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Components from wheat roots modify the bioactivity of ZnO and CuO nanoparticles in a soil bacterium

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ABSTRACT

ZnO and CuO nanoparticles (NPs) have widespread commercial uses and their impact on agricultural systems is unresolved. This study examined whether the metabolites washed from wheat (*Triticum aestivum* L.) roots modulated the metabolic response to the NPs of a biosensor generated in the root colonizer, *Pseudomonas putida* KT2440. The root wash components boosted light output of the biosensor consistent with their catabolism. Dose-dependent and rapid inhibition of cell metabolism occurred with both ZnO and CuO NPs in water suspensions but high light output was maintained in root wash. Root wash also protected biosensor output in challenges with Zn ions. However the root wash components did not protect culturability or biosensor light output upon exposure to Cu ions. Imaging by atomic force microscopy suggested that root wash materials coated the NPs. We deduced that the response of a microbe to these metal oxide NPs could be negated by components released from roots.

1. Introduction

Zinc oxide (ZnO) and copper oxide (CuO) nanoparticles (NPs) are used in many commercial applications including antimicrobial formulations. Recent studies suggest the use of these NPs as fungicides in agriculture and the food industry: treatments with ZnO or CuO NPs inhibit growth of fungal plant pathogens, *Botrytis cinerea*, *Penicillium expansum*, *Fusarium graminearum and Phytophthora infestans* (He et al., 2011; Dimkpa et al., 2013a; Giannousi et al., 2013). Consequently, although NPs may be formulated for use in agriculture as crop protectants, their impact on nontarget soil microbes is not fully known.

Both CuO and ZnO NPs cause bacterial cell death at doses that vary with the microorganism (Dimkpa et al., 2011b; Gajjar et al., 2009; Jones et al., 2008; Liu et al., 2009; Mortimer et al., 2008; Ren et al., 2009). Commercial sources of CuO and ZnO NPs result in dose-dependent and rapid changes in light output from a microbial biosensor constructed in the beneficial, root-colonizing bacterium, *Pseudomonas putida* KT2440 (Gajjar et al., 2009). Energy status in this bacterium is indicated by the degree of light output from a fusion of *luxAB* genes, encoding a luciferase, with a heavy metal-activated promoter (Miller et al., 2009). The decline in light output with CuO NPs and ions (0.1–10 mg Cu/L) during a 60 min treatment is consistent with cellular toxicity as shown by assessment of cell culturability (Gajjar et al., 2009). Culturability assays show that *P. putida* KT2440 biosensor (Gajjar et al., 2009), as well as another beneficial root-colonizing strain, *P. chlororaphis* O6 (Dimkpa et al., 2011b), has a high tolerance in short term studies to both ZnO NPs and Zn ions. With ZnO NPs, light output from the biosensor increases at low doses (<1 mg Zn/L) followed by a decline as dose increased to >7 mg Zn/L in 60 min exposure studies (Gajjar et al., 2009). With Zn ions, 0.5 mg Zn/L activates yet 1 mg/L inhibits light output. We associate increased light output to metal activation of the promoter when the concentration of ions is below the toxic level (Gajjar et al., 2009; Miller et al., 2009).

We are interested in understanding how factors in soils will influence NP responses in plant-associated microbes. In this paper we address how components released from plant roots influence the responses of the biosensor to ZnO and CuO NPs. To our knowledge, there are no published studies where researchers have examined the effects of soil components from exudation from plant roots on the activity of plant-associated microbes when challenged by NPs. We obtained these metabolites from the roots of wheat (*Triticum aestivum* L.) seedlings. They would be available as nutrients to the bacteria in the rhizosphere (Child et al., 2007). Root exudates are demonstrated to influence the rhizosphere populations (Yang and

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Crowley, 2000; Jin et al., 2006; Haichar et al 2008). Also, certain metabolites from the root surfaces are likely to change the bioactivity of metals because of their ability to form complexes with free metal ions (McLean et al., 2013). Thus the responses of the bacterial cells may differ when they are suspended in root wash components than in water where metal complexation would be minimal.

To determine how the root wash components interacted with the NPs, imaging was performed with atomic force microscopy (AFM) to detect changes in particle size. Also modifications to the surface charge of particles were measured for the NPs suspended in root wash, because neutralization of charge is associated with agglomeration of particles (Jiang et al., 2009; Bian et al., 2011; Zhao et al., 2012; Dimkpa et al., 2013a). Ion release from metal oxide NPs is demonstrated to be important in their microbial reactivity (Dimkpa et al., 2011b). Consequently to understand the contribution of Zn and Cu ions released from the NPs to the responses, the soluble metals present in the cell suspensions after the biosensor assay was determined. The root wash material was assayed for its inorganic and organic composition to allow modeling of free ion versus the complexed metal to better understand bioreactivity. Biosensor assays were run with NPs and ions in water as a control and in the root wash to determine the effect of the components released from plant roots on the response of the microbe.

2. Materials and methods

2.1. Characterization of NPs

The CuO and ZnO NPs were obtained from Sigma Chemical Company (MO, USA). The metal composition of the NPs, determined after acid digestion by inductively-coupled mass plasma spectrometry (ICP-MS), is reported in Dimkpa et al. (2012). Aggregation of these NPs in water has been demonstrated by dynamic light scattering (Dimkpa et al., 2012) and by field flow fractionation techniques (Gajjar et al., 2009). AFM analysis of freshly prepared suspensions in root wash followed the procedures as described in Dimkpa et al. (2011a) with a Nanoscope III Bioscope (Digital Instruments, Inc.). Determination of colloid charge in root wash involved measurements of zeta potential as described by Dimkpa et al. (2011b).

Metal release from the NPs during the bioassay was determined by ICP-MS after incubation of cells in water or root wash for 60 min. The bioassay suspensions were centrifuged and the ICP-MS assays were run on the supernatants generated by two successive centrifugations of the NP suspensions at 15,500 g for 30 min. Centrifugation at this time and speed would remove NPs as small as 2 nm radius based on the size and density of the NPs and the viscosity of the liquid using the equation developed by Svedberg and Nichols (1923). Zinc and Cu concentrations in the supernatants were analyzed by ICP-MS (Agilent 7500c).

2.2. Root wash preparation and characterization

Root wash was prepared from washing, with sterile distilled and deionized water, the roots of 7-day old wheat seedlings grown in sterile vermiculite (Dimkpa et al., 2013b). The root wash was analyzed for pH (Method 4500H, APHA, 1999), electrical conductivity (EC) (Method 2510 B, APHA, 1999), trace elements by ICP-MS (Method 3125, APHA, 1999), and major cations and anions by ion chromatography (Dionex IC 3000) (Method 4110 B, APHA, 1999). Volatile fatty acids were analyzed using ion chromatography (Dionex, Application Note 123) with a gradient elution from 1 mM to 60 mM NaOH. A trap column (Dionex, IonPac ATC-HC), guard column (Dionex, AG11), analytical column (Dionex, IonPac AS11), and Anion Self-Regenerating Suppressor (Dionex, ASRS-ULTRA), prepared the sample for measurement with an EC detector (Dionex, CD20). Dissolved organic carbon was determined by combustion with IR detection (Method 5310B, APHA, 1999) using a Teledyne Tekmar Apollo Combustion TOC Analyzer. Protein content was determined using Micro BCA Protein Assay Kit (Thermo Scientific, USA) with bovine serum albumen as the standard. Neutral sugars were determined using the phenol-sulfuric acid method (Dubois et al., 1956) with glucose as the standard. The concentration of free ion and complexed Cu and Zn when added at defined levels to root wash was modeled using the thermodynamic geochemical computer program GEOCHEM (Parker et al., 1995).

2.3. Biosensor and culturability studies

The *P. putida* biosensor strain was stored in 15% glycerol at −80 °C and grown to stationary phase in liquid minimal medium (MM) (Gajjar et al., 2009). After regrowth of overnight cultures in MM, cells suspended to 1×10^8 cells ml^{-1} in sterile deionized distilled water or root wash were examined for light output. The NPs or ions at defined concentrations (20, 200 and 250 mg Zn/L from ZnO NPs; 10, 20, 250 mg Cu/L from CuO NPs; 10, 20, 200 mg Zn/L from Zn ions or 0.1, 0.3, 0.5 mg Cu/L as Cu ions) were added directly to the water or the root wash immediately before adding the cells. Data were collected at ten minute intervals for 60 min in a LMaxII384 Molecular Device Luminometer (Molecular Devices Corporation, Sunnyvale CA) with Softmax Pro V. 4.7 software. Each treatment was performed in at least three different experiments each with triplicate readings of one sample. Culturability of the cells after 60 min exposure was determined by dilution plating onto LB agar medium and counting colonies after 2 days incubation at 26 °C.

2.4. Statistical analysis

A one-way analysis of variance (ANOVA) (OriginPro 8.6) was used to determine significant differences among treatments involving different dilutions of root wash. A Tukey's means comparison was performed to further explore differences with a significant ($p \leq 0.05$) ANOVA result. Data relating to the effects of NPs and ions in water or root wash is presented as means with standard deviation resulting from three replicates. Because of the complexity of these bioassays, the pattern of response is discussed. The significance of the data is described by overlap of the error bars.

3. Results

3.1. Characterization of the NPs: evolution of NP size in root wash and solubility of ions

Analysis of the root wash revealed that a mixture of inorganic and organic materials were present; soluble Cu and Zn were detected (Table 1). The organic materials included proteins, sugars, organic acids and short chain fatty acids that are typical of fermentation products (Table 1). Suspension of the NPs in 1:10 diluted root wash at pH 7.2, reduced the charge on visible colloids: CuO NPs produced colloids with charges of −22.0 mV in water and −14.0 ± 1.8 mV in root wash; for ZnO NPs, the charge in water was −24.0 mV and in root wash −10.9 ± 1.9 mV. AFM analysis (Fig 1) confirmed the presence of individual particles of nanosize in water suspensions, but also showed formation of agglomerates in the presence of root wash. Together with the reduction in surface charge, these images suggested that the root wash materials likely coated and caused aggregation of the NPs).

3.2. Release of ions from NPs

The levels of soluble Zn and Cu at the end of the 60 min-bioassays performed in water and in root wash suspensions are

Table 1
Root wash analysis. Data are the mean of two independent assays of the root wash with standard deviations. Metals assayed but detected at or below detection limit were Al, V, Mn, Co, Ni, As, Se Cd, Pb. Trace levels of lactate, butyrate, valerate, and fumarate were detected.

Compound or element	mg/L
Total organic carbon	2600 ± 300
Neutral sugars	119 ± 5.3
Protein	137 ± 31.2
pH	9.2
EC (mS/cm)	2.14
Cr	0.11 ± 0.01
Fe	0.10 ± 0.01
Cu	0.12 ± 0.01
Zn	0.15 ± 0.04
Ca	20 ± 6
Mg	34 ± 4
Na	332 ± 28
K	76 ± 15
Phosphate	2.5 ± 1.6
Chloride	482 ± 5.7
Nitrate	2 ± 0.6
Acetate	51.5 ± 7.9
Succinate	28.1 ± 3.9
Malate	33.8 ± 3.5
Oxalate	3.7 ± 0.5
Isocitrate	12.7 ± 1.9
Isobutyrate	68.6 ± 9.6
Isovalerate	6.2 ± 1.1

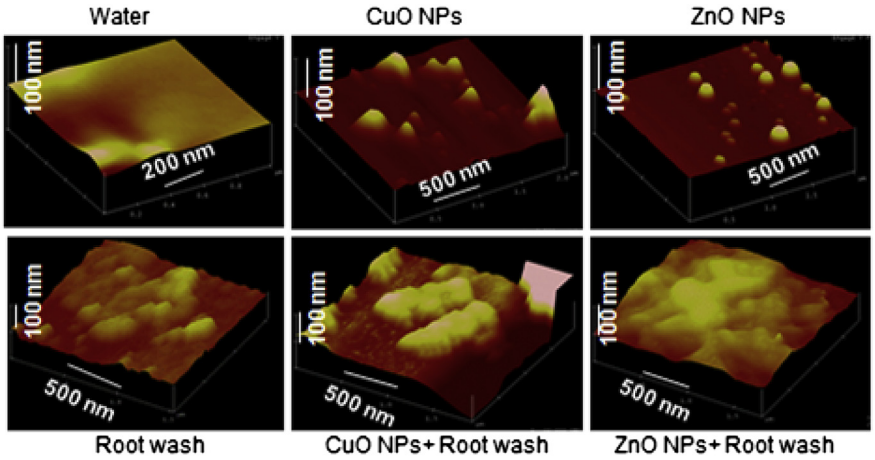

Fig. 1. A. AFM images of CuO NPs and ZnO NPs from suspensions of 500 mg metal/L in water or in 1:10 diluted root wash after 60 min. Controls for water and the root wash are shown. Images are typical of at least 10 different views for each preparation. Bars show dimensions in nm.

shown in Table 2. The levels of Cu ranged from 0.39 to 1.2 mg/L and for Zn 0.4–3 mg/L: the presence of root wash increased soluble Cu with addition of 250 mg Cu/L as NPs, but decreased soluble Zn levels at the two lower concentration of NPs tested. Contributions of Zn released from impurities in the CuO NPs (<0.001 mg/L) and Cu released from impurities in the ZnO NPs (0.015 mg/L) would not have an impact on the biosensor (Dimkpa et al., 2012). Geochemical modeling (GEOCHEM-PC, Parker et al., 1995), based on the materials detected in the root wash (Table 1), and metal concentrations similar to the soluble levels measured from the bioassays (Table 2), indicated that a majority of Zn in the cell suspensions would remain as the free ion whereas Cu would be chelated with isocitrate (Table 3).

3.3. Biosensor analyses: effects of exposure to root wash

Cells of the biosensor produced light, measured as relative light units (RLU), at higher levels than in water when suspended in root wash at 1:5–1:50 dilutions during the 60 min time course. Dilution of the root wash by 100-fold did not increase light output Fig. 2 over the control value until 40 min. Studies to determine the effects of components in the root wash on biosensor output showed that chloride ions, added as KCl (50 and 200 mg/L), had no effect on RLUs (Supplemental Fig. 1A). Acetate, provided at 20 and 50 mg/L as sodium acetate, caused a slow increase in RLU to two-fold compared to that of cells suspended in water, with output peaking at 40–60 min (Supplemental Fig. 1B). This increase in RLU was similar to the fold increases observed with the root wash.

After a 60 min-exposure, culturable cells were recovered from the root wash and water suspensions at similar densities (Table 4A). However, root wash when used at a dilution of 1:20 supported cell growth of the biosensor; from an inoculation density of 1×10^3 colony forming units/ml, cell density increased to 3×10^7 cfu/ml after 48 h shake culture at 26 °C (data not shown).

3.4. Biosensor responses to ZnO NPs and Zn ions in water versus root wash

Suspension of cells in 1:10 diluted root wash with 20 mg Zn/L ZnO NPs resulted in rapid and high levels of light output that was maintained throughout the 60 min assay (Fig 3 A). The levels were over ten-fold higher than for cells suspended in water or in root wash without ZnO NPs (Fig 3 A). After an initial boost in light output, treatment with 200 and 250 mg/L of ZnO NPs resulted in low light outputs with time (Fig 3 A). None of these treatments caused changes to the culturability of the cells as observed from the colony numbers growing after a two-day incubation on the plate medium (Table 4B).

Cells suspended in water containing 10 mg/L Zn ions showed activation of light output (Fig 3 B). With 20 mg/L Zn ions there was no light output from water suspensions of the cells after 10 min and with the 200 mg/L dose no light output above background was observed even at the initial time of sampling; these findings confirmed the toxicity from exposure in water suspensions

Table 2
Solubilization of metals from the NPs in water or root wash (1:10 dilution) after 1 h of suspension in the presence of cells. Soluble metals were analyzed from the supernatants of the cell suspensions after 60 min with or without the shown concentrations of NPs. The method involved pelleting the NPs and assays of the resulting solution by ICP-MS as described in Materials and methods. Data are means of two independent studies with standard errors. Values followed by the same letter within a column are not significantly different ($p = 0.05$).

Treatment (mg/L NPs)	Cu (mg/L)	Zn (mg/L)
Water	0.0005	0.001 A
Root wash	0.05 ± 0.02 A	0.005 A
Water and CuO NPs (10)	0.4 ± 0.1 B	–
Root wash and CuO NPs (10)	0.2 ± 0.1 B	–
Water and CuO or ZnO NPs (20)	0.4 ± 0.1 B	1.2 ± 0.2 B
Root wash and CuO or ZnO NPs (20)	0.4 ± 0.1 B	0.4 ± 0.1 D
Water and ZnO NPs (200)	–	1.1 ± 0.1 B
Root wash and ZnO NPs (200)	–	0.8 ± 0.2 D
Water and CuO or ZnO NPs (250)	0.6 ± 0.2 B	3.0 ± 0.6 C
Root wash and CuO or ZnO NPs (250)	1.2 ± 0.1 C	1.9 ± 0.8 C

Table 3
Geochemical prediction of the speciation of Zn (1 or 10 mg/L) or Cu (0.11 or 0.31 mg/L) added as ions to 1:10 dilutions of root wash at pH 7.2. The analysis was performed with GEOCHEM according to Parker et al. (1995).

Specie	Concentration (mg/l)			
	Zn		Cu	
	1	10	0.11	0.31
Free metal	0.535	7.2	0	0
Metal-hydroxide	0.09	1.2	0	0
Metal-isocitrate	0.29	0.38	0.11	0.31
Zn-malate	0.03	0.29	0	0
Zn-oxalate	0.06	0.22	0	0
Insoluble phosphate	0	0.58	0	0

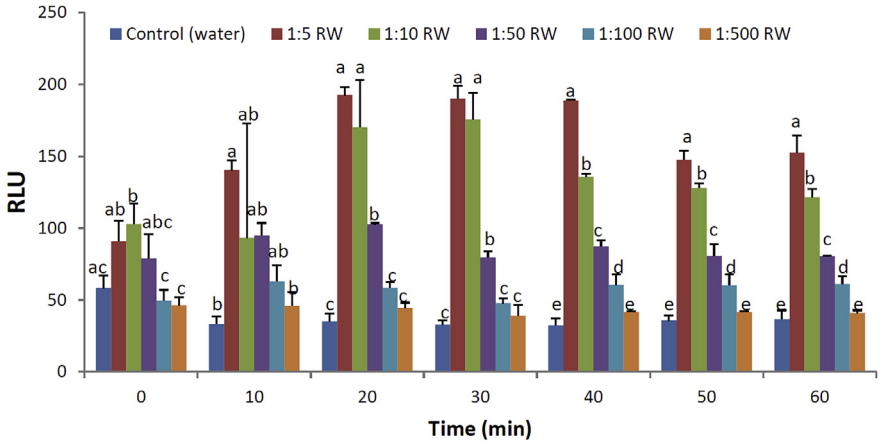

Fig. 2. Effect of different dilutions of root wash (RW) on light output from the biosensor cells. The data are the means of the relative light units (RLU) and standard deviations for three replicates from one study typical of three independent studies with the same patterns of effect. The different letters on the bars show significant differences among the treatments determined separately for each time point by one-way ANOVA ($p = 0.05$).

observed in our former findings (Gajjar et al., 2009). However, when cells were suspended in root wash there was activation of light output with the 10 and 20 mg/L Zn ion doses; the pattern of activation differed between doses, being high and maintained for the 10 mg/L treatment compared to levels that increased with time for the 20 mg/L dose (Fig. 3 B). Light output from the cells suspended in root wash with 10 mg/L exceeded ten-fold activation throughout the time course. Differences in light output were significant between cell suspensions in water and in root wash with the 10 and 20 mg/L Zn ion treatments. The 200 mg ions/L treatment generated little to no light output in the presence or absence of the root wash. At this Zn concentration, culturability of the cells did not differ between treatments whether cells were suspended in water or root wash (Table 4B).

3.5. Biosensor responses to CuO NPs and Cu ions in water versus root wash

In water suspension, addition of 10 mg Cu/L from CuO NPs caused little change to the light output of the cells, whereas a decrease with time was observed with 20 mg Cu/L from the CuO NPs (Fig 4 A). In contrast to the data reported in Gajjar et al. (2009), where with a previous batch of CuO NPs, also from Sigma Aldrich, culturability was not observed at above 10 mg/L, culturability of the cells was little affected at 20 mg Cu/L with this source of NPs (Table 4C). The higher NP dose, 250 mg/L, gradually decreased light output to become zero at 60 min (Fig 4 A) and reduced culturability from 4×10^7 cells/ml to 2×10^4 cells/ml (Table 4C).

With cells suspended in the root wash, the levels of light output in the presence of 10 and 20 mg/L Cu from CuO NPs were similar to those of the root wash alone (Fig 4 A), suggesting that cells did not respond to the NPs but only to the root wash. Even with 250 mg/L CuO NPs loss in light output was less in the root wash than for the cells in water suspension (Fig 4A). The culturability studies for the cells exposed in root wash showed that there was protection; culturable cells were recovered at the 250 mg/L dose at 0.2×10^7 cells/ml compared to 0.002×10^7 cells in water suspension (Table 4C).

Cu ions at 0.1 mg/L produced little change in light output for cells in water suspension relative to cells with no Cu exposure, but the higher doses of 0.3 and 0.5 mg/L Cu ions reduced light output initially and with time (Fig 4 B). In water there was little effect on culturability of 0.1 and 0.3 mg/L Cu ions but culturability was eliminated with 0.5 mg/L Cu ions (Table 4C). In contrast to challenge with Zn, there was little difference in changes in light output with time when cells were suspended in root wash and challenged with Cu ions compared to the water suspensions (Fig 4 B). At some time points light output levels were not different whether cells were in water or root wash. The treatments of 0.3 and 0.5 mg/L Cu ions in root wash resulted in loss in culturability of the cells (Table 4C).

Table 4A
Culturability of biosensor in root wash at defined dilutions with sterile distilled deionized water. Data are means with standard errors of two separate studies each with two replicates. The root wash (RW) was used at the different dilutions in sterile distilled water to suspend the bacteria for 1 h with shaking at room temperature. Samples were plated onto LB plates to determine culturability.

Treatment	Cell culturability (cells $\times 10^7$/ml)
Water	6.0 ± 1.0
Root wash (1:5)	2.1 ± 0.3
Root wash (1:10)	1.0 ± 0.2
Root wash (1:50)	2.3 ± 0.4
Root wash (1:100)	9.3 ± 1.4
Root wash (1:500)	6.0 ± 1.0

4. Discussion

AFM imaging indicated that both the CuO and ZnO NPs contained particles of nanosize when in water suspensions, with agglomeration and coating after suspension in root wash for 60 min. Similar increases in size were imaged using AFM and seen by DLS analysis for Ag NPs suspended in root wash material compared to water (Dimkpa et al., 2013b). Aggregation into larger particles could in part be due to reduction in the surface charge: suspension of the CuO and ZnO NPs in root wash reduced the negative surface charge of developed colloids, consistent with the binding of positively-charged materials present in the root wash. This reduction in charge would reduce repulsion of like-charged particles and increase aggregation. Attempts to use dynamic light scattering measurements to confirm changes in particle size were foiled because the presence of aggregates in the root wash materials prevented the gathering of reproducible data.

Although aggregates were observed in the treatments, the NP suspensions were bioreactive, confirming the studies of Gajjar et al. (2009). However here we note variability in the antimicrobial activity of two lots of commercially produced CuO NPs. As reported in Gajjar et al. (2009), the commercial CuO NPs at 10 mg Cu/L

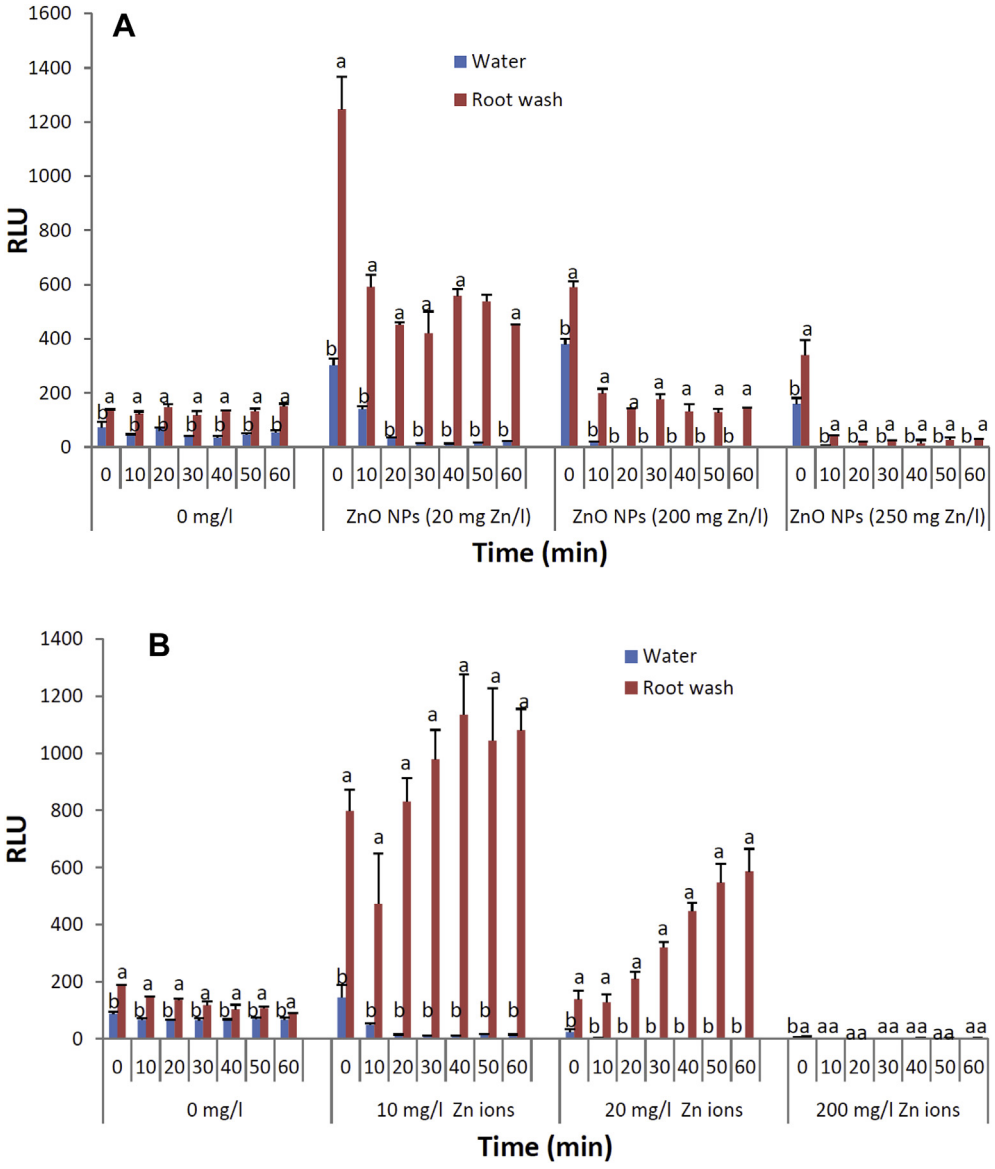

Fig. 3. Effects on light output from the biosensor cells of the presence or absence of root wash and (A) ZnO NPs and (B) Zn ions. The data are the means of the RLU and standard deviations for three replicates from one study typical of three independent studies with the same patterns of effect.

eliminated light output by 60 min and reduced culturability from 10^8 to 10^4 cfu/ml. The source of CuO NPs used in this preparation however reduced cell culturability to 10^4 cfu/ml in water suspension only by exposure to a much higher dose, 250 mg/L. The sensitivity of the biosensor cells to Cu ions was similar between the studies suggesting that the differences in toxicity were not due to changes in biosensor sensitivity. ICP-MS assay for the CuO NPs from the source used in this study revealed contamination with an array of inorganic impurities (iron, silicon, aluminum, Mn and Zn) at varying concentrations to extents less than 0.1% by weight (Dimkpa et al., 2012). We lack a similarly detailed analysis of the impurities in the lot used by Gajjar et al. (2009). Variability between lots of NPs has been reported by other workers: differences in composition between lots of TiO_2 NPs affected properties studied by Liu et al. (2011).

Catabolism of organic materials in the root wash by the biosensor cells was indicated by the increase in light output in a dose-dependent manner during the 60 min bioassay by cells suspended in 1:5, 1:10, 1:20 and 1:50 dilutions. Also the root wash supported increased cell density of the biosensor in a growth study. These data indicate the importance of metabolites from the root surface as nutrient sources for the rhizosphere microbes, as demonstrated by other studies (e.g.Yang and Crowley, 2000; Jin et al., 2006; Haichar et al 2008).

Table 4B
Effects on cell culturability of exposure of biosensor for 60 min to ZnO NPs or Zn ions in the presence or absence of root wash. Data are the means and standard deviations obtained from three or more studies for each treatment. There is no significant difference in culturability between treatments at $p = 0.05$. NA = not applicable.

Treatment (mg Zn/L)	ZnO NPs (cells × 10^7/ml)	Zn ions (cells × 10^7/ml)
Control	4.0	4.0
Root wash	3.5	2.6
Zn (10) in water	NA	1.2
Zn (10) in root wash	NA	1.4
Zn (20) in water	3.2	2.3
Zn (20) in root wash	2.0	2.2
Zn (200) in water	2.6	1.6
Zn (200) in root wash	2.9	1.0
Zn (250) in water	4.0	NA
Zn (250) in root wash	4.0	NA

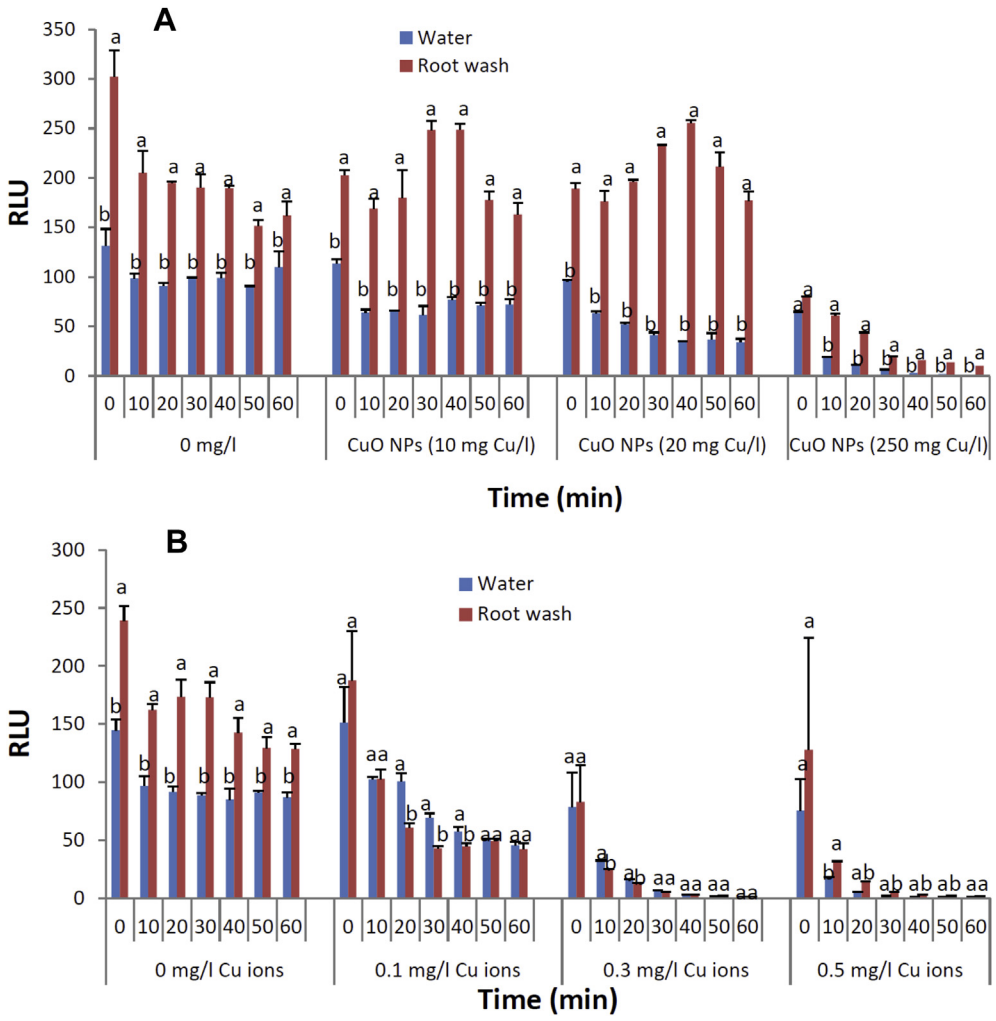

Fig. 4. Effects on light output from the biosensor cells of the presence or absence of root wash and (A) CuO NPs and (B) Cu ions. RLU relative light units. The data are the means of the RLU and standard deviations for three replicates from one study typical of three independent studies with the same patterns of effect.

Suspension in root wash protected the bacterium from the changes in metabolism caused by exposure to ZnO and CuO NPs. The sensitivity and timing of the changes as revealed by the biosensor differed between these NPs and the metal ions. Root wash materials permitted much higher light output with exposure to 20 mg Zn/L ZnO NPs than in the assays with root wash alone. Increased light output could have resulted from a combination of mechanisms. One mechanism involves the activation of the metal-activated promoter of the *luxAB* fusion cassette by cellular detection of levels of Zn ions above background, as discussed in Gajjar et al. (2009) and Miller et al. (2009). This is evident in the initial time samples for light output from cells when exposed to ZnO NPs in water being higher than that in water alone (Fig. 3 A). Studies by Gajjar et al. (2009), and confirmed during the studies for this paper (data not shown), revealed that treatments with 0.05 and 0.1 mg/L Zn ions in water lead to strong activation of light output. These findings were consistent with sensing of increased cytoplasmic Zn.

For cells suspended in root wash, there would be an increase in cellular energy available from the catabolism of the metabolites present in the root wash. This effect was demonstrated in the bioassay where addition of acetate in the absence of NPs caused increased light output of the biosensor. Higher energy output of the cells would permit functioning of the metal efflux pump systems that require ATP as part of the efforts to achieve metal homeostasis (Lemire et al., 2013). In *Escherichia coli*, changes in Zn at the femtomolar level effected gene expression changes involved in homeostasis (Outten and O'Halloran, 2001). The functioning in isolate KT2440 of efflux pumps has been cited in the regulation of cellular

Table 4C
Effects on cell culturability of exposure for 60 min to CuO NPs or Cu ions in the presence or absence of root wash. Data are the means and standard deviations obtained from three or more studies for each treatment. Asterisks (*) denotes culturability that is significantly different at $p = 0.05$ from the control cells. NA = not applicable.

Treatment (mg Cu/L)	CuO NPs (cells × 10^7/ml)	Cu ions (cells × 10^7/ml)
Control	4.5	4.0
Root wash	3.5	2.6
Cu (0.1) in water	NA	4.5
Cu (0.1) in root wash	NA	0.4
Cu (0.3) in water	NA	1.3
Cu (0.3) in root wash	NA	0.04*
Cu (0.5) in water	NA	0.0*
Cu (0.5) in root wash	NA	0.002*
Cu (10) in water	3.7	NA
Cu (10) in root wash	3.6	NA
Cu (20) in water	3.0	NA
Cu (20) in root wash	4.0	NA
Cu (250) in water	0.002*	NA
Cu (250) in root wash	0.2*	NA

Zn (Hynninen et al., 2010). Cellular detection for Cu is more sensitive than for Zn, with homeostasis at zeptomolar levels (Changela et al., 2003). The presence of Cu-inducible ATP-driven efflux pump for Cu ions in isolate KT2440 was demonstrated by proteomics (Miller et al., 2009). Thus, enhanced ability to withstand the metal stress through ATP-dependent efflux could be involved in increasing the light output and we are now investigating activation of efflux pumps by NPs.

We propose that the extensive activation of light output in cells exposed to 20 mg Zn/L from ZnO NPs in root wash was due to the contribution of higher energy levels, maintenance of Zn homeostasis and promoter activation. From the studies with biosensor activation with added ions, the treatment with 20 mg Zn/L from the ZnO NPs would be consistent with the cell detecting Zn at the levels achieved by addition of 0.05–0.5 mg Zn/L ions. The soluble Zn measured in the incubation solutions was about 1 mg/L for the 20 mg/L ZnO NPs exposure in water, with a trend for reduced levels in root wash. Modeling by GEOCHEM of an addition of 1 mg/L Zn ions to root wash revealed formation of Zn complexes with hydroxide and isocitrate that would alter the potential for bioactivity compared to the free ions. For Zn, no correlation could be made between biosensor output and culturability due to the high tolerance of isolate KT2440 to this metal. We have demonstrated (Martineau et al. unpublished) that adaption of cells to Zn through prior growth with Zn ions occurs in isolate KT2440. We assume that the changes in light output seen in the biosensor reflect an initial but transient toxicity by Zn.

With the Cu treatments, loss in culturability correlated with time-dependent reduction in light output for treatments with 0.5 mg/L Cu ions and 250 mg Cu/L from CuO NPs, whether in root wash or water. With exposure to 250 mg Cu/L as NPs, the greater loss in culturability in water than in root wash, could reflect that physical coating of the NPs by root wash components offered protection to the cells. The responses of cells to exposure to Cu in root wash compared to water also would be complicated by the formation of Cu complexes with acids such as isocitrate that were bioavailable as demonstrated by McLean et al. (2013). At doses of 10 and 20 mg/L CuO NPs in root wash, light output was similar to the levels observed with cells suspended only in root wash. Thus, it appeared that the cells were unable to detect the CuO NPs, although the soluble Cu levels were above the 0.1 mg/L levels shown to have an impact when Cu ions were presented to cells suspended in water. The protection by root wash components for the CuO NPs but not ions could be explained by their aggregation and coating. In studies of the amelioration of the antimicrobial effects of Ag NPs in soil on the soil microbe *P. chlororaphis* O6, protection was attributed in part to potential coating of NPs with humic acids in the soil (Calder et al. 2012).

Our findings illustrate that components exuded from wheat roots, and thus part of soil chemistry, have the potential to ameliorate the toxicity of the NPs towards a soil bacterium. Predictions based on the root wash composition suggested that for Zn at low levels the metal would remain mostly as the free ion whereas for Cu it would be complexed with organic acids. The protection observed with the NPs could be explained in part by aggregation and possibly coating of the particles, reducing their bioactivity through physically shielding the NPs and altering ion delivery to the bacterial cells.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2013.12.022.

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