Environmental Pollution 215 (2016) 322–330

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Single versus repeated applications of CuO and Ag nanomaterials and their effect on soil microflora☆

Karsten Schlich [a, *], Lukas Beule [b], Kerstin Hund-Rinke [a]

[a] Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Auf dem Aberg 1, 57392 Schmallenberg, Germany
[b] University of Göttingen, Department of Soil Science of Temperate Ecosystem, Büsgenweg 2, 37077 Göttingen, Germany

ARTICLE INFO

Article history:
Received 24 February 2016
Received in revised form
10 May 2016
Accepted 12 May 2016
Available online 21 May 2016

Keywords:
Silver nanomaterials
Copper oxide nanomaterials
Ecotoxicology
Soil
Microbial activity

ABSTRACT

Nanomaterials enter the terrestrial environment via the repeated application of sludge to soils over many years. The goal of this investigation was to compare the effects of CuO and Ag nanomaterials on soil microorganisms after a single application and after repeated applications ultimately resulting in the same test concentrations. The effect on soil microorganisms was determined using the ammonium oxidation (ISO 15685), enzymatic activity patterns (ISO 22939) and MicroResp™ tests on days 28, 56 and 84. The comparability of single and repeated applications of ion-releasing nanomaterials depended on the test endpoint and duration. No significant differences between single and repeated applications were observed when testing nitrifying microorganisms and exoenzymes, but differences were observed in the substrate-induced respiration test. The three test systems used together provide more comprehensive information about the impact of different nanomaterials on the soil microflora and its diversity.

1. Introduction

Nanomaterials are used in many consumer products because they have diverse and beneficial properties. For example, silver nanomaterials (Ag-NMs) and copper oxide nanomaterials (CuO-NMs) are used in medical products, cosmetics, textiles, household goods, paints and coatings due to their antibacterial activity. The "Nanotechnology Consumer Products Inventory" (CPI, 2015) published in 2015 listed 441 products containing Ag-NMs, which are the most widely used nanomaterials (Vance et al., 2015). CuO-NMs are often found in wood preservatives, lubricants, health and fitness products, food and beverage supplements, electronics and computers (CPI, 2015; DaNa 2.0, 2015).

Many studies have shown that nanomaterials are released from consumer products (Voelker et al., 2015; Benn and Westerhoff, 2008; Lorenz et al., 2012; Kaegi et al., 2010; Quadros et al., 2013) and are exposed to different environmental transformation processes that modify their properties (Levard et al., 2011; Lowry et al., 2012a; Impellitteri et al., 2013; Kaegi et al., 2013). The pristine and modified nanomaterials may have different effects on living organisms (Reinsch et al., 2012; Hund-Rinke and Schlich, 2014). The release of nanomaterials into the environment often occurs via point-source accumulation in sewage sludge (a by-product of sewage treatment) applied as agricultural fertilizer. In Germany, 2 million tons of dry sewage sludge solids are produced annually by municipal wastewater treatment plants, and approximately 30% of this sludge is applied to farmland. In Spain, Portugal, France and the UK, the majority of the sewage sludge produced by wastewater treatment plants is applied to agricultural land (Wiechmann et al., 2012).

The influence of Ag-NMs introduced to the soil via sewage sludge on the activity of soil microorganisms has been tested using Ag-NMs remaining in sewage sludge after passing through a simulated sewage treatment plant, revealing long-term effects on soil microorganisms lasting more than 140 days (Schlich et al., 2013a). In a more realistic scenario, different kinds of nanomaterials find their way into the soil over several years via sewage sludge and other routes such as leaching from wood preservatives or facades. There is little information about the fate of nanomaterials in soil, whether they are released into the environment via sewage sludge or other pathways, because there are no appropriate detection methods for nanomaterials in complex

☆ This paper has been recommended for acceptance by B. Nowack.

* Corresponding author.
E-mail addresses: karsten.schlich@ime.fraunhofer.de (K. Schlich), lukas.beule@stud.uni-goettingen.de (L. Beule), kerstin.hund-rinke@ime.fraunhofer.de (K. Hund-Rinke).

http://dx.doi.org/10.1016/j.envpol.2016.05.028

media such as soil (von der Kammer et al., 2012). The retention of nanomaterials in soil depends on the soil properties (Coutris et al., 2012; Hoppe et al., 2014; Cornelis et al., 2010) and an increase of effects over time cannot be excluded (Schlich et al., 2013a) and must be considered for regulatory purposes. Currently the aspect of increasing concentrations of a traditional chemical over a period of time is not considered in the test approaches requested in the scope of regulation. Ion releasing nanomaterials differ from traditional chemicals. Nanomaterials as Ag-NMs and CuO-NMs release ions continuously over time and it cannot be excluded that the adaptation of the soil microflora and its activity after several exposures via run-off, air or sewage sludge differs from the adaptation in the presence of one single exposure.

The goal of this study was to compare the effects of Ag-NMs and CuO-NMs on soil microflora after a single application and repeated applications in three steps over a test period of 84 days. The test approach considered the current procedure described in various OECD test guidelines (OECD Guideline 216, 2000; OECD Guideline 217, 2000) regarding standard incubation periods of 28 days and an extended by two further incubation intervals of 28 days. The test concentrations for both application strategies were chosen to achieve the same final concentration after 56 days, followed by a further incubation period to extend the test duration to 84 days. Due to the comparability of our approach with the existing approaches in the OECD test guidelines (OECD Guideline 216, 2000; OECD Guideline 217, 2000) the acceptance by regulatory authorities is expected to be increased. The approach should provide basic information on the need of a repeated exposure in contrast to the common approach of a single exposure. It cannot be excluded that a modification of the incubation intervals might affect the changes in microbial activity to a small, negligible extent. The effect of the nanomaterials on ammonia-oxidizing bacteria (ISO Guideline 15685, 2012), the functional biodiversity in soil based on enzyme activity patterns (ISO/TS Guideline 22939, 2010), and microbial respiration based on the MicroResp™ approach (MicroRespTM, 2015), was investigated after 28, 56 and 84 days. The three methods address different endpoints as well as different microbial groups or enzyme patterns. Although most ammonia-oxidizing bacteria are autotrophic, the MicroResp™ system detects active heterotrophic microorganisms and the enzymatic activity patterns test detects microbial exoenzymes. This diverse approach provided more comprehensive information about the microbial community. Ag-NMs and CuO-NMs were chosen because their toxicity is based predominantly on the long-term release of ions (Semisch et al., 2014; Lee et al., 2012). Pristine materials were used to be in line with the approach used for regulatory purposes.

Although several studies have tested the impact of Ag-NMs against different soil microorganisms e.g. ammonia-oxidizing bacteria, data about the toxicity of CuO-NMs are limited. In the studies that have been published, the nanomaterials were applied in a single concentration, whereas a more realistic exposure scenario involves repeated applications. This study for the first time compares single and repeated applications that ultimately result in the same overall concentrations, and determines their effects on soil microorganisms.

2. Materials and methods

2.1. Test soil

The experiments were carried out using a reference soil (RefeSol) whose physicochemical properties are listed in Table 1. RefeSol soils were selected as reference soils by the German Federal Environment Agency (Umweltbundesamt UBA) and they match the properties stated in various OECD terrestrial ecotoxicological guidelines (e.g. tests with plants and soil microflora). 50 kg dry matter (dm) of soil were sampled 1–4 weeks before the test. If the soil was too wet for sieving it was dried at room temperature to 20–30% of the maximum water holding capacity (WHC_{max}) with periodic turning to avoid surface drying. If the tests did not start immediately after sieving, the soil was stored in the dark at 4 °C under aerobic conditions (ISO Guideline, 18512, 2007).

Table 1
Physicochemical properties of reference soils.

Parameter	RefeSol 01A[a]
Soil type	Dystric cambisol
Properties	Loamy sand, medium acid, very light humic
Sand [%]	73
Silt [%]	22
Clay [%]	5
pH ($CaCl_2$)	5.51
Corg [%]	1.10
CEC_{eff} [$mmol_c$/kg]	37.9
WHC_{max} [mL/kg]	292

[a] Arable land; CEC = cation exchange capacity; WHC_{max} = maximum water-holding capacity.

2.2. Nanomaterials

The Ag-NM NM-300K was selected for the OECD Sponsorship Programme (Organisation for Economic Co-operation and Development, 2007) and was used in the EU FP7 project Marina (http://www.marina-fp7.eu/). NM-300K is a colloidal silver dispersion with a silver concentration of 10% (w/w), and a particle size of ~15 nm with a narrow size distribution (99%). A second particle size of 5 nm, which is much less abundant (1%), was identified by transmission electron microscopy. The particles are dispersed in a mixture of a stabilizing agents (NM-300K DIS) comprising 4% (w/w) each of polyoxyethylene glycerol trioleate and polyoxyethylene sorbitan monolaurate (Tween-20) (Klein et al., 2011). The CuO-NM was selected in the EU FP7 project SUN (http://www.sun-fp7.eu/) and was provided by PlasmaChem GmbH as CuO-NM powder (CAS: 1317-38-0). The CuO-NM had a primary particle size of 15–20 nm, a Brunauer–Emmett–Teller (BET) surface area of 47 m^2/g and a purity of 99.9%.

The time schedule of the different application regimes and subsequent ecotoxicological studies is shown in Fig. 1.

2.3. Application of nanomaterials

The test materials were applied to the soil as previously described (Hund-Rinke et al., 2012; Schlich et al., 2013b). An application scheme for the single and stepwise repeated applications of Ag-NMs and CuO-NMs to achieve the final test concentrations of 1.67 and 5.00 mg/kg dry matter (dm) for Ag-NMs and 333 and 1000 mg/kg dm for CuO-NMs is presented in Table 2.

For the single application nanomaterials were added as previous described for Ag-NM dispersion and nanomaterials in its powder form (CuO-NMs) (Schlich et al., 2012, 2013b) to the soil, which was then homogenized thoroughly. The single application procedure described above was also used for the repeated application, which was carried out three times (at 4-week intervals) within a period of 84 days. A defined amount of soil was removed from the incubation vessels at each time point (5% and 1% of the soil mass for the Ag-NM and CuO-NM applications, respectively). The removed soil was air dried for three days and then used as the carrier soil for the subsequent application. Samples were taken for the ecotoxicological studies immediately before the next application step. For the incubation of the test soil, each treatment was adjusted to 45%

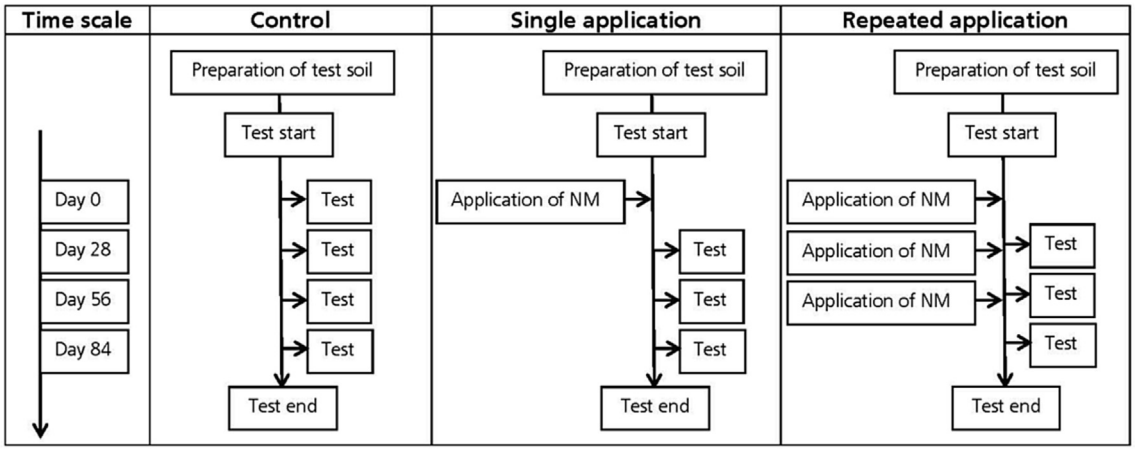

Fig. 1. Time schedules of the single and stepwise repeated applications and the subsequent ecotoxicological experiments.

Table 2
Strategy for the single application and stepwise repeated application of Ag-NMs and CuO-NMs.

	Single application [mg/kg dm soil]		Repeated application [mg/kg dm soil]	
Nanomaterial	Ag-NM	CuO-NM	Ag-NM	CuO-NM
Soil conc. 1	1.67	333	0.56	111
Soil conc. 2	5.00	1000	1.67	333
Date of application	d0 (at once)		d0, 28, 56	

WHC_{max}. The above procedure was carried out at each single test concentration and the control treatments to ensure that each test sample was a homogeneous mixture of the test materials and soil, as confirmed by inductively coupled plasma optical emission spectroscopy (ICP-OES) (Schlich et al., 2013b).

2.4. Ecotoxicological tests

We used a combination of two test guidelines to assess the effect of Ag-NMs and CuO-NMs on ammonia-oxidizing bacteria. Previous studies have shown that ISO Guideline 15685 (ISOGuideline 15685, 2012) is more suitable than OECD Guideline 216 (OECD Guideline 216, 2000) for measuring the toxicity of ion-releasing nanomaterials such as Ag-NMs and CuO-NMs (Hund-Rinke and Schlich, 2014). Incubation was therefore carried out according to OECD Guideline 216 (OECD Guideline 216, 2000), but the short-term potential ammonia oxidation test as recommended by ISO Guideline 15685 (ISOGuideline 15685, 2012) (measuring nitrite concentration) was used to investigate microbial activity. The purpose of this method is to measure the ammonia oxidation potential, which provides an indication of the size of the ammonia-oxidizing bacterial community. Nitrite levels of the supernatant were determined using an Epoch™ spectrophotometer (BioTek® Instruments, Inc., Vermont, USA).

Functional microbial diversity was measured by enzyme activity patterns in soil samples according to ISO/TS Guideline 22939 (ISO/TS Guideline 22939, 2010) using four fluorogenic substrates as indicators. In this test, 2 g dry matter (dm) soil for each concentration of nanomaterial was mixed with 200 mL ultra-high-quality (UHQ) water and homogenized for 3 min at 9600 rpm with a homogenizer. Substrates for the nitrogen cycle (L-alanine-AMC, L-leucine-AMC), the carbon cycle (4-MUF-nonanoate) and the phosphorus cycle (4-MUF-phosphate) were prepared at concentrations of 0.1 mM/L. Tests were carried out in 96-well microtiter plates containing 50 μL soil suspension, 50 μL buffer and 100 μL substrate. Following the preparation of the microtiter plates, the fluorescence was measured with a Synergy MX spectrophotometer (BioTek Germany, Bad Friedrichshall, Germany) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm.

The MicroResp™ test (MicroRespTM, 2015) was used to determine microbial respiration activity after the addition of selected carbon sources thus targeting the metabolism and activity of soil microorganisms (Campbell et al., 2003). The test included indicator substrates for the nitrogen cycle (L-alanine and L-leucine), the carbon cycle (nonanoic acid) and the phosphorus cycle (phenylphosphate disodium salt). Treated soil samples and carbon sources were transferred to 96-deep-well plates, and a 96-well detection plate containing a cresol, potassium chloride and sodium bicarbonate indicator was assembled onto a deep-well plate using a MicroResp™ seal. Absorbance at 570 nm was measured using the Epoch™ spectrophotometer. The respiration activity caused by the added carbon source increased the rate of CO_2 production, which caused the indicator to change from pink to yellow. The amount of CO_2 produced in μg CO_2 (g dm * h)$^{-1}$ was calculated as the difference between the values determined after 0 and 6 h.

2.5. Chemical analysis of silver and copper in soil

The silver and copper content of the test soil was measured at the end of the test to confirm that the anticipated concentration of Ag-NMs and CuO-NMs in the soil was achieved. Two samples were obtained from each application batch. Digestion was carried out according to ISO Guideline 11466 (Guideline 11466, 1995) and DIN EN Guideline 13346 (Guideline, 2001).

Prior to digestion/extraction, the soil was dried at 105 °C until the weight was constant for at least 12 h, and 3 g (for the quantification of Cu) or 0.5 g (for the quantification of Ag) of the homogenized material was mixed with 28 mL aqua regia and incubated at room temperature for at least 12 h without agitation. The mixture was then heated under reflux for 2 h, cooled to room temperature and then carefully made up to 100 mL (for the quantification of Cu) or 50 mL (for the quantification of Ag) with 3% HNO_3 (suprapur® quality, supplied by Carl Roth, Karlsruhe, Germany) and filtered prior to analysis (0.45 μm syringe filter, Supor membrane, Pall Corporation, NY, USA). Ag and Cu concentrations in aqueous samples of digested soil were measured by ICP-OES using an Agilent 720 device (Agilent Technologies, Waldbronn, Germany). Silver was detected at 328.068 nm and copper at 324.754 nm.

2.6. Treatment of data

The data were treated for each measuring time separately. The statistically differences were calculated based on the replicates. Statistical analysis was carried out using ToxRatPro v2.10 software for ecotoxicity response analysis (ToxRat Solutions GmbH, Alsdorf, Germany). Shapiro-Wilk's test on normal distribution, the Levene-test on variance homogeneity and a one-sided *t*-test ($p < 0.05$) were applied. Percent inhibition was calculated based on mean values.

3. Results

All experiments were carried out twice. Because of the large data sets and the comparability of the outcomes, only the results of the first experiment are presented herein. Any substantial differences between the results of the two replicate experiments are discussed in the text.

3.1. Chemical analysis

Soil was sampled from each of the different treatments at the end of the test. The chemical analysis of Ag and Cu in soil samples following single and repeated applications of the nanomaterials is presented in SI Table 1. Duplicate samples were analyzed to determine the Cu and Ag concentrations. The soil treatments were intended to achieve nominal concentrations of 1.67 and 5.00 mg/kg dm soil for Ag-NM and 333 and 1000 mg/kg dm soil for CuO-NM either by a single application or by the stepwise application of lower concentrations over 84 days, with applications on days 0, 28 and 56. The recovery in the different treatments ranged from 86.1% to 119% for Ag-NM and from 91.8% to 101% for CuO-NM. The mean empirical concentrations thus agreed with the anticipated nominal concentrations.

3.2. Potential ammonium oxidation (ISO 15685:2012)

The transformation of ammonia was expressed as nitrite production. The ammonia oxidation data and the inhibition caused by the nanomaterials are summarized in SI Table 2. The effects of nanomaterials were compared to controls as summarized in Fig. 2 (Ag-NM) and Fig. 3 (CuO-NM), confirming that ammonia-oxidizing bacteria were inhibited by both Ag-NM and CuO-NM in the soil. A previous study has shown that the dispersant used to prepare Ag-NM has no independent effect on the soil microflora (Hund-Rinke and Schlich, 2014). The activity of ammonia-oxidizing bacteria in the control soil was constant over the entire test period of 84 days (SI Table 2). The Ag-NM treatment was significantly more toxic than the CuO-NM towards the ammonia-oxidizing bacteria.

3.2.1. Ag-NMs

To determine the impact of Ag-NMs on ammonia-oxidizing bacteria, a single application was tested at two concentrations (low concentration = 1 × 1.67 mg/kg dm soil, high concentration = 1 × 5.00 mg/kg dm soil, administered on day 0) and a repeated application was tested at two equivalent concentrations delivered in three parts (low concentration = 3 × 0.56 mg/kg dm soil, high concentration = 3 × 1.67 mg/kg dm soil, administered on days 0, 28 and 56). The single application of Ag-NMs at the high concentration caused a statistically significant concentration-dependent inhibition of 88.4% compared to control soil after 28 days whereas the low concentration caused a statistically nonsignificant 12.3% inhibition over the same period (Fig. 2). For the repeated application treatment, the first application of Ag-NMs at the low concentration caused a statistically significant stimulation (23.8%) of ammonia-oxidizing activity by day 28, before the second application, whereas the first application of Ag-NMs at the high concentration caused a statistically nonsignificant 24.0% inhibition of ammonia-oxidizing activity after 28 days, which is in a comparable range to the effect of the single application (12.3%) at the low concentration (Fig. 2).

The single and repeated treatments were compared again on day 56, before the third application in the stepwise protocol. In the single application treatments, inhibition increased between days 28 and 56 from 12.3% to 65.2% at the low concentration, and from 88.4% to 96.4% at the high concentration (Fig. 2). In the repeated application treatments, inhibition increased between days 28 and

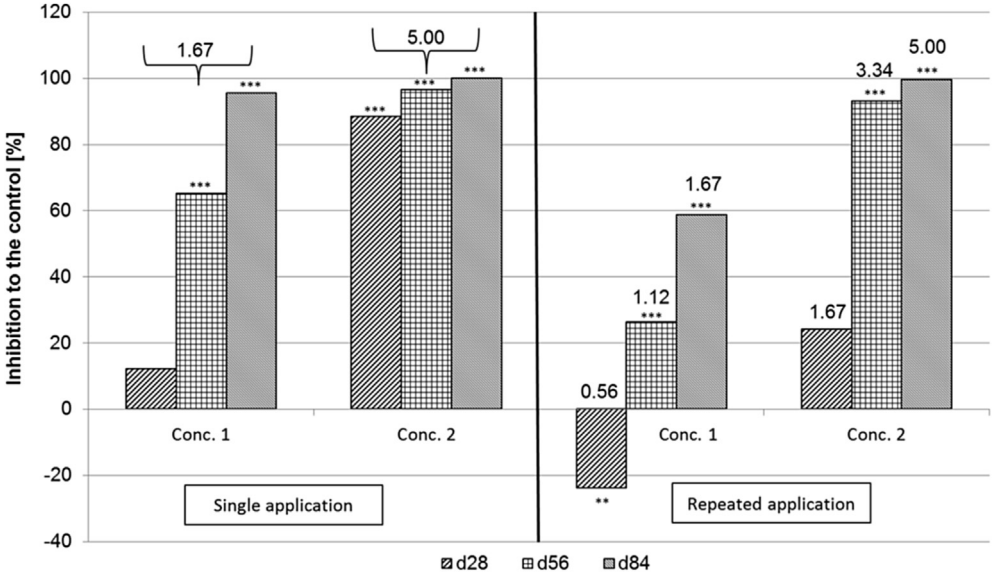

Fig. 2. Inhibition of the activity of ammonia-oxidizing bacteria in soil caused by Ag-NMs. Comparison of effects based on single and repeated applications at high and low concentrations. Single application, low concentration = 1 × 1.67 mg/kg dm soil (conc. 1), high concentration = 1 × 5.00 mg/kg dm soil (conc. 2). Repeated application, low concentration = 3 × 0.56 mg/kg dm soil (conc. 1), high concentration = 3 × 1.67 mg/kg dm soil (conc. 2). Asterisks indicate the statistical significant difference to control: * $0.05 \geq P \geq 0.01$; ** $0.01 \geq P \geq 0.001$; ***$P \leq 0.001$.

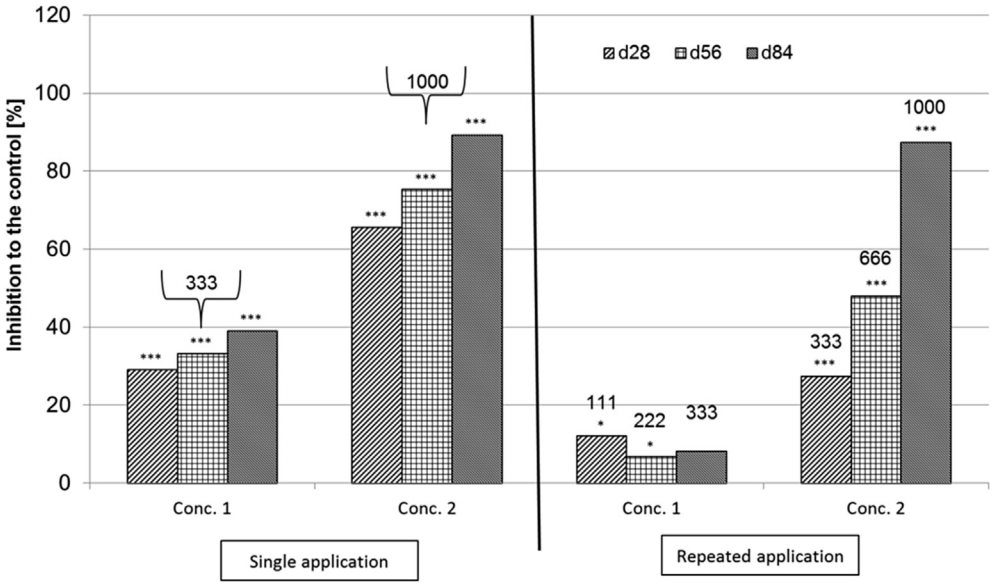

Fig. 3. Inhibition of the activity of ammonia-oxidizing bacteria in soil caused by CuO-NMs. Comparison of effects based on single and repeated applications high and low does. Single application, low concentration = 1 × 333 mg/kg dm soil (conc. 1), high concentration = 1 × 1000 mg/kg dm soil (conc. 2). Repeated application, low concentration = 3 × 111 mg/kg dm soil (conc. 1), high concentration = 3 × 333 mg/kg dm soil (conc. 2). Asterisks indicate the statistical significant difference to control: * $0.05 \geq P \geq 0.01$; ** $0.01 \geq P \geq 0.001$; ***$P \leq 0.001$.

56 from −23.8% to 26.4% at the low concentration, and from 24.0% to 93.1% at the high concentration (Fig. 2).

By day 84, when the final samples were tested, the level of inhibition achieved in the single application treatments had reached 95.6% for the low concentration and 100% for the high concentration, whereas the level of inhibition achieved in the repeated application treatments showed a statistically significant increase to 58.8% inhibition for the low concentration and an increase to 99.6% inhibition for the high concentration (Fig. 2). The activity of the ammonia-oxidizing bacteria was therefore inhibited more potently by a single concentration of Ag-NMs at low or high concentrations than the equivalent amount administered in three parts, representing cumulative dosing.

3.2.2. CuO-NMs

To determine the impact of CuO-NMs on ammonia-oxidizing bacteria, a single application was tested at two concentrations (low concentration = 1 × 333 mg/kg dm soil, high concentration = 1 × 1000 mg/kg dm soil, administered on day 0) and a repeated application was tested at two equivalent concentrations delivered in three parts (low concentration = 3 × 111 mg/kg dm soil, high concentration = 3 × 333 mg/kg dm soil, administered on days 0, 28 and 56). The single application of CuO-NMs at both the high and low concentrations caused statistically significant concentration-dependent inhibition (65.5% and 28.0%, respectively) compared to control soil after 28 days (Fig. 3). In the repeated application treatments, the first delivery of CuO-NMs at both the high and low concentrations caused statistically significant concentration-dependent inhibition (12.0% and 27.2%, respectively) compared to control soil after 28 days (Fig. 3). The inhibition caused by single and repeated applications (both at a concentration of 333 mg/kg dm soil) after 28 days was therefore similar at 28.9% and 27.2%, respectively (Fig. 3).

By day 56, the level of inhibition caused by the single application treatment at the low concentration had increased from 28.9% to 33.1%, whereas at the high concentration the level of inhibition had increased from 65.5% to 75.2% (Fig. 3). For the repeated application treatment, there was no significant change between days 28 and 56 following the low concentration (12.0% vs 6.7%) whereas the high concentration caused an increase from 27.2% to 47.9% over the same period (Fig. 3).

By day 84, the level of inhibition caused by the single application treatment had increased to 38.9% at the low concentration and to 89.2% at the high concentration (Fig. 3). In the repeated application treatments, the level of inhibition remained stable at the low concentration (8.2%) but increased to 87.4% at the high concentration, i.e. the low repeated concentration caused 10-fold less inhibition than the high repeated concentration after 84 days (Fig. 3). The single and repeated applications achieved similar levels of inhibition at the high concentration after 84 days, whereas the single application of a low concentration achieved a significantly higher level of inhibition than the comparable repeated application.

3.3. Enzyme activity patterns (ISO/TS 22939)

Enzyme activity patterns were analyzed using four fluorogenic substrates, according to ISO/TS Guideline 22939 (ISO/TS Guideline 22939, 2010). The transformation of the fluorogenic substrates represents the degradation of macromolecules representing the carbon, nitrogen and phosphorus cycles by specific exoenzymes in the soil. The effects of Ag-NMs and CuO-NMs at the low and high concentrations described above (compared to control soils) in terms of enzyme inhibition are summarized in Table 3. The enzyme activities (μmol/L per g dm per h) in soils treated with Ag-NMs and CuO-NMs are presented in SI Table 3.

3.3.1. AgNM

After 84 days, degradation of 4-MUF-nonanoate was unaffected by Ag-NMs at the low concentration in both the single and repeated application treatments. However, at the high concentration, the enzymes were inhibited by 31.2% after a single application and 35.7% after repeated applications, representing a similar overall effect by the end of the test.

Degradation of 4-MUF-phosphate was inhibited by only 7.0% (statistically nonsignificant) when the low concentration was applied as a single application, but by 37.6% when the same

Table 3
Percentage inhibition of the functional biodiversity of test soils by Ag-NMs and CuO-NMs in terms of enzyme activity patterns, showing statistical differences between test soils and untreated control soils (*0.05 ≥ p ≥ 0.01; **0.01 ≥ 0.001; ***p ≤ 0.001).

Ag-NM: Single application						Ag-NM: Repeated application					
Conc. [mg/kg]	Day	4-MUF-nonanoate (C-cycle)	L-Alanine-AMC (N-cycle)	L-Leucine-AMC (N-cycle)	4-MUF-phosphate (P-cycle)	Conc. [mg/kg]	Day	4-MUF-nonanoate (C-cycle)	L-Alanine-AMC (N-cycle)	L-Leucine-AMC (N-cycle)	4-MUF-phosphate (P-cycle)
1.67	28	−5.7	−9.8	46.4***	11.5*	**0.56**	28	−20.2***	−28.5***	19.2**	−8.6
	56	−4.8	15.7**	−1.4	15.6**	**1.12**	56	−7.4	9.3	7.5	−10.6*
	84	−4.1	23.1***	11.4*	7.0	**1.67**	84	−0.9	19.7***	7.3	37.6***
5.00	28	8.8	28.8***	41.1***	12.0*	**1.67**	28	−0.5	−27.0***	−8.9	−25.7***
	56	23.4***	59.7***	50.9***	15.8**	**3.34**	56	31.0***	43.7***	36.0***	23.3***
	84	31.2***	64.2***	26.5***	28.5***	**5.00**	84	35.7***	63.5***	69.2***	38.3***
CuO-NM: Single application						**CuO-NM: Repeated application**					
Conc. [mg/kg]	Day	4-MUF-nonanoate (C-cycle)	L-Alanine-AMC (N-cycle)	L-Leucine-AMC (N-cycle)	4-MUF-phosphate (P-cycle)	Conc. [mg/kg]	Day	4-MUF-nonanoate (C-cycle)	L-Alanine-AMC (N-cycle)	L-Leucine-AMC (N-cycle)	4-MUF-phosphate (P-cycle)
333	28	−23.9***	−4.6	28.9***	22.2***	**111**	28	−4.0	10.2	28.5***	8.9*
	56	−12.5*	12.6*	23.5***	35.6***	**222**	56	0.6	12.2*	12.9**	25.0***
	84	−18.1***	10.2*	14.2	44.4***	**333**	84	−13.4***	15.8**	24.4**	45.0***
1000	28	3.4	7.4	48.7***	39.5***	**333**	28	−15.4*	−3.5	17.5**	18.6**
	56	10.6*	28.0***	32.6***	49.3***	**666**	56	7.0	14.9*	26.8***	39.7***
	84	−0.6	19.5**	28.4**	60.3***	**1000**	84	4.1	12.5**	18.1	57.5***

concentration was applied as repeated applications (Table 3). At the high concentration, comparable levels of inhibition were observed after the single application (28.5%) and the repeated application treatment (38.3%), again representing a similar overall effect by the end of the test (Table 3).

The degradation of the amino acids L-alanine-AMC and L-leucine-AMC as representatives of the nitrogen cycle was more sensitive to the presence of Ag-NMs than either the representatives of the carbon or phosphorus cycles. For L-alanine-AMC, the low concentration of Ag-NMs had a comparable tendency in the effects after 28, 56 and 84 days regardless of whether the nanomaterial was applied as a single application or repeated applications. The high concentration showed an effect after 28 days as a single application (28.8%) and no inhibition after the first concentration in the repeated application treatment. However, the single and repeated application treatments showed similar effects after 56 days (59.7% vs 43.7% inhibition) and 84 days (64.2% vs 63.5%). At the end of the test, the level of inhibition achieved by the low concentration of Ag-NMs was 23.1% and 19.7% for the single and repeated applications, respectively, and the level of inhibition achieved by the high concentration was 64.2% and 63.5% for the single and repeated applications, respectively (Table 3).

The inhibition of L-leucine-AMC degradation was less pronounced than the inhibition of L-alanine-AMC degradation but the trends were comparable at least after day 56. There were some discrepancies between the duplicate experiments, e.g. at the end of the test the high concentration caused 26.5% inhibition after a single application but 69.2% after repeated applications reported here. In the second experiment the level of inhibition after 84 days was comparable in single and repeated application treatment (data not shown).

3.3.2. CuO-NM

In contrast to the profound impact of Ag-NMs on the degradation of the amino acids, CuO-NMs showed the strongest impact on the degradation of the substrate 4-MUF-phosphate representing the phosphorus cycle. In the 84 days following the single application of CuO-NMs, inhibition increased continuously from 22.2% to 44.4% at the low concentration and from 39.5% to 60.3% at the high concentration. Comparable increases were observed during the repeated application treatment although with a lower starting point, i.e. from 8.9% to 45% at the low concentration and from 18.6% to 57.5% at the high concentration. All these values were concentration-dependent and statistically significant compared to control samples.

The CuO-NMs appeared to have no impact on the degradation of MUF-nonanoate representing the carbon cycle even at the highest concentration. The degradation of L-alanine-AMC and L-leucine-AMC was inhibited, but the effect of inhibition declined over time for the single application treatment (slight effects in the case of L-alanine-AMC) while remaining more stable in the repeated application treatment due to an apparent 'topping up' effect. L-leucine-AMC degradation appeared to be more sensitive to CuO-NM than L-alanine-AMC degradation and in the single application treatment the loss of inhibition effect was linear, from 28.9% (day 28) to 14.2% (day 84) at the low concentration and from 48.7% (day 28) to 28.4% (day 84) at the high concentration (Table 3). The repeated applications interrupted this recovery process resulting in a more stable profile.

3.4. MicroResp approach

The substrate-induced respiration activity of soil microorganisms was measured using the MicroResp™ system on days 28, 56 and 84. The degradation of L-alanine and L-leucine (representing the nitrogen cycle), nonanoic acid (representing the carbon cycle) and phenylphosphate disodium (representing the phosphorus cycle) were affected by both Ag-NMs and CuO-NMs as summarized in Table 4. The respiration activity in ng CO_2 (g dm * h)$^{-1}$ in the soil treated with Ag-NMs or CuO-NMs is presented in SI Table 4.

3.4.1. AgNM

The concentration-dependent inhibition of substrate-induced respiration was observed regardless of the application regime. For the single application treatment, the strongest inhibition was generally observed at the first measuring point (28 days) followed by a decline, e.g. as shown by the inhibition of nonanoic acid degradation at the high concentration of Ag-NMs, which fell from 26.5% at 28 days to 9.8% at 84 days. In the repeated application treatment, the low concentration had no statistically significant effects, whereas the high concentration resulted in an increase in inhibition between 28 and 56 days and the value remained steady thereafter, e.g. the degradation of L-alanine was inhibited by just 3.0% after 28 days rising to 26.2% after 56 days and then to 33.2% at

Table 4
Percentage inhibition of the functional biodiversity of test soils by Ag-NMs and CuO-NMs in terms of the MicroResp™ approach, showing statistical differences between test soils and untreated control soils (*0.05 ≥ p ≥ 0.01; **0.01 ≥ 0.001; ***p ≤ 0.001).

Ag-NM: Single application						Ag-NM: Repeated application					
Conc. [mg/kg]	Day	Nonanoic acid (C-cycle)	L-Alanine (N-cycle)	L-Leucine (N-cycle)	Phenyl-phosphate disodium (P-cycle)	Conc. [mg/kg]	Day	Nonanoic acid (C-cycle)	L-Alanine (N-cycle)	L-Leucine (N-cycle)	Phenyl-phosphate disodium (P-cycle)
1.67	28	19.2**	0.9	13.7**	−5.7	**0.56**	28	−1.7	−6.4	3.2	−15.7
	56	13.9**	−11.0*	−16.2**	−24.3***	**1.12**	56	9.4	7.0*	−3.0	−7.9
	84	5.6	15.6**	6.7	4.5	**1.67**	84	4.3	10.7*	3.7	1.6
5.00	28	26.5***	25.2***	15.0**	5.8	**1.67**	28	20.2**	3.0	1.7	−21.6
	56	23.8***	22.0**	13.9*	11.8*	**3.34**	56	27.2***	26.2***	25.9***	14.7**
	84	9.8	20.2**	4.6	8.2	**5.00**	84	18.6**	33.2***	17.1**	18.5**
CuO-NM: Single application						**CuO-NM: Repeated application**					
Conc. [mg/kg]	Day	Nonanoic acid (C-cycle)	L-Alanine (N-cycle)	L-Leucine (N-cycle)	Phenyl-phosphate disodium (P-cycle)	Conc. [mg/kg]	Day	Nonanoic acid (C-cycle)	L-Alanine (N-cycle)	L-Leucine (N-cycle)	Phenyl-phosphate disodium (P-cycle)
333	28	12.1*	16.0**	12.5*	4.0	**111**	28	1.5	10.7	5.7	−1.8
	56	3.6	−2.0	3.5	12.4*	**222**	56	−9.2	−16.7	1.1	12.9*
	84	10.2*	10.6*	7.8	3.1	**333**	84	22.6**	21.7**	19.7**	11.4*
1000	28	8.6	2.9	3.1	−1.9	**333**	28	3.9	40.3***	5.7	15.4**
	56	1.9	1.4	1.4	5.4	**666**	56	11.8*	7.7	15.3*	21.7**
	84	12.3*	14.6**	6.9	6.7	**1000**	84	28.9***	24.4***	14.3*	8.6

the end of the test.

3.4.2. CuO-NM

A single application of low or high concentrations of CuO-NMs did not have a statistically significant effect on substrate-induced respiration, as shown by the ≤16% inhibition of the degradation of all substrates (Table 4). Although several statistically significant inhibitions were calculated they are not considered as remarkable as no clear tendency in the course of the inhibition was observed. However, the repeated application treatments achieved inhibitory effects of up to 22.6% at the low concentration and up to 28.9% at the high concentration in the case of nonanoic acid, which was the only substrate to show evidence of concentration-dependent inhibition.

4. Discussion

4.1. General ecotoxicity of Ag-NMs and CuO-NMs

In general, Ag-NMs and CuO-NMs were more toxic towards ammonia-oxidizing bacteria than aerobic heterotrophic microorganisms (determined by substrate-induced respiration), which agrees with previous studies involving Ag-NMs (Hund-Rinke and Schlich, 2014; Schlich et al., 2013a; Hänsch and Emmerling, 2010; Schlich and Hund-Rinke, 2015). A previous study on the effect of CuO-NM on the microbial community structure over 48 h revealed a decline in the abundance of bacteria at a concentration of 0.1% w/w (1000 mg/kg dm soil) (Frenk et al., 2013). At a concentration of 1% w/w (10,000 mg/kg dm soil) the CuO-NMs also affected bacterial hydrolytic activity, oxidative potential and the community composition and size (Hänsch and Emmerling, 2010). The effect of CuO-NMs was strongly dependent on soil properties, as previously reported for Ag-NMs (Schlich and Hund-Rinke, 2015; Frenk et al., 2013). Another study investigated the effect of nano-CuO on bacterial growth (Rousk et al., 2012). Dissolution was found to be the principal mechanism of toxicity and direct acute toxicity towards soil bacteria was observed although the bulk material was not toxic. Here again, toxicity was strongly dependent on soil properties. These results show that CuO-NMs affect the soil microbial community mainly at higher test concentrations (333–1000 mg/kg dm soil) in contrast to Ag-NMs which are also active at lower concentrations (1.67–5.00 mg/kg dm soil). If nanomaterials reach the soil, their fate is influenced by different natural processes that may cause homo-aggregation or hetero-aggregation, thus affecting their toxicity (Cornelis et al., 2014). The application of nanomaterials as powder (here CuO-NM) or as a dispersion (here Ag-NM) may also influence these processes by affecting the rate of ion release. Differences in the toxicity of Ag-NMs and CuO-NMs may therefore reflect their physicochemical properties.

The concentration-dependent inhibitory effect observed herein increased during the test period, as previously reported for the inhibition of ammonia-oxidizing bacteria by Ag-NMs (Schlich et al., 2013a; Schlich and Hund-Rinke, 2015). The increasing toxicity of the Ag-NMs during the test period may reflect the long-term release of Ag^+ ions (Schlich and Hund-Rinke, 2015). Ag-NMs are oxidized in the soil and release Ag^+ ions that are toxic towards the soil microflora (Lowry et al., 2012b). This slow ion release model is consistent with the results of the current study, in which repeated applications of Ag-NMs at the low concentration (3 × 0.56 mg/kg dm soil) are less toxic than the equivalent concentration (1.67 mg/kg dm soil) presented as a single application. When the Ag-NMs are applied in a single concentration, the residence time is 84 days, whereas stepwise dosing reduces the residence time of the highest concentration of particles to 28 days if the final application is made on day 56, resulting in the lower release of Ag^+ ions.

The inhibitory effects of Ag-NMs and CuO-NMs on enzyme activity have been reported in previous studies (Josko et al., 2014; Kim et al., 2013; Peyrot et al., 2014; Shin et al., 2012). Citrate-coated Ag-NMs had an adverse effect on urease, acid phosphatase, arylsulfatase and β-glucosidase, representing general microbial activity (Shin et al., 2012). The urease activity (representing the nitrogen cycle) was most sensitive towards Ag-NMs. The effect of polyacrylate-stabilized Ag-NMs has been tested on enzyme activity patterns according to ISO 22939 (Peyrot et al., 2014) using the degradation of 4-MUF-phosphate, 4-MUF-sulfate, 4-MUF-glucopyranoside and L-leucine-AMC to represent the phosphorus, sulfur, carbon and nitrogen cycles, respectively. The degradation of 4-MUF-phosphate (by phosphomonoesterase) and L-leucine-AMC (by leucine aminopeptidase), as also tested in the current study, was significantly inhibited by the Ag-NMs, whereas the degradation of 4-MUF-sulfate and 4-MUF-glyucopyranoside was inhibited to a lesser extent. These studies (Peyrot et al., 2014; Shin et al., 2012) confirm the adverse effects of Ag-NMs on the phosphorus cycle (4-MUF-phosphate) and nitrogen cycle (L-leucine-AMC)

described in the current report. The previously reported sensitivity of urease to Ag-NMs by Shin et al. (Shin et al., 2012) is consistent with the enzyme activity patterns (ISO/TS Guideline 22939, 2010) described herein, because the degradation of L-alanine-AMC and L-leucine-AMC by aminopeptidase, which also is part of the nitrogen cycle, showed the highest sensitivity to the Ag-NMs tested in this study.

CuO-NMs were reported to have an adverse effect on dehydrogenase, urease, acidic phosphatase and alkaline phosphatase activity during an incubation period of 24 days and 196 days in two different soils (Josko et al., 2014). The outcome was proposed to reflect the extended contact time between the CuO-NMs and clays or natural organic matter, or the adaptation of microorganisms to stress factors (Josko et al., 2014). CuO-NMs were also found to inhibit soil enzyme activity (dehydrogenase, acidic phosphatase and β-glucosidase) at a test concentration of 1000 mg/kg dm soil after one week exposure (Kim et al., 2013). The inhibition of phosphatase reported in these studies (Josko et al., 2014; Kim et al., 2013) corresponds to the adverse effects of CuO-NMs on phosphomonoesterase activity, representing the phosphorus cycle in this investigation. Furthermore, the concentration-dependent inhibition of urease activity following a single application of CuO-NMs (Josko et al., 2014; Kim et al., 2013) is comparable to the inhibition of alanine and leucine aminopeptidases reported here. All substrates contain ammonium groups and are representatives for the nitrogen cycle. The extension of the incubation period for CuO-NMs at a concentration of 100 mg/kg dm soil by Josko et al. (Josko et al., 2014) caused the inhibition of urease activity to decline, in agreement with the effect on leucine aminopeptidase presented here. In contrast to the previously reported reduced inhibition of phosphatase activity on day 196 by Josko et al. (Josko et al., 2014), the inhibition of phosphomonoesterase increased over time in this study, which may reflect the different incubation periods of 196 and 84 days and the corresponding differences in contact time.

4.2. Single vs. repeated applications

The repeated application treatments achieved the same final concentrations as the single application treatments but used a stepwise dosing strategy so that the nanomaterials accumulated over a defined period of time. The contact time at the final concentration is therefore shorter for the repeated application strategy compared to the single application, but the repeated application treatment is likely to be more environmentally relevant given the continuous entry of nanomaterials into the environment. The different contact periods between single and repeated applications limits the comparability of both approaches. Comparing the results of the stepwise exposure to the single exposure seems problematic, as the soil microorganisms are exposed to the different concentrations for different exposure periods. Currently the continuous entry into the environment is not considered at all within ecotoxicological testing for regulation. From the authors point of view the testing of a stepwise approach is the only way to investigate if the actual testing regime in accordance to the different guidelines is appropriate also for nanomaterials.

The inhibition of ammonia-oxidizing bacteria (ISO Guideline 15685, 2012) was stronger for the single application, which is the standard procedure described in the test guidelines. In contrast, the repeated application treatments had a stronger impact on substrate-induced respiration (MicroResp™) compared to the single application, although the final test concentrations were identical. These trends were observed for both the Ag-NMs and CuO-NMs. The difference between the systems mainly reflects the microorganisms that are tested. The substrates of the MicroResp™ test system are used as a carbon source by many microbial species, whereas the potential ammonia oxidation activity is limited to a rather small group of mainly autotrophic ammonia-oxidizing bacteria. Respiration activity can be maintained by replacing sensitive microorganisms with more resistant ones, resulting in limited effects following a single disturbance, but repeated disturbances have a more severe effect that is difficult to overcome (Prosser, 2012; Allison and Martiny, 2008). Accordingly, respiration in soil already contaminated with Zn was shown to be more susceptible to further contamination with Cu than a non-contaminated soil (Klimek, 2012). The microbial community in the contaminated soil was already depleted in terms of genetic diversity, which led to more pronounced adverse effects following the additional disturbance. The stronger adverse effects of repeated application reflect the restructuring of a microbial population already weakend in terms of genetic diversity (Allison and Martiny, 2008). However, the ammonia-oxidizing bacteria are a specialized group of microorganisms capable of nitrogen transformation, and are much less diverse than the community of heterotrophic microorganisms. Repeated disturbances targeting the ammonia-oxidizing bacteria appear to have a weaker impact than disturbances targeting the more diverse heterotrophic microorganisms. If the resistance of the ammonia-oxidizing bacteria is extremely high and therefore less influenced by a repeated application cannot be sufficiently explained by the presented data. The enzyme activity patterns (ISO/TS Guideline 22939, 2010) reflect the activity of exoenzymes that are stabilized by soil components (Allison, 2006). The comparable effects of single and repeated applications on the activity of exoenzymes indicate that this activity is less susceptible to the time course and mainly influenced by the total concentration of nanomaterials.

5. Conclusions

The comparability of single and repeated applications of ion-releasing nanomaterials depends on the test endpoint and duration. Repeated applications ultimately resulting in the same test concentrations as a single application do not provide further information if nitrifying microorganisms and exoenzymes are tested. However, differences between single and repeated applications become apparent when substrate-induced respiration is considered. The toxicity of ion-releasing nanomaterials is specific to the material, and is likely to be based on parameters such as the rate of ion release and agglomeration behavior. For regulatory purposes, the test duration is an important factor and should be extended to observe the effects of long-term ion release from nanomaterials.

Overall, the potential ammonia oxidation activity was the test parameter with the highest sensitivity among the three test systems and this provides important information concerning the toxicity and bioavailability of the test material. The MicroResp™ approach indicates the effect of nanomaterials on the microbial community and can report the potential recovery of microbial populations due to the replacement of sensitive, damaged microorganisms with more resistant ones. The enzyme activity patterns reveal the activity of exoenzymes in the bulk soil, and this indicates the rate of ion release by nanomaterials because exoenzyme activity adapts more slowly than the microbes producing the enzymes. The three test systems together therefore provide comprehensive information about the impact of different nanomaterials on the soil microflora and its diversity.

Acknowledgements

This study was funded by the European framework program project SUN (CP-FP 604305) and MARINA (CP-FP 263215). The authors would like to thank Theo Görtz, Katja Mock, Ruben Schlinkert,

Marco Kraas, Katharina Kroll and Maria Brüggemann for assistance with the laboratory work, Dr. Thorsten Klawonn for the performance of chemical analysis, and Dr Richard M. Twyman for editing the manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2016.05.028.

References

Allison, S.D., 2006. Soil minerals and humic acids alter enzyme stability: implications for ecosystem processes. Biogeochemistry 81 (3), 361–373.

Allison, S.D., Martiny, J.B.H., 2008. Resistance, resilience, and redundancy in microbial communities. Proc. Natl. Acad. Sci. U. S. A. 105 (Suppl. 1), 11512–11519.

Benn, T.M., Westerhoff, P., 2008. Nanoparticle silver released into water from commercially available sock fabrics. Environ. Sci. Technol. 42, 4133–4139.

Campbell, C.D., Chapman, S.J., Cameron, C.M., Davidson, M.S., Potts, J.M., 2003. A Rapid Microtiter Plate Method To Measure Carbon Dioxide Evolved from Carbon Substrate Amendments so as To Determine the Physiological Profiles of Soil Microbial Communities by Using Whole Soil. Appl. Environ. Microbiol. 69 (6), 3593–3599.

Cornelis, G., Kirby, J.K., Beakc, D., Chittleborough, D., McLaughline, M.J., 2010. A method for determination of retention of silver and cerium oxide manufactured nanoparticles in soils. Environ. Chem. 7 (3), 298–308.

Cornelis, G., Hund-Rinke, K., Kuhlbusch, T.A.J., Van den Brink, N., Nickel, C., 2014. Fate and bioavailability of engineered nanoparticles in soils: a review. Environ. Sci. Technol. http://dx.doi.org/10.1080/10643389.2013.829767.

Coutris, C., Joner, E.J., Oughton, D.H., 2012. Aging and soil organic matter content affect the fate of silver nanoparticles in soil. Sci. Total Environ. 420, 327–333.

CPI, 2015. Woodrow Wilson Center for Scholars Project on Emerging Nanotechnologies Consumer Products Inventory of Nanotechnology Products. Available from: http://www.nanotechproject.org/cpi.

DaNa 2.0, 2015. Information about Nanomaterials and Their Safety Assessment.

DIN EN Guideline 13346, 2001. Characterization of Sludges - Determination of Trace Elements and Phosphorus - Aqua Regia Extraction Methods. International Organization for Standardization, Berlin, Germany.

Frenk, S., Ben-Moshe, T., Dror, I., Berkowitz, B., Minz, D., 2013. Effect of metal oxide nanoparticles on microbial community structure and function in two different soil types. PLoS One 8 (12), e84441.

Hänsch, M., Emmerling, C., 2010. Effects of silver nanoparticles on the microbiota and enzyme activity in soil. J. Plant Nutr. Soil Sci. 173 (4), 554–558.

Hoppe, M., Mikutta, R., Utermann, J., Duijnisveld, W., Guggenberger, G., 2014. Retention of sterically and electrosterically stabilized silver nanoparticles in soils. Environ. Sci. Technol. 48 (21), 12628–12635.

Hund-Rinke, K., Schlich, K., 2014. The potential benefits and limitations of different test procedures to determine the effects of Ag nanomaterials and AgNO3 on microbial nitrogen transformation in soil. Environ. Sci. Eur. 26 (1).

Hund-Rinke, K., Schlich, K., Klawonn, T., 2012. Influence of application techniques on the ecotoxicological effects of nanomaterials in soil. Environ. Sci. Eur. 24 (30).

Impellitteri, C.A., Harmon, S., Silva, R.G., Miller, B.W., Scheckel, K.G., Luxton, T.P., Schupp, D., Panguluri, S., 2013. Transformation of silver nanoparticles in fresh, aged, and incinerated biosolids. Water Res.

ISO Guideline 11466, 1995. Soil Quality - Extraction of Trace Elements Soluble in Aqua Regia. International Organization for Standardization, Geneve, Switzerland.

ISO Guideline 18512, 2007. Soil Quality - Guidance on Long and Short Term Storage of Soil Samples. International Organization for Standardization, Genf, Schweiz.

ISO/TS Guideline 22939, 2010. Soil Quality - Measurement of Enzyme Activity Patterns in Soil Samples Using Fluorogenic Substrates in Micro-well Plates. International Organization for Standardization, Genf, Schweiz.

ISO Guideline 15685, 2012. Soil Quality - Determination of Potential Nitrification and Inhibition of Nitrification - Rapid Test by Ammonium Oxidation. International Organization for Standardization, Genf, Schweiz.

Josko, I., Oleszczuk, P., Futa, B., 2014. The effect of inorganic nanoparticles (ZnO, Cr_2O_3, CuO and Ni) and their bulk counterparts on enzyme activities in different soils. Geoderma 232.

Kaegi, R., Sinnet, B., Zuleeg, S., Hagendorfer, H., Mueller, E., Vonbank, R., Boller, M., Burkhardt, M., 2010. Release of silver nanoparticles from outdoor facades. Environ. Pollut. 158 (9), 2900–2905.

Kaegi, R., Voegelin, A., Ort, C., Sinnet, B., Thalmann, B., Krismer, J., Hagendorfer, H., Elumelu, M., Mueller, E., 2013. Fate and transformation of silver nanoparticles in urban wastewater systems. Water Res. 47 (12), 3866–3877.

Kim, S., Sin, H., Lee, S., Lee, I., 2013. Influence of metal oxide particles on soil enzyme activity and bioaccumulation of two plants. J. Microbiol. Biotechnol. 23 (9), 1279–1286.

Klein, C.L., Comero, S., Stahlmecke, B., Romazanov, J., Kuhlbusch, T.A.J., Doren, E.V., Temmerman, P.-J.D., Maat, J., Wick, P., Krug, H., Locoro, G., Hund-Rinke, K., Kördel, W., Friedrichs, S., Maler, G., Werner, J., Lingsinger, T., Gawlik, B.M., 2011. NM-Series of Representative Manufactured Nanomaterials NM-300 Silver Characterisation, Stability, Homogeneity (JRC Scientific and Technical Reports).

Klimek, B., 2012. Effect of long-term zinc pollution on soil microbial community resistance to repeated contamination. Bull. Environ. Contam. Toxicol. 88 (4), 617–622.

Lee, Y.J., Kim, J., Oh, J., Bae, S., Lee, S., Hong, I.S., Kim, S.H., 2012. Ion-release kinetics and ecotoxicity effects of silver nanoparticles. Environ. Toxicol. Chem. 31 (1), 155–159.

Levard, C., Reinsch, B.C., Michel, F.M., Oumahi, C., Lowry, G.V., Brown, G.E., 2011. Sulfidation processes of PVP-coated silver nanoparticles in aqueous solution: impact on dissolution rate. Environ. Sci. Technol. 45 (12), 5260–5266.

Lorenz, C., Windler, L., von Goetz, N., Lehmann, R.P., Schuppler, M., Hungerbuhler, K., Heuberger, M., Nowack, B., 2012. Characterization of silver release from commercially available functional (nano)textiles. Chemosphere 89 (7), 817–824.

Lowry, G.V., Gregory, K.B., Apte, S.C., Lead, J.R., 2012. Transformations of nanomaterials in the environment. Environ. Sci. Technol. 46 (13), 6893–6899.

Lowry, G.V., Espinasse, B.P., Badireddy, A.R., Richardson, C.J., Reinsch, B.C., Bryant, L.D., Bone, A.J., Deonarine, A., Chae, S., Therezien, M., Colman, B.P., Hsu-Kim, H., Bernhardt, E.S., Matson, C.W., Wiesner, M.R., 2012. Long-term transformation and fate of manufactured ag nanoparticles in a simulated large scale freshwater emergent wetland. Environ. Sci. Technol. 46 (13), 7027–7036.

MicroResp^{TM}. 2015. www.microresp.com.

OECD Guideline 216, 2000. OECD Guideline for the Testing of Chemicals. Test No. 216: Soil Microorganisms: Nitrogen Transformation Test. Organisation for Economic Co-operation and Development, Paris.

OECD Guideline 217, 2000. OECD Guideline for the Testing of Chemicals. Test Guideline 217: Soil Microorganisms: Carbon Transformation Test. Organisation for Economic Co-operation and Development, Paris.

Organisation for Economic Co-operation and Development, 2007. Safety of Manufactured Nanomaterials - Sponsorship Programme for the Testing of Manufactured Nanomaterials. http://www.oecd.org/science/nanosafety/sponsorshipprogrammeforthetestingofmanufacturednanomaterials.htm.

Peyrot, C., Wilkinson, K.J., Desrosiers, M., Sauve, S., 2014. Effects of silver nanoparticles on soil enzyme activities with and without added organic matter. Environ. Toxicol. Chem. 33 (1), 115–125.

Prosser, J.I., 2012. Ecosystem processes and interactions in a morass of diversity. FEMS Microbiol. Ecol. 81 (3), 507–519.

Quadros, M.E., Pierson, R.t., Tulve, N.S., Willis, R., Rogers, K., Thomas, T.A., Marr, L.C., 2013. Release of silver from nanotechnology-based consumer products for children. Environ. Sci. Technol. 47 (15), 8894–8901.

Reinsch, B.C., Levard, C., Li, Z., Ma, R., Wise, A., Gregory, K.B., Brown Jr., G.E., Lowry, G.V., 2012. Sulfidation of silver nanoparticles decreases Escherichia coli growth inhibition. Environ. Sci. Technol. 46 (13), 6992–7000.

Rousk, J., Ackermann, K., Curling, S.F., Jones, D.L., 2012. Comparative toxicity of nanoparticulate CuO and ZnO to soil bacterial communities. PLoS One 7 (3), e34197.

Schlich, K., Hund-Rinke, K., 2015. Influence of soil properties on the effect of silver nanomaterials on microbial activity in five soils. Environ. Pollut. 196, 321–330.

Schlich, K., Terytze, K., Hund-Rinke, K., 2012. Effect of TiO_2 nanoparticles in the earthworm reproduction test. Environ. Sci. Eur. 24.

Schlich, K., Klawonn, T., Terytze, K., Hund-Rinke, K., 2013. Hazard assessment of a silver nanoparticle in soil applied via sewage sludge. Environ. Sci. Eur. 25 (17).

Schlich, K., Klawonn, T., Terytze, K., Hund-Rinke, K., 2013. Effects of silver nanoparticles and silver nitrate in the earthworm reproduction test. Environ. Toxicol. Chem. 32 (1), 181–188.

Semisch, A., Ohle, J., Witt, B., Hartwig, A., 2014. Cytotoxicity and genotoxicity of nano - and microparticulate copper oxide: role of solubility and intracellular bioavailability. Part. Fibre Toxicol. 11.

Shin, Y.J., Kwak, J.I., An, Y.J., 2012. Evidence for the inhibitory effects of silver nanoparticles on the activities of soil exoenzymes. Chemosphere 88 (4), 524–529.

Vance, M.E., Kuiken, T., Vejerano, E.P., McGinnis, S.P., Hochella, M.F., Rejeski, D., Hull, M.S., 2015. Nanotechnology in the real world: redeveloping the nanomaterial consumer products inventory. Beilstein J. Nanotechnol. 6, 1769–1780.

Voelker, D., Schlich, K., Hohndorf, L., Koch, W., Kuehnen, U., Polleichtner, C., Kussatz, C., Hund-Rinke, K., 2015. Approach on environmental risk assessment of nanosilver released from textiles. Environ. Res. 140, 661–672.

von der Kammer, F., Ferguson, P.L., Holden, P.A., Masion, A., Rogers, K.R., Klaine, S.J., Koelmans, A.A., Horne, N., Unrine, J.M., 2012. Analysis of engineered nanomaterials in complex matrices (environment and biota): general considerations and conceptual case studies. Environ. Toxicol. Chem. 31 (1), 32–49.

Wiechmann, B., Dienemann, C., Kabbe, C., Brandt, S., Vogel, I., Roskoch, A., 2012. Klärschlammentsorgung in der Bundesrepublik Deutschland.