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Macroscopic Observation of Soil Nitrification Kinetics Impacted by Copper Nanoparticles: Implications for Nanotechnology for Micronutrient Fertilizer

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ABSTRACT

The potential agricultural use of metal nanoparticles (NPs) for slow-release micronutrient fertilizers is beginning to be investigated by both industry and regulatory agencies. However, the impact of such NPs on soil biogeochemical cycles is not clearly understood. In this study, impacts of a commercially-available copper NPs on the soil nitrification kinetics was investigated via batch experiments. The X-ray absorption near edge structure spectroscopy analysis showed that the NPs readily oxidized to Cu(II) and strongly retained in soils with minimum dissolution (<1% of total mass). The Cu^{2+} (aq) at 1mg/L showed the beneficial effect on the nitrification, an increase in the rate of nitrification kinetics (V_{max}). However V_{max} was negatively impacted by ionic Cu at 10-100mg/L and CuNP at 1-100 mg/L. The trace metal toxicity of soil nitrifiers seems to be critical in the soil nitrification processes. Among CuNPs, the suppressed nitrification kinetics was concentration dependent at >10mg/L. The reaction products of surface oxidation such as the release of ionic Cu seem to play an important role in suppressing the nitrification process. Considering the potential use of copper NPs as a slow-release micronutrient fertilizer, further studies are needed in heterogeneous soil systems.

1. INTRODUCTION

With the rise of nanotechnology within the past decade, nano-fertilizers have been considered for use in agricultural fields (DeRossa et al. 2010). While this technology continues to advance, the possibility for slow-release micronutrients resulting from the nano-sized solid state of these products is appealing for some agricultural systems. In particular, hydrological regimes impacted by climate change could alter the mobility of micronutrients, influencing the plant growth and microbially mediated biochemical cycles of nutrients (e.g., N, P). The advent of nanotechnology could increase the feasibility of the long-desired agricultural goal of slow-release fertilizers, which are both more cost-efficient and environmentally sound (Lin et al. 2006; Ray et al. 1997). The physical state of nanoparticles (NPs) as nano-sized solid metal rather than dissolved ions has the potential to allow for a controlled release over time in soil solutions. Trace metals such as Cu and Zn, essential micronutrients for crops and microbial growth (Barden et al. 1987), are commonly produced NPs. Like many micronutrients, these metal-based NPs have the potential to be beneficial to plants and/or microorganisms by preventing deficiency; however, an overdose of metal NPs can lead to toxicity. Benefits of metallic Cu(0)NPs (CuNPs) to plants include increased shoot: root ratio in lettuce seedlings (Shah et al. 2009). However, CuNPs can cause unique adverse reactions in plants not accounted for by ionic copper. For instance, seedlings of mung beans and wheat grown on a CuNP-impregnated agar exhibited diminished root and shoot

length beginning at concentrations of 200 mg/L (Lee et al. 2008). Metallic CuNPs are known to cause less oxidative stress on plants than free copper ions; plants take advantage of this fact by synthesizing metallic CuNPs through Cu^{2+} reduction in the rhizosphere (Manceau et al. 2008). In a study of hydroponic zucchini plant growth in the presence of CuNPs at 1,000 mg/L, Stampoulis and coworkers found that metallic CuNPs resulted in a slower rate of plant growth compared to the control. Interestingly, they found that 1,000 mg/L of bulk Cu had approximately the same effect as 10 mg/L $CuNO_3$, and that 1,000 mg/L of CuNPs had a similar effect as 100 mg/L $CuNO_3$ (Stampoulis et al. 2009). This provides further evidence that NPs do not have the same oxidative impact on plant growth as ionic micronutrients. Other metallic micronutrient NPs have shown similar results. At 2000 mg/L, zinc NPs almost completely inhibited root growth of radish, rape, ryegrass, lettuce, and cucumber seedlings with a lesser, but still significant inhibition of corn seedling root growth (Lin et al. 2007). This same study also investigated aluminum NPs, which only diminished root growth in corn, and showed no effect on the other plant seedlings (Lin et al. 2007).

While the direct impact of metal-based NPs on soil bacteria has not been extensively studied, CuNPs have been shown to affect the growth of common environmental bacteria including *Escherichia coli* and *Staphylococcus aureus* in a pure-culture environment, especially at concentrations in excess of 2% w/w (20,000 mg/kg) (Cioffi et al. 2005). Although silver NPs are well-known for their toxicity to microorganisms (VandeVoort and Arai, 2012; VandeVoort, et al. 2014a; VandeVoort et al. 2014b), CuNPs were shown to impact the survival of the common environmental bacteria *E. coli* and *Bacillus subtilis* at slightly lower concentrations (60 mg/L) than AgNPs (70 mg/L) (Yoon et al. 2007). It has been documented that CuNPs are likely available to microorganisms in soil environments. A study by Kumar and coworkers documented a significant difference in substrate utilization compared to the control system by an arctic soil bacterial community when CuNPs were present at 66 mg/kg, indicating a shift in the bacterial community even at this relatively low concentration. This change was not at marked as the change in substrate utilization caused by the same concentration of AgNPs (Kumar et al. 2011). The impacts of other nano-micronutrients have been poorly investigated. Aluminum NPs appeared to have minimal, if any, impact on the metabolic activity of *Vibrio fischeri* (Doshi et al. 2008). In a study of metal oxide NPs, both titanium dioxide and Zn oxide NPs negatively impacted the soil bacterial community of a grassland, as demonstrated through decreased genotype richness, substrate induced respiration, and extractable soil DNA over time periods up to 60 days (Ge et al. 2011). These metal oxide NPs may induce more oxidative impacts than metallic, zero-valent metal NPs.

While these studies suggest both beneficial and toxicological effects of metal NPs, it remains difficult to extrapolate such results to evaluate the potential use of metal NPs as nano-fertilizers since agricultural soils are often ignored in the most of experimental systems. This study aims to investigate the effects of metallic CuNPs as a nano-fertilizer component on the complex nitrogen

cycle in agricultural soils. The nitrogen cycle in soil systems is essential to the growth of successful crop species. In particular, the nitrification process is of particular importance. In the objective of this study was to examine the effect of CuNPs to the soil nitrification kinetics using batch biogeochemical experiments. Metallic Cu(0)NPs were chosen as a model CuNP. One can expect that dissolution and sorption of CuNPs in soils that control the bioavailability of Cu to soil bacteria. For this reason, the dissolution experiments of CuNPs were performed in conjunction with adsorption isotherm experiments of Cu^{2+}(aq) and CuNPs onto soils.

2. Methods and Materials

2.1. Materials

Fresh surface sandy loam soil from the Toccoa series (coarse-loamy, thermic typic Udifluvents) was used in this study, sourced from the Organic Farm in Clemson, South Carolina, USA. Soil was maintained at field capacity for nitrification experiments. For sorption experiments, soil was air dried and sieved to 2 mm prior to use. This soil was limed to pH 6.5 based on exchangeable acidic cation content (i.e., H^+ and Al^{3+}) prior to use and was maintained at field capacity. The near neutral pH was chosen to facilitate the nitrification process. Physicochemical and mineralogical characterization of the soil is discussed in our previous work (VandeVoort and Arai, 2012). Briefly, the soil has a native cation exchange capacity of 7.4 $cmol_c/kg$, contains 1.5% organic matter, and has mineralogy dominated by quartz, kaolinite, hydroxyl interlayer vermiculite, gibbsite, hematite, and goethite. Metallic Cu(0) NPs (average particle size: 35 nm, uncoated) were purchased from Nanostructured and Amorphous Materials, Inc. (Houston, Texas, USA). For the following experiments, CuNP suspensions were freshly prepared for each use in a 2,000 mg/L solution. They were immediately sonified for 30 sec to ensure complete suspension of particles prior to its use. All reagents were prepared with ACS-grade chemicals and Milli-Q distilled, deionized water (18.2 MΩ).

2.2. Copper Nanoparticle Dissolution Experiments

Dissolution of CuNPs was conducted under 0.01 M ionic strength (*I*) using either $NaNO_3$ or Na_2SO_4. To evaluate the ionic Cu toxicity, one should track the extent of Cu dissolution from the CuNP. The dissolution was assessed in these two electrolytes that were used in this study. It was to investigate the impact of nitrate (i.e., a product of nitrification) during the nitrification experiments. Sulfate was used as the background electrolyte for all analyses to prevent nitrate interference with nitrification analyses. Care was taken to ensure complete suspension of particles in the sample media, including a sonification of a fresh CuNP solution prior to each experiment. Copper NP concentrations ranged from 5 to 1,000 mg/L, and solutions were created from stock solutions described above. pH was maintained at 7±0.3, and adjusted with NaOH when necessary. Dissolution experiments were conducted for 48 hrs in batch mode. Samples were centrifuged at 11,000 rpm for 29 min to ensure settling of all solid CuNPs before samples were measured for Cu^{2+} concentration using an ion specific electrode (ISE). A separate set of dissolution experiments were also conducted under the same conditions in the presence of 20 mM MOPSO buffer used for the sorption experiments. Since many organic buffers contain

active sites that could function as potential ligands for Cu^{2+}, and since dissolution experiments maintained stable pH even without the addition of a buffer, the full suite of dissolution experiments was conducted without MOPSO.

2.3. Copper Nanoparticle and Cu(II)(aq) Adsorption Experiments

The sorption of whole CuNPs was tested on Toccoa soil in a batch mode, using a ratio of 1 g soil to 20 mL solution with a reaction time of 2 days. Since the nitrification experiments will be conducted in soil sulurry, it id difficult to track the dissolved Cu and CuNPs because of sorption reaction. To assess the adsorption capacity of soils, the as\dsorption experiments were conducted. Copper NPs were added at concentrations ranging from 5 to 1,000 mg/L. pH was maintained at 7 (±0.3 pH units) using 20 mM 3-Morpholino-2-hydroxypropanesulfonic acid (MOPSO) buffer solution, and ionic strength was maintained at 0.01 M using Na_2SO_4 salt. Solutions were centrifuged at 11,000 rpm for 29 minutes. Stokes' Law was used to calculate this centrifugation time to ensure that larger soil particles would settle out of solution, but any free CuNPs would remain in solution. Aliquot samples were acidified with HNO_3 prior to total Cu analysis using ICP-AES. Aliquots without acidification were also tested for potential dissolved Cu^{2+} using an ISE. A difference between total Cu and dissolved Cu^{2+} was used to evaluate the CuNP sorption. Because of the gradual dissolution of metallic CuNP during the nitrification experiments, sorption experiments of Cu^{2+} was also conducted under the same conditions. Cu(II) solutions as $CuSO_4$ were centrifuged at the same rate to maintain consistency and analyzed for $[Cu^{2+}]$ and $[Cu]_{total}$ using both ISE and ICP-AES methods, respectively. In the final mass balance calculation, it was assured that there was a negligible contribution from the background Cu in soils.

2.4. Batch Nitrification Kinetic Experiments

The kinetic rate of nitrification was assessed through the oxygenated-shaken slurry method (Hart et al., 1994). This slurry method was chosen because it is a well-accepted method in the soil science filed and has been already demonstrated in the soil nitrification experiments using the same soil (Masrahi et al. 2014). The maximum nitrification rate, (V_{max}), is determined over a 24-hr period. This value was used as an indicator to estimate the nitrification in these soils.

To maintain bacterial populations, a nutrient solution containing 1 mM $NH_4H_2PO_4$ and 0.25 mM $(NH_4)_2SO_4$ was created to ensure that NH_4^+ was the limiting nutrient. This solution was maintained at an ionic strength of 0.01 M using Na_2SO_4. Each sample was housed in a 125 mL Erlenmeyer flask. To each sample, 50 mL of the solution described above was added to 9 g soil, which had been limed and was maintained at field capacity. Copper NP flasks were dosed with the appropriate amount of freshly-prepared CuNP stock solution to achieve concentrations of 1, 10, or 100 mg/L CuNPs. Ionic Cu flasks were dosed with an appropriate amount of 1,000 mg/L $CuSO_4$ as Cu^{2+} solution to achieve 1, 10, or 100 mg/L as $[Cu^{2+}]$. Based on preliminary experiments, 100 mg/L Cu^{2+} samples were not feasible as no nitrification was observed. Concentrations of 1 mg/L Cu^{2+} were also avoided as this was not above the background concentration of copper in this soil. Each flask was sealed with vented Parafilm to allow gas exchange and was placed on an orbital shaker at 180 rpm. This vigorous shaking maintained the oxygenated environment throughout the experiments. 10 mL samples were obtained at 2, 4, 22,

and 24 hrs. At each sampling time, suspensions were centrifuged at 8,000 x *g* for 8 min, and the supernatant frozen until nitrate analysis was conducted. Each condition was replicated a minimum of eight times to ensure accurate results.

Nitrate was analyzed through a salicylic acid colorimetric technique (Cataldo et al., 1975). Subsamples of 0.80 mL held in 8 mL glass cuvettes were reacted with 0.32 mL of 5% salicylic acid dissolved in sulfuric acid, followed by 7.6 mL of 1.7 M NaOH. After solutions cooled for 30 min, they were measured for absorbance at 420 nm using a spectrophotometer, and concentration was determined using a standard curve. Based on solution volume and soil mass, concentrations were converted to units of mg N/kg soil.

2.5. Statistical Analysis of Batch Nitrification Kinetic Experiments

The V_{max} of each sample was determined from the linear regression analysis of nitrate concentration per kg soil per hour. An overall one-way analysis of variance was conducted to determine whether the variance between groups was greater than the variance within groups. To compare individual conditions, two-tailed t-tests were used to determine significant differences.

2.6. X-ray Absorption Near Edge Structure Spectroscopy (XANES) Analysis

To better understand the changes in Cu speciation of Cu(0)NPs in water and soils, XANES analysis was conducted at beamline X11A at National Synchrotron Light Source (NSLS), Upton, NY. The monochromator consisted of two parallel Si(111) crystals with a vertical entrance slit of 0.4 mm and a horizontal entrance slit of 1cm. Using the CuNP dissolution method, freshly hydrated (~3hrs) CuNPs in 0.1M $NaNO_3$ solutions were centrifuged and loaded on a mylar tape. A soil sample was prepared using the batch nitrification method (total Cu as CuNP: 500 mg/kg). After 4mo under oxic condition, a centrifuged soil sample was loaded on a polycarbonate sample holder covered with a 0.2 mm poly film on the front according to the method described in our previous work (Arai, 2011). The concentration was chosen to meet the detection limit of XAS measurements at a bending magnet BL X11A at NSLS. An incident of X-ray beam was calibrated at the first inflection point of the first derivative peak of a Cu foil spectrum at 8979eV. The Cu K-edge XANES spectra were collected at 8900-9200 eV in fluorescence mode at room temperature using a Ge13 detector. Reference spectra of Cu reference compounds ($Cu(I)_2O$, Cu(II)O, and $Cu(OH)_2$) (Sigma, Mo, USA) and unreacted Cu(0)NP were also collected in transmission mode. All samples were loaded in a N_2 filled glove bag right before the measurements. Total of two to three spectra were collected for each soil sample. No beam induced reduction was observed during the measurements. The XAS data were normalized according to the method described in the previous work (Arai, 2011). Because of the formation of insoluble Cu compounds, the XANES data were processed using the linear combination of reference compounds fit the data range of 8920– 9100 eV. In this analysis, the self-absorption correction function in SIXpack was used and no negative fit (Webb, 2005). The energy shifts of reference compounds were not allowed during the fit.

3. Results and Discussion

3.1. Copper Nanoparticle Dissolution Experiments

Dissolution experiments of CuNPs were conducted in sulfate media because an additional nitrate input to nitrification experiments could alter a baseline in evaluating nitrification kinetics. For this reason, sulfate-based salts were used for the background electrolyte in the nitrification experiment. We anticipate both nitrate and sulfate are commonly present in soil solutions. A comparison of dissolution data in two electrolytes is shown in Fig 1. At low concentrations (< 10mg/L), CuNPs displayed maximum dissolution of approximately 0.4 mg/L (~8%) under a sulfate background and approximately 0.2 mg/L (~4%) under nitrate background (Fig 1). Under both nitrate and sulfate backgrounds, as the total concentration of CuNPs increases, the percent dissolved Cu decreases predictably. The small difference in dissolution extent between nitrate and sulfate backgrounds only shows a significant difference at very high $[Cu]_{total}$ (500 mg/L) concentrations. The sulfate background promoted CuNP dissolution to a greater extent (~0.9 mg/L dissolution) than did the nitrate background (~0.35mg/L dissolution), as shown by a 2-tailed t-test ($p < 0.05$). The extent of CuNP dissolution is important because the release of Cu ions are associated with the production of reactive oxygen species and as well as DNA damage in bacteria (Bondarenko et al., 2012).

It was found that sulfate ion resulted in slightly heightened Cu^{2+} dissolution from CuNPs at very high [Cu] (Fig 1). A similar trend has been observed by other researchers. In a study of CuO microparticles (<80 μm), 0.5 M H_2SO_4 resulted in a higher kinetic rate of dissolution of the particles when compared to the same concentration of HNO_3, and displayed a lower activation energy for dissolution (Habbache et al., 2009). With very high [Cu] in the 500 mg/L CuNP experiments, it is likely that these effects are magnified. The effects of sulfate on CuNP dissolution could also be due to the higher affinity of Cu^{2+}, a borderline acid on the hard/soft acid/base scale, for sulfur-containing compounds.

Copper and CuO NPs have been reported to have a wide range of dissolution values, indicating that the rate and extent of dissolution is impacted not only by the background ions of the solution, but also by a variety of particle-specific factors such as diameter, production quality, and initial amount of surface-sorbed Cu^{2+} (Misra et al., 2012). For this reason, a careful analysis of CuNP sorption is essential in evaluating the toxicity to soil biota. A study by Griffitt et al. (2007) found ~25% dissolution of CuNPs (particle size: 80-450 nm, specific surface area: 30.77 m^2/g) after 48 hrs at concentrations of 1.25 mg/L (Griffitt et al., 2007). The pattern of diminished Cu^{2+} dissolution with increasing CuNP concentration was noted by Baek and An (Baek et al., 2011) while studying CuO and other metal oxide NPs, providing evidence of particle-specific factors, rather than simply dissolution, controlling NP toxicity (Baek et al., 2011). The relatively low extent of dissolution of these CuNPs, in addition to their toxicity even at low concentrations suggests that the toxicity of these NPs may be due more to whole-particle effects rather than due to the evolution of Cu^{2+} ions from the surfaces of these particles, as also suggested by other researchers studying CuO NPs (Baek et al., 2011). This is in contrast to the toxicity pattern displayed by silver NPs under both oxidizing and reducing conditions on the same soils studied here. The AgNPs appear to exhibit toxicity largely based on the amount of Ag^+ that is dissolved from their surfaces (VandeVoort and Arai, 2012; Masrahi et al. 2014). Overall, the CuNPs used in our sorption and nitrification experiments appear to be quite stable, compared to other commercially-available forms.

3.2. CuNP and Cu^{2+}(aq) Sorption Experiments

The results of the sorption experiments are shown in Fig 2, which quantifies the amount of Cu sorbed onto soil surfaces compared to the amount of total copper free in solution. All CuNP sorption samples (Fig 2a), when measured for Cu^{2+} on the ISE, were below the detection limit. The instrument was calibrated to measure concentrations as low as which is 0.0006 mg/L. No plateau in CuNP sorption was reached through this experiment. The isotherm displays a nearly linear shape up to C_{eq} = 0.5 mg/L, and the trend became a slightly non-linear at C_{eq} >0.5 mg/L.

Ionic Cu^{2+} displayed an S-curve isotherm, as shown in Fig 2b. Again, no plateau in Cu^{2+} sorption was reached through this experiment. Both sorption isotherms were modeled using the pseudo-equilibrium Freundlich model, as shown in Figs 2c and 2d, and quantified in Table 1. The affinity of CuNP for soil surfaces was extremely large as compared to the affinity of Cu^{2+} for soil surfaces, as indicated by the K_d values for both.

In the presence of soils, the dissolution of CuNPs may be different from what is experienced under more controlled aqueous conditions. While Cu^{2+} was measured in all sorption samples using an ISE, the concentration was below detection limit or ISE (detection limit = 0.0006 mg/L Cu^{2+}) or no significant concentration was present in soil solution. It is likely that dissolved Cu^{2+} from the CuNP surfaces was adsorbed in soils and or formation of insoluble compounds. In general, metallic NPs exhibit strong sorption onto soil surfaces, or indeed, any solid surface with which they come into contact e.g., (VandeVoort and Arai, 2012; Darlington et al. 2009). For this reason, it was not surprising to see the strong sorption of CuNPs onto Toccoa soils (linear range C_{eq} up to 0.5 mg/L in Fig 2). A slightly non-linear increase at C_{eq} >0.5 mg/L might indicate the aggregation of CuNPs in soil particles. In our previous AgNPs sorption investigation in the same soils, the high retention capacity of uncoated AgNPs in the same soils was attributed to the sorption of AgNP aggregates in soils (VandeVoort et al. 20014b).

In contrast to the CuNPs, Cu^{2+} displays moderate sorption in the soils (Fig 2 and Table 1). The beginnings of an S-shaped sorption isotherm displayed by Cu^{2+} onto the soil (Fig 2b) is common for Cu^{2+} ion onto mineral soil, as has been noted by other researchers, though it appears that we have not yet reached the sorptive capacity of Cu^{2+} onto this soil. This indicates that while the sorptive capacity of soil organic matter may have been reached, there are additional sites on the mineral components of the soil for Cu^{2+} sorption (Sposito, 2008).

3.3. XANES analysis

In order to better interpret the nitrification potential results below, XANES measurements were conducted to see the potential oxidation of Cu(0) during hydration and the nitrification experiments in soils. It is important to note that XAS spectra of soil without CuNPs were also taken using a GE 13 detector, and the results show negligible edge jump. This suggests that the spectra of CuNP reacted soil represent the Cu speciation of CuNPs in soils. Fig. 3 and Table- 2 show the results of LC fit XANES analysis of freshly hydrated Cu(0)NPs and aged CuNPs in soils. The results of the LC fit was mainly contributed by CuNPs, Cu(II)O and $Cu(OH)_2$ and $Cu(I)_2O$. $CuSO_4$, $CuCl_2$, and $Cu(NO_3)_2$(aq) were also considered in the fit, however, the fit excluded these fractions. It is clear that Cu(0)NPs were contained Cu(I) prior to the hydration

(vertical dashed line A in Fig 3). The NPs were further readily oxidized in 0.1M NaNO3 to Cu(I) and Cu(II) forming oxide and hydroxide phases. Approximately 51% was the original CuNPs following by $Cu(OH)_2$, CuO and Cu_2O. The partially oxidized CuNPs was further oxidized to Cu(II) in soils. After 4 mo, only 39% of NPs have remained, and the rest was Cu(II) species (a vertical line B in Fig 3).

3.4. Batch Nitrification Kinetic Experiments

The results of a one-way ANOVA showed that the source of variance was largest between conditions, rather than within conditions ($p < 10^{-10}$). The variance of each condition is shown in Fig 4. Strong nitrification was observed in the control condition (Fig 5) along with a linear correlation between the concentration of nitrate as N and time, as documented by the high R^2 value. Experimental conditions ranged from strong, linear nitrification, as in 1 mg/L Cu^{2+} (Fig 6d) to a variable, non-linear relationship between the concentration of nitrate as N and time, as in 100 mg/L Cu^{2+} (Fig 6f). Full results and V_{max} values are shown in Fig 6 and Table 3.

Based on the ANOVA results, sources of variance between individual conditions were assessed using two-tailed t-tests. All Cu additions, with the exception of 1 mg/L Cu^{2+}, negatively impacted the ability of the native soil organisms to complete nitrification in this soil as compared to the control, $p < 0.05$ (Fig 6, Table 4). Due to large variations in results, some conditions were repeated more than the standard eight times (i.e., Control and 10 mg/L CuNP). Within the CuNP conditions, the V_{max} values of 1 and 10 mg/L CuNP conditions were not significantly different from one another, while they were both significantly lower than the control (Table 4). Similarly, within the Cu^{2+} conditions, the V_{max} values of 10 and 100 mg/L Cu^{2+} did not significantly differ from one another but were both significantly lower than the control (Table 4). The V_{max} of 100 mg/L CuNP was the highest of all of the CuNP conditions, significantly higher than that of the 1 or 10 mg/L CuNP values. It was significantly lower, however than the control V_{max} value. Amongst the CuNP conditions, V_{max} increased as CuNP concentration increased. Within the Cu^{2+} conditions, V_{max} decreased as Cu^{2+} concentration increased.

3.5.Impacts of Cu^{2+} and CuNPs on soil nitrification

To evaluate the effect of CuNPs on the nitrification process, the effects of Cu^{2+} is first discussed because dissolved Cu^{2+} is a dissolution product of CuNP in soils. Because of any soil experiments exhibit some standard deviations in the results, the statistical analysis was carefully conducted using a large group of nitrification experiments. As the XANES analysis indicated, more than 50% of Cu in the NPs is present as Cu(II). At 1 mg/L Cu^{2+}, the rate of nitrification kinetics gradually (though not significantly) increased (Figs 5 and 6d, Table 3). This trend supports the importance of Cu as a micronutrient in soils. The nitrifiers were supplied with an essential micronutrient through this addition, instead of a toxicant. Low concentrations of Cu^{2+}, under 10 mg/L, have been shown to enhance microbial growth in activated sludge, a community with a strong component of nitrifying bacteria (Cabrero et al., 1998). Copper has also been suggested to provide micronutrient levels of Cu^{2+} to bacteria at low concentrations (Raffi et al., 2010).

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However, the toxicity observed from 10 mg/L Cu^{2+} was greater than would be expected from the literature values (Lees, 1948; Fait et al. 2006). As shown in Table 3, soils treated with 10 mg/L Cu^{2+} displayed a significantly lower V_{max} value than the control. At 100 mg/L Cu^{2+}, nitrification essentially stopped in some replications and continued at a significantly lower rate in others. This high variability is shown in the varied data shown in Fig 5f and Table 3. The low R^2 value also indicates high variability and a potentially toxic condition, at least in some replications. This result is to be expected, as this high concentration will likely pose toxicity to the vast majority of bacteria in the batch reactor (Lees, 1948; Fait et al., 2006). Because of the high concentration, there is no statistical difference in V_{max} between 10 and 100mg/L Cu^{2+} (Table-4).To begin to impact the nitrification process, the Cu^{2+} concentration in the soil must be quite large. A 1948 study by Lees documented a 13% inhibition of nitrification when a solution containing 64 mg/L Cu^{2+} was percolated through the soil, also noting that Cu^{2+} showed some toxicity (Lees, 1948). Similarly, in a more recent study, it was documented that Cu^{2+} required a relatively high concentration (above 250 mg/kg) to cause at least half of the soil bacterial nitrifying community to be affected (Fait, Broos et al., 2006). In summary, soils treated with ≥10 mg/L Cu^{2+} displayed a significantly lower V_{max} value than the control.

Through the course of the nitrification experiment, all CuNP conditions exhibited significantly lower V_{max} values than the control condition (Table-3 and - 4). The overall negative response of CuNPs is consistent with other observations in the literature (e.g., Raffi et al. 2010). In this study, there is no statistical difference in V_{max} between CuNP 1 and 10 mg/L (Table-4). But statistical difference was observed between 10 and 100 mg/L systems (Table-4). The concentration independent effects were observed. Shah and Belozerova (2009) investigated the impact of CuNPs (Shah and Belozerova, 2009), among other metallic NPs, on the overall soil microbes in situ, based on the microbial use of various substrates. They found that CuNPs negatively impacted the ability of microorganisms to utilize some commonly available substrates at concentrations of CuNPs as low as 130 mg/kg.

Although we observe the variations in V_{max} in the CuNP data with respect to the ionic Cu data, the statistical analysis (Table-4) showed that the toxicity of the CuNP is not statistically different from the respective concentration of the ionic Cu^{2+} system. With increasing the concentration of CuNP, what is offsetting the similar toxicological response in the kinetic rate of nitrification, V_{max}? The concentration of dissolved Cu^{2+} is always greater, though nearly all Cu ions undergo adsorption in soils, in the ionic Cu systems compared to the respective CuNP system. The reasonable explanation is the potential production of reactive oxygen species (ROS) from CuNP. The surface oxidation enhanced ROS production has been reported by several researchers (Bondarenko et al., 2012; Shi et al., 2012). Although the detection of ROS was difficult in our soil slurry systems because of interference from dissolved organics and other ions in soil filtrates, ROS via the surface oxidation of CuNP cannot be ignored. This could explain the similar suppressed V_{max} among the same concentration of Cu^{2+} and CuNP.

4. Conclusion

This study evaluated the effects of metallic CuNPs, as a nano-fertilizer, on the soil nitrification process. While CuNPs strongly retained in soils, they showed negative effects on nitrification kinetics ay 1-100 mg/L. As evident in the XANES analysis, the metallic Cu(0)NP readily

oxidized to Cu(II)-oxide and –hydroxides with increasing aging time under oxic condition. The dissolution of Cu^{2+} as well as potential ROS production could explain the suppressed nitrification kinetic rate. A window of [Cu^{2+} (aq)] for a beneficial effects to soil nitrifer seems very small. The results suggest the delivery of Cu incorporated nano-fertilizer must be carefully evaluated with respect to its trace metal toxicity to microorganisms in various agricultural soils.

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Abbreviations

CuNP: Copper nanoparticles

ISE: Ion selective electrode

MOPSO: 3-Morpholino-2-hydroxypropanesulfonic acid

NP: nanoparticles

XAS: X-ray absorption spectroscopy

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Table 1. Freundlich equation isotherm parameters for Freundlich models shown in Figs 2c and 2d. K_d indicated distribution constant for the adsorbant, calculated from the inverse log of the intercept. *n* is the conversion factor, calculated from the inverse of the slope.

Copper species	Intercept	K_d	Slope	*n*	R^2
CuNP	4.646	44,210	1.362	0.7341	0.9782
Cu(II)SO_4	2.426	266.5	0.6088	1.642	0.9644

Table-2: The results of linear combination of reference compound fit of Cu K edge XANES spectra shown in Fig 3.

Sample	% Cu(0)NP	% Cu(II)O	%$Cu(I)_2O$	%$Cu(OH)_2$	Reduced Chi square
		Bulk XAS Analysis			
Freshly hydrated Cu(0)NPs	51.19±0.5	20.98±0.5	22.4±0.5	6±0.5	0.00101
4mo aged Cu(0)NPs in soil	39.04±0.7	11.03±0.7	---------	52.3±0.7	0.00063

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Table 3. Nitrification kinetics V_{max} and linear regression values for all conditions.

$Cu]_{total}$ and Cu species	N	Average V_{max} (mg NO_3^--N kg $soil^{-1}$ hr^{-1}) value	Average intercept of linear regression	R^2 of linear regression line
Control	17	0.645(±0.190)	2.62	0.961
1 mg/L Cu^{2+}	9	0.703(±0.236)	11.7	0.908
10 mg/L Cu^{2+}	8	0.247(±0.0430)	10.4	0.947
100 mg/L Cu^{2+}	8	0.279(±0.224)	16.0	0.0837
1mg/L CuNP	12	-0.0489(±0.205)	15.1	0.132
10 mg/L CuNP	20	0.132(±0.347)	76.8	0.757
100 mg/L CuNP	13	0.400(±0.163)	4.90	0.911

Table 4. *p* values from a t-test matrix of average kinetic rate (V_{max}) for each condition compared to one another. Squares marked "x" indicate a duplicate t-test, and are not included.

$[Cu]_{total}$ and Cu species	Control	1 mg/L CuNP	10 mg/L CuNP	100 mg/L CuNP	1 mg/L Cu^{2+}	10 mg/L Cu^{2+}
1 mg/L CuNP	0.000285	x	x	x	x	x
10 mg/L CuNP	<0.0001	0.0637	x	x	x	x
100 mg/L CuNP	0.00106	0.00255	0.00588	x	x	x
1 mg/L Cu^{2+}	0.534	<0.0001	<0.0001	0.00506	x	x
10 mg/L Cu^{2+}	<0.0001	<0.0001	0.565	0.00227	0.00035	x
100 mg/L Cu^{2+}	0.0253	0.0424	0.382	0.382	0.0152	0.804