zn<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> or Zn associated Fe oxy/hydroxides, respectively, regardless of the form added (ENPs or metal salts) (Ma et al., 2014). After mixing these sewage sludges with soil, it was therefore consistent that leaching of metals, GHG emissions and soil microbial community structure showed similarities between M and ENP treatments. Determination of the forms of Zn and Ag in these soil/sewage sludge mixtures did not fall within the scope of this paper and we therefore do not know whether further transformations occurred during soil incubation. However, total amounts leached still provide information about their availability and environmental fate.

The effect of transformations of ZnO and Ag ENPs and their metal salts occurring in sewage sludges was to stabilize the

particles (Ma et al., 2014; Whitley et al., 2013), likely resulting in the relatively small amounts of Zn and Ag leached from the soil/sewage sludges. This would subsequently lead to lower amounts of metals reaching the ground water or accumulating in surface waters via run-off, compared to adding pristine ENPs or metal salts directly to the lysimeters. The main pathway for organismal exposure to these metals would therefore be through soil exposure and uptake. We showed that soil microbial community structure may be affected by addition of sewage sludge enriched either with ENPs or metal salts, with fungal communities being reduced compared to bacterial communities. Since fungi are important in the decomposition of organic material, this could have an impact on nutrient cycling, and subsequently on plant growth. Also, certain groups of bacteria may be affected directly, which may have been the case in the observed decrease in CH<sub>4</sub> emissions and changes in nitrogen cycling. Further research will focus on the impact of ENPs and related metal salts on soil invertebrates and plant growth. It will be of special interest whether these transformed ENPs can be taken up by plants and thereby enter the food chain.

#### 3.5. Conclusions

A realistic approach was taken to study leaching of Zn and Ag ENPs and their metal salts, by adding these to the influent of a waste water treatment plant and mixing the resulting fully processed sewage sludges with soil. As hypothesized, total leaching amounts were very low and there was no difference between ENP and salt forms of Zn and Ag. Inorganic N leaching indicated inhibition of nitrification by metals at the start of the experiment and GHG emissions indicated an impact on methanogens. However, these microbial impacts were not accompanied by major changes in microbial community structure, although PLFA profiling indicated a reduction in the fungal component upon metal exposure. Overall, leachate composition and soil microbial community responses were very similar in soils treated with sludges enriched with either ENPs or metal salts at the WWTP.

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#### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.envpol.2015.12.063>.

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