UC Santa Barbara

UC Santa Barbara Previously Published Works

Title

(1)H NMR and GC-MS Based Metabolomics Reveal Defense and Detoxification Mechanism of Cucumber Plant under Nano-Cu Stress.

Permalink

https://escholarship.org/uc/item/42r9w94b

Journal

Environmental science & technology, 50(4)

ISSN

0013-936X

Authors

Zhao, Lijuan
Huang, Yuxiong
Hu, Jerry
et al.

Publication Date

2016-02-01

DOI

10.1021/acs.est.5b05011

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

This is an open access article published under an ACS AuthorChoice License, which permits copying and redistribution of the article or any adaptations for non-commercial purposes.

Environmental Science & Technology

Article

pubs.acs.org/est

^{1}H NMR and GC-MS Based Metabolomics Reveal Defense and Detoxification Mechanism of Cucumber Plant under Nano-Cu Stress

Lijuan Zhao,[†,⊥] Yuxiong Huang,[†,⊥] Jerry Hu,[‡] Hongjun Zhou,[§] Adeyemi S. Adeleye,[†,⊥] and Arturo A. Keller*[,†,⊥]

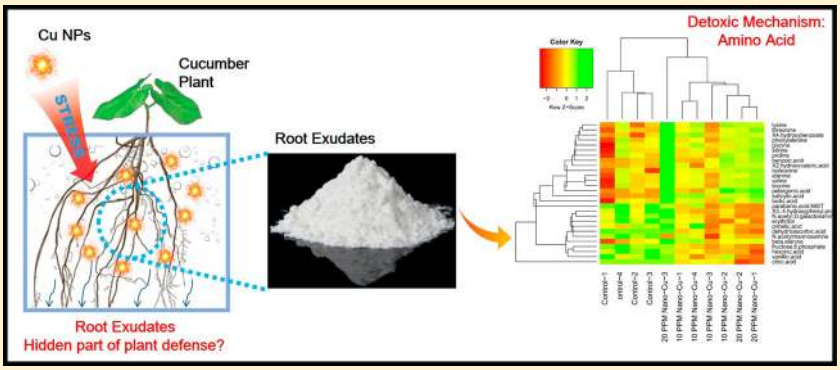
[†]Bren School of Environmental Science and Management, University of California, Santa Barbara, California 93106-5131, United States

[‡]Materials Research Laboratory, University of California, Santa Barbara, California 93106-5050, United States

[§]Neuroscience Research Institute and Molecular, Cellular and Developmental Biology, University of California Santa Barbara, Santa Barbara, California 93106, United States

[⊥]University of California, Center for Environmental Implications of Nanotechnology, Santa Barbara, California 93106, United States

Supporting Information



ABSTRACT: Because copper nanoparticles are being increasingly used in agriculture as pesticides, it is important to assess their potential implications for agriculture. Concerns have been raised about the bioaccumulation of nano-Cu and their toxicity to crop plants. Here, the response of cucumber plants in hydroponic culture at early development stages to two concentrations of nano-Cu (10 and 20 mg/L) was evaluated by proton nuclear magnetic resonance spectroscopy (^{1}H NMR) and gas chromatography–mass spectrometry (GC-MS) based metabolomics. Changes in mineral nutrient metabolism induced by nano-Cu were determined by inductively coupled plasma-mass spectrometry (ICP-MS). Results showed that nano-Cu at both concentrations interferes with the uptake of a number of micro- and macro-nutrients, such as Na, P, S, Mo, Zn, and Fe. Metabolomics data revealed that nano-Cu at both levels triggered significant metabolic changes in cucumber leaves and root exudates. The root exudate metabolic changes revealed an active defense mechanism against nano-Cu stress: up-regulation of amino acids to sequester/exclude Cu/nano-Cu; down-regulation of citric acid to reduce the mobilization of Cu ions; ascorbic acid up-regulation to combat reactive oxygen species; and up-regulation of phenolic compounds to improve antioxidant system. Thus, we demonstrate that nontargeted ^{1}H NMR and GC-MS based metabolomics can successfully identify physiological responses induced by nanoparticles. Root exudates metabolomics revealed important detoxification mechanisms.

INTRODUCTION

Copper and its compounds have been known to have the ability to inhibit fungi since ancient times[1] and have been used widely in agriculture as fungicides,[2] algaecides,[3] pesticides,[4] and herbicides.[5] There are at least 209 pesticide products registered in California that use copper oxide as an active ingredient.[4] In addition, due to steady increase of drug resistance of bacteria, synthesis and application of novel antibacterial/anti antifungal Cu nanoparticles (NPs) has increased.[6] Besides antibacterial applications, Cu NPs (which includes nano-Cu as well as nano copper oxides) also have application as additives of livestock and poultry feed.[7] There is increasing concern about the potential for bioaccumulation and toxicity of Cu NPs after their release to the environment. It has been shown in several studies that nano-Cu triggers reactive oxygen species (ROS) generation and induces oxidative stress in cells, bacteria, and zebrafish.[8,9] However, very few studies have focused on the toxicity of Cu NPs on terrestrial plants, especially crop plants. Lee et al.,[10] documented that Cu NPs are toxic to mung bean

Received: October 12, 2015
Revised: January 4, 2016
Accepted: January 11, 2016

 © XXXX American Chemical Society

DOI: 10.1021/acs.est.5b05011
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

(*Phaseolus radiatus*) and wheat (*Triticum aestivum*) at concentrations of 335 and 570 mg/L, respectively. Hong et al.[11] reported that even at the level of 5−20 mg/L, Cu NPs significantly reduced the root length of alfalfa and lettuce and altered their nutrient uptake.

Cucumber plants are generally more sensitive to contaminants and their bioaccumulation is higher than many other plants due to their high transpiration rate. Our previous study showed both CeO_2 and ZnO NPs triggered more physiological changes in cucumber plants compared to corn plants, which have lower transpiration rates.[12,13] Here we hypothesized that nano-Cu would induce physiological responses in cucumber plants. To evaluate this, we selected metabolomics studies as a novel approach to understand plant−nanoparticle interactions. We selected a ^{1}H nuclear magnetic resonance (NMR)-based environmental metabolomics platform to detect the induced alteration, because NMR can simultaneously detect a variety of metabolites with simple sample preparation.[14,15] In addition, compared with other "omics", metabolomics reveals effects downstream of DNA[15] and simultaneously provides a non-specific assessment of the end result of multiple biological responses.[16] Therefore, ^{1}H NMR has been employed to evaluate the toxicity of a large variety of environmental contaminants on different organisms.[17−21] The toxicity and toxicity mechanism of titanium dioxide NPs to earthworms[16] and rats[22] was evaluated via an environmental metabolomics platform using ^{1}H NMR.

Furthermore, most previous studies of the effect of various NPs to plants have concentrated on toxicity. Potential detoxification mechanisms have been less reported. It is well-known that approximately 30−40% of all photosynthetically fixed carbon will be transferred to the rhizosphere[23,24] as root exudates, including organic acids, amino acids, sugars, proteins, phenolic compounds, and CO_2.[25] These compounds play an important role in plant stress tolerance and external exclusion of pollutants.[23] Considerable evidence exists that plants up-regulate some organic acids, including amino acids, to chelate or complex toxic metals (e.g., Al, Cd, Zn, Fe, Cu), to hinder their translocation to aboveground plant tissues.[26−30] Murphy et al.[31] reported copper ions induce citrate production in root secretions. Our hypothesis is that root exudates may play an important role in NP mobility and bioavailability, as well as up- or down-regulation of metabolite levels due to toxic effects and to induce detoxification.

Therefore, the aims of the present study were to investigate the uptake, translocation, bioaccumulation, and toxicity of nano-Cu in cucumber tissues. We used nontargeted ^{1}H NMR and GC-MS based metabolomics to evaluate the physiological changes induced by nano-Cu in cucumber plants. Mineral nutrient metabolism was also evaluated by determining the elemental content in different tissues.

EXPERIMENTAL SECTION

Cu NPs. Detailed characterization of nano-Cu (U.S. Research Nanomaterials) employed here is presented in a previous study.[32] Briefly, the primary particle size is 40 nm and the hydrodynamic diameter is 2590 ± 1138 nm in deionized (DI) water, due to considerable aggregation. The surface charge expressed as zeta potential is +10.9 ± 4.0, −29.4 ± 0.8, and −40.8 ± 1.7, at pH 4, 7, and 11, respectively.

Seed Germination and Plant Growth. Cucumber (*Cucumis sativus*) seeds were surface-disinfected in a 4% NaClO solution for 30 min with continuous stirring, then rinsed 3 times with sterile nanopure water (NW), and immersed in NW for 24 h. Subsequently, the seeds were transferred to autoclaved paper towels for germination.[33] In each paper towel, 10 drops of antibiotic solution (Sigma 5955) were added to prevent fungal growth. The rolled paper towel with seeds was placed in a Mason jar with NW at the bottom and kept in the dark for 4 days, followed by 1 day exposure in light.

After germination, the young plants were transferred to a magenta box and grown hydroponically in modified Hoagland nutrient solution in a greenhouse. The temperature in greenhouse was maintained at 28 °C by day and 20 °C by night. The nutrient solution was renewed every 7 days. However, between these 7-day periods, tap water was used to refill the magenta boxes to compensate for the water taken up via transpiration. To collect sufficient amounts of secreted compounds, the young unexposed cucumber plants were allowed to grow for 2 weeks with continuous aeration. On day 14, the cucumber seedlings were exposed to half strength Hoagland nutrient solution containing different concentrations (0, 10, and 20 mg/L) of nano-Cu for 1 week. These levels are consistent with expected concentrations from pesticide use or in biosolids.[34,35] Each treatment was replicated four times, and each magenta box contained 9 cucumber seedlings.

Cu and Nutrient Elements Analysis. At harvest, the cucumber plants were thoroughly washed with DI water and oven-dried for 3 days at 70 °C. Dried tissues were digested with a mixture of 4 mL of H_2O_2 and 1 mL of plasma pure HNO_3 (v/v 4:1) using a microwave oven system (Multiwave Eco, Anton Par) at 180 °C for 1 h. The standard reference materials (Peach leaves, NIST 1547, USA; Spinach, leaves, NIST 1570A) were also digested and analyzed as samples. The recoveries for all elements were between 90 and 99%.[12] Cu and other mineral elements were analyzed using ICP-MS (7900 Agilent, USA).

Root Exudates Collection and Analysis. The procedure for collection of root exudates was modified from that described by Zhao et al.[36] Specifically, at the end of exposure to nano-Cu, the cucumber seedlings were removed from the magenta box, and the roots were washed thoroughly with DI water. The seedlings were then incubated in a new magenta box containing 100 mL of autoclaved 0.1 mM $CaCl_2$ solution for 4 h for root exudate collection. The solution was aerated continuously during the 4-h period of collection. After 4 h, the 0.1 mM $CaCl_2$ solution, which then contained the secreted metabolites, was filtered immediately using a syringe filter (0.45-μm) to remove all the microorganisms. The filtrate was immediately freeze-dried under a vacuum system to obtain the powdered root exudate.

NMR Sample Preparation. The leaves of cucumber plants exposed to different levels of nano-Cu were collected after day seven. The leaves' tissues were immediately frozen using liquid nitrogen, ground, and then freeze-dried for 2 days using a lyophilizer (Labconco, MO, USA). The extraction method by Kim et al.[37] was followed. Specifically, 50 mg of the frozen dried leaf tissues was mixed with 0.75 mL of CD_3OD and 0.75 mL of KH_2PO_4 buffer in D_2O (pH 6.0) containing 0.1% TSP (w/w). The mixture was vortexed for 1 min, and ultrasonicated for 20 min at room temperature. After sonication, the mixture was centrifuged for 15 min. The entire 0.8 mL of supernatant was transferred to a 5-mm NMR tube. Extraction of the freeze-dried root exudate samples followed the same method.

NMR Analysis and Data Processing. The ^{1}H NMR spectra of cucumber leaf extracts and root exudates were

DOI: 10.1021/acs.est.5b05011
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

measured on a Bruker AVANCE III 800 MHz SB NMR Spectrometer. Specifically, one-dimensional (1D) ^{1}H NMR spectra of leaf extracts and root exudates were obtained using a 14.5-μs 90° pulse, 3-s relaxation delay, 20-ppm spectral width, and 2-s acquisition time, with 200 transients of 64k data points collected over a 16-min data accumulation time. The residual water signal was suppressed with presaturation during the relaxation delay. The resulting spectra were phase and baseline corrected and then chemical shift was calibrated using the Bruker Topspin 3.1 software.

The 1D NMR spectra were converted to an appropriate format for multivariate analysis using MNOVA (Version 10, Mestrelab Research, S.L). For NMR data, the chemical shift region of significance between 0.5 and 10.0 ppm was divided into small bins of equal width (0.04 ppm bin size) with the region from 4.7 to 4.85 ppm excluded to eliminate the small residual signal from water. Finally, about 240 buckets were generated for each sample. Principal components analyses (PCA) of the binned NMR data were conducted with the PCA module of MNOVA. For the bins with significant changes across a given set of samples, the responsible metabolites were identified with Chenomx NMR Suite 7.0 (Chenomx Inc.).

Statistical Analyses of Other Data. For the assay of biomass, Cu concentration, and mineral nutrient concentration, significant differences between treatment means were evaluated by one-way ANOVA followed by Tukey-Kramer posthoc tests, performed by SPSS. The effects of nano-Cu treatment were considered statistically significant when $p < 0.05$. Data are presented as mean ± standard errors ($n = 4$).

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis of Root Exudates. The freeze-dried root exudate samples were subjected to gas chromatography time-of-flight mass spectrometer-mass spectrometry (GC-TOF-MS) analysis at the Genome Center Core Services, University of California Davis, to identify the metabolites. Description of the instrument has been reported previously.[38] The sample extraction method and chromatography sample preparation were previously reported by Fiehn et al.[39]

RESULTS AND DISCUSSION

Impact of Nano-Cu on Cucumber Growth. After 7 days exposure to 10 and 20 mg/L of nano-Cu, cucumber plants exhibited significant decrease in root length compared to the control. Exposure to 10 and 20 mg/L of nano-Cu also resulted in root biomass reduction by 11.7% and 30.2%, respectively (Supporting Information Figure S1), but only the effect at 20 mg/L was statistically significant ($p \leq 0.05$). Similar results have been reported in zucchini, squash, wheat, lettuce, and alfalfa.[10,11,40,41] Stampoulis et al.[40] observed reduced root elongation of zucchini seeds after exposure to 1000 mg/L Cu NPs. Musante and White reported that exposure to Cu NPs at 100 and 500 mg/L resulted in significant biomass reduction of squash.[41] Lee et al. also found Cu NPs at 200 mg/L affected wheat root elongation.[10] More recently, Hong et al.[11] reported that Cu NPs at a concentration of 20 mg/L significantly reduced the root length of alfalfa and lettuce. It has been reported that copper toxicity results from inhibition or activation of some enzymes in the root zone.[42] For instance, Kennedy and Gonsalves reported Cu inhibited ATPase activity in the plasma membrane of *Zea mays* roots.[43] Previous studies showed copper toxicity triggered oxidative damage and increased the antioxidative enzymes.[44−46]

Compared to the roots, nano-Cu had no statistically significant impact on stem and leaf biomass. This is consistent with previous reports that roots are the most vulnerable organ under nano-Cu stress.[10,47,48] Lee et al. reported that nano-Cu at 200 mg/L affected the root length of wheat, while the threshold to induce shoot length reduction was 800 mg/L. $CuSO_4$ was observed to inhibit root growth but not shoot growth in rice seedlings.[47] Fernandes and Henriques[48] attributed the differential effect of Cu on root and shoot growth to the fact that Cu is mainly bioaccumulated in roots compared to shoots. However, Hong et al. demonstrated that the Cu toxicity is a species-specific response.[11] They showed that although the Cu concentration was high in alfalfa shoots, compared to lettuce, there was no shoot length reduction.[11]

Cu Uptake and Translocation. In all tissues including root, stem, and leaves, Cu concentration in plants treated with 10 and 20 mg/L of nano-Cu were significantly higher than that in the control (Figure 1). This indicates that copper (in either form) was taken up by and transported from root to stem and leaves within 7 days. The distribution patterns of Cu in

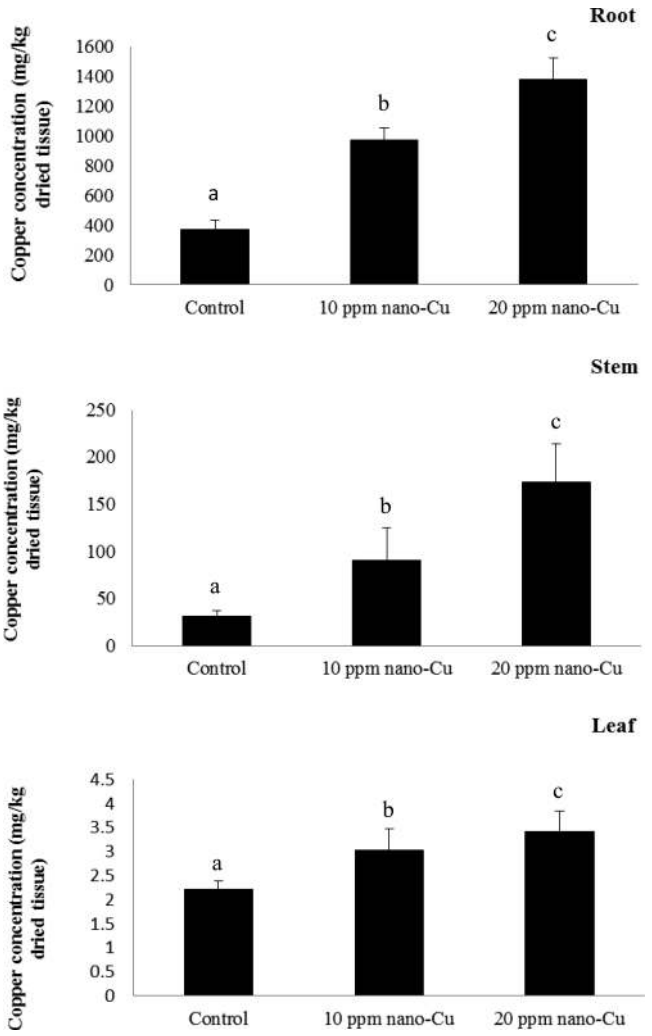


Figure 1. Cu bioaccumulation in tissues of cucumber plants exposed to nano-Cu at different concentrations (0, 10, and 20 mg/L) for 7 days in hydroponic media. All data show the mean of a total of four replicates (nine plants per replicate). Error bars represent standard deviation. Different letters stand for statistical differences at $p \leq 0.05$ (Tukey's HSD multiple comparison at $p \leq 0.05$).

DOI: 10.1021/acs.est.5b05011
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

different tissues in both control and nano-Cu treated plants are similar: Cu was predominant in roots (89−92%), followed by stems (8−11%), and leaves (0.2−0.5%). This indicates that Cu was sequestered primarily in the root compartment, and only a small percentage was transported to upper tissues. This is consistent with previous reports showing that Cu is located primarily in roots of alfalfa and lettuce.[11]

It is interesting to note that the stem/root translocation factors (Cu in stems/Cu in roots) have the tendency to increase with increasing nano-Cu concentration, while the leaf/stem translocation factors (Cu in leaves/Cu in stems) decrease (Figure S2). This indicates cucumber plants tend to retain/sequester more Cu in their stems. The mechanism underlying this phenomenon is complex, and still unclear. We hypothesize that this is a detoxification or active protective process for the plants to avoid damage to the more vulnerable tissue of roots and leaves. It has been shown that copper in xylem sap is almost 100% bound to amino acids.[49−52] The stem may secret more ligands, e.g., carboxyl and amino groups, organic acids, glutathione, cysteine, to complex or chelate with Cu when it is in excess.[53] Liao et al.[52] showed evidence that more than 99.6% of total Cu in the xylem sap of tomato plants was in a complexed form, with the xylem sap producing amino acids to chelate Cu and hinder its translocation to leaves. These findings with Cu are consistent with previous reports that plants are able to minimize the adverse effects of excess metals by regulating their distribution and translocation within their organs or cells.[53,54]

Effects of Nano-Cu on Mineral Nutrient Metabolism. As shown in Table 1, in the presence of nano-Cu, nutrient elements were markedly affected. The concentrations of Na, P, S, K, Mo, and Zn decreased in all cucumber tissues in exposed plants. Except K in roots (p = 0.073) and leaves (p = 0.087), and Na in leaves (p = 0.168), all the decreases were statistically significant ($p \leq 0.05$). In addition, nano-Cu decreased Fe and Mg uptake in roots, but not in stems and leaves. Reduced uptake of P, S, and Fe in lettuce and alfalfa by nano-Cu was also observed in hydroponic systems.[11] Because the applied nano-Cu concentrations in the two studies are similar, cucumber seems to be more sensitive to nano-Cu than lettuce or alfalfa, probably because of its higher transpiration rate that carries more Cu to plant cells. Our previous study showed that $nCeO_2$ and nZnO NPs decreased Cu and Mo content in cucumber fruits; however, neither $nCeO_2$ nor nZnO affected mineral element accumulation in corn cobs.[12,13]

The decrease of K concentration in all tissues may be the result of leakage mediated by ion channels. Murphy demonstrated that Cu promotes K^+ efflux rather than inhibiting K^+ uptake in *Arabidopsis* seedlings.[31] Several studies have shown that iron uptake is decreased by excess Cu.[55−57] Hong et al. also found nano-Cu significantly decreased Fe uptake in lettuce. Waters and Armbrust[58] found that high Cu supply inhibited the activity of ferric reductase, which is an indicator of Fe demand. This led to decreased demand for Fe by the plant, which subsequently led to less Fe accumulation.

Decreased P is due to the formation of Cu−phosphate complexes at the root surface.[59] However, some studies[11,60] showed the up-regulation of S in the presence of nano-Cu or other stressors, which is quite different from our finding. It is possible that nano-Cu damages the root cell membrane and leads to leakage of many ions. These elements participate in respiration and photosynthesis and in some enzyme system functions. The decrease of these minerals may lead to other

Table 1. Effects of Nano-Copper on Mineral Accumulation in Cucumber Tissues (mg/kg Dry Weight)[a]

	Na		P		S		K		Mg		Mn		Mo		Zn		Fe		Al	
									Root											
control	12846	a	1012	a	8104	a	36700	a	11921	a	127	a	328	a	761	a	362	a	141	a
10 ppm	10736	ab	825	b	5711	b	38022	a	10416	a	85	a	89	b	295	b	149	b	155	a
20 ppm	9078	b	791	b	4570	b	30579	a	8038	b	133	a	121	b	287	b	159	b	223	a
p value	**0.002**		**0.012**		**0**		0.073		**0.003**		0.162		**0.006**		**0.001**		**0.002**		0.242	
									Stem											
control	10939	a	1132	a	7684	a	85883	a	10904	a	39	a	155	a	82	a	46	a	28	a
10 ppm	6978	b	969	ab	6012	b	67068	b	10270	a	36	a	96	b	54	b	33	a	35	ab
20 ppm	6740	b	989	b	5865	b	67971	b	10043	a	36	a	131	ab	57	ab	37	a	51	b
p value	**0**		**0.033**		**0.001**		**0.002**		0.24		0.793		**0.019**		**0.023**		0.096		**0.04**	
									Leaf											
control	356	a	129	a	875	a	2472	a	26499	a	19	a	25	ab	17	a	6	a	9	a
10 ppm	346	a	117	b	859	b	2417	a	26186	a	18	ab	24	b	14	b	6	a	3	a
20 ppm	318	a	101	b	835	b	2286	a	24217	a	16	b	23	ac	11	b	6	a	3	a
p value	0.168		**0.009**		**0**		0.087		0.575		**0.019**		**0.01**		**0.006**		0.551		0.416	

[a]Data are average of four replicates. Different letters stand for statistical differences at $p \leq 0.05$.

DOI: 10.1021/acs.est.5b05011
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

metabolic changes. For example, Cu-induced Fe deficiency can contribute to decreased leaf chlorophyll content and reduced photosynthesis.[61] Reichman[42] reported that the chlorotic symptoms on young leaves of plant experiencing Cu toxicity could be an induced Fe-deficiency.

Effect of Nano-Cu on Overall Metabolic Profile of Cucumber Leaves. Principal component analysis (PCA) was performed as a first step to provide a general overview of trends, grouping, and outliers in the ^{1}H NMR data.[14,16,19] PCA of the metabolomics data set extracted from 12 cucumber leaf samples produced three principal components (PCs) which explained more than 66.9% of the total variance (PC1, PC2, and PC3 explained 34.1%, 18.8%, and 14.0%, respectively). The score plots from PC1 and PC2 reflect that leaf tissues from the control and nano-Cu treated plants were clearly separated from each other by PC2 (Figure S3) reflecting differences in metabolic profiles. However, no difference was found between 10 and 20 ppm treatments, indicating that nano-Cu at either level changed the pattern of metabolites in cucumber leaves.

The PCA loading plot identified the regions of the NMR spectra that contribute to the observed differences in PC scores. Further, bins with high weighting score were selected and subjected to one-way ANOVA to identify metabolites significantly regulated due to nano-Cu stress. A total of 25 bins were found to be significantly altered in cucumber leaves in response to nano-Cu exposure (p-value $\leq$0.05) (data not shown).

Identification of Metabolites and Metabolite Pathways Altered by Nano-Cu. In total, 22 metabolites were identified as significantly altered using Chenomx (Table 2). Most of the altered metabolites are secondary metabolites. Among the five up-regulated metabolites, 4-aminobutyrate (GABA), acetylglucosamine, and phenyllactate have previously been shown to be related to stress response. GABA is a nonprotein amino acid. In plants there are numerous observations of a rapid accumulation of GABA in response to biotic and abiotic stress.[62−64] Zulak reported that GABA levels were rapidly increased in elicitor-treated opium poppy cell cultures.[65] Acetylglucosamine, an amino-sugar, plays an important role in cell signaling. It has been reported that inflammation induced by bacterial infection can result in increased release of amino sugars from mammalian host cells.[66] Phenyllactate and p-hydroxyphenyllactate were found to decrease ROS production in both mitochondria and neutrophils.[67]

Those metabolites were further analyzed with MetaboAnaylst 2.0 (http://www.metaboanalyst.ca/MetaboAnalyst/) to identify the major perturbed metabolic pathways induced by nano-Cu. The pathway impact value threshold was set as 0.1.[49] Results showed that none of the pathways were disturbed by nano-Cu in cucumber leaves.

Root Exudate Metabolomics Analysis. Extracts of root exudate from control and nano-Cu treated plants were analyzed by ^{1}H NMR followed by PCA analysis. PCA analysis revealed clear separation in the root exudate metabolomics profiles collected from control and nano-Cu treated cucumber plants along PC1 (Figure S4). The PCA loadings identify the regions of NMR spectra that contribute to the differences in PC1 (Figure S5 A−C). In the region of organic acids (δ 3.00−0.00), a number of bin areas increased in the presence of nano-Cu, indicating nano-Cu and released Cu^{2+} increased the level of a number of amino acids or organic acids (Figure S5 A). The aromatic region (δ 10.00−6.00) comprises many characteristic signals of secondary metabolites,[37] which play a crucial role in plant defense to environmental stress.[68] As shown in Figure S5 C, nano-Cu/Cu ions altered the pattern of some secondary metabolites. We further identified those bins to corresponding compounds. However, due to the extremely low concentration and high baseline, it was difficult to link those bin areas to specific compounds using the Chenomx NMR Suite. Thus, GC-MS was used for identification and quantification. A total of 156 metabolites in root exudates were identified by GC-MS.

To visualize the general differences between control and nano-Cu treated plants, the 156 identified metabolites were normalized and analyzed by Partial Least Squares Discriminant Analysis (PLS-DA) using online resources,[69] which is a supervised clustering method to maximize the separation between groups. The score plot (Figure 2) shows that cucumber root exudate exposed to different concentrations of nano-Cu are clearly separated along the first principal axis (PC1), which explained 30.4% of the total variability. This indicates nano-Cu considerably altered the metabolic profiles of cucumber root exudate, which is consistent with the NMR data. Using parameters of variable importance in projection (VIP) score,[70] a total of 56 metabolites were found to be responsible for this separation (data not shown). For the metabolites of

Table 2. Discriminating Metabolites of Control and Nano-Copper Treated Cucumber Leaves

Up-Regulated Metabolites		
metabolite	classification	pathway
4-aminobutyrate (GABA)	amino acids	alanine, aspartate and glutamate metabolism
acetylglucosamine	amides	amino sugar and nucleotide sugar metabolism
3-phenyllactate	aromatics	phenylalanine and tyrosine metabolism
nicotinurate	amido acids, aromatics	tryptophan metabolism
glutaric acid monomethyl ester	carboxylic acids	food components
Down-Regulated Metabolites		
metabolite	classification	pathway
N-carbamoylaspartate	amido acids	pyrimidine metabolism
desaminotyrosine	carboxylic acids, phenols	phenylalanine and tyrosine metabolism
N-acetyltyrosine	amido acids, phenols	phenylalanine and tyrosine metabolism
N-carbamoyl-beta-alanine	amides	pyrimidine metabolism
thymidine	nucleotides and nucleosides	pyrimidine metabolism
cytidine	nucleotides and nucleosides	pyrimidine metabolism
melatonin	aromatics	tryptophan metabolism
kynurenine	amines	tryptophan metabolism
caprate	fatty acids	fatty acid biosynthesis
O-acetylcarnitine	amido acids	lipid metabolism
carnosine	amines	histidine metabolism
NADH	amines	monosaccharides and disaccharides
riboflavin	amides, alcohols and polyols	riboflavin metabolism
urea	amides	arginine and proline metabolism
epicatechin	alcohols and polyols, phenols	flavonoid biosynthesis
chlorogenate	hydroxy acids, phenols	food components

DOI: 10.1021/acs.est.5b05011
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

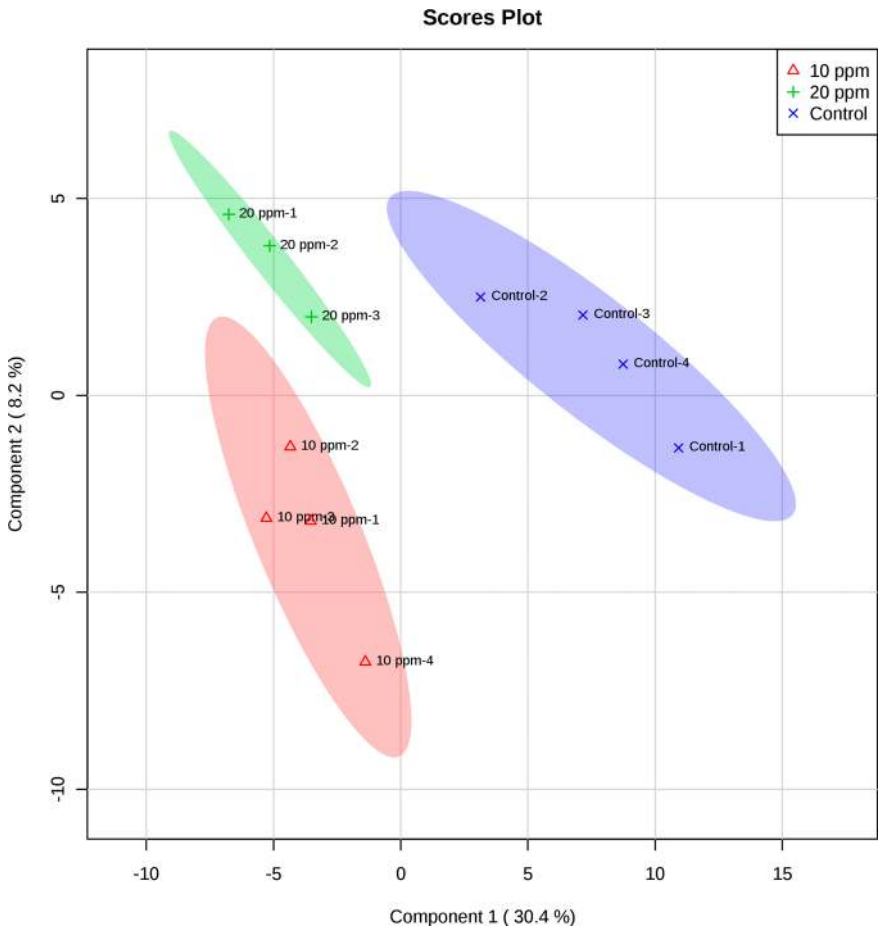


Figure 2. Scores plot (PC1 vs PC2) of partial least-squares-discriminant analysis (PLS-DA) of metabolites in cucumber root exudates. Two-week-old cucumber plants were exposed to different concentrations of nano-Cu (0, 10, 20 mg/L) for 1 week. Root exudates were collected for 4 h at the end of nano-Cu exposure and analyzed by GC-TOF-MS.

interest, hierarchical clusters analysis (HCA) was performed by grouping the samples into clusters based on the similarity of their metabolite abundance profiles. Figure 3 presents the resulting heat map for the selected metabolites, which indicates that some metabolites, including lysine, threonine, phenylalanine, glycine, serine, proline, isoleucine, alanine, valine, leucine, beta-alanine, 4-hydroxybenzoate, benzoic acid, 2-hydroxyvaleric acid, pelargonic acid, salicylic acid, lactic acid, were up-regulated by nano-Cu. However, some metabolites were down-regulated by nano-Cu, including parabanic acid, 3-(4-hydroxyphenyl) propionic acid, *N*-acetylmannosamine, erythritol, pimelic acid, dehydroascorbic acid, *N*-acetyl-D-galactosamine, fructose-6-phosphate, hexonic acid, vanillic acid, and citric acid. In the following section, we categorized those metabolites into five groups: amino acids, organic acids, fatty acid, phenolic and other compounds, and discuss them separately.

Amino Acids. Eleven amino acids, including Alanine (Ala), beta alanine (β-Ala), Glycine (Gly), Isoline (Ile), Leucine (Leu), Lysine (Lys), Phenylalanine (Phe), Proline (Pro), Serine (Ser), Threonine (Thr), and Valine (Val), were significantly up-regulated in response to nano-Cu in a dose-dependent way (Figure 3 and Figure S6). The increased exudation of amino acids is likely an active defense response of the cucumber plant. The up-regulated amino acids can provide many binding sites for copper, hindering the translocation from the root cell membrane. Previous studies showed that amino acids play an important role in chelating Cu^{2+}.[52,71] It is reported that amino acid complexes formed with other metals, e.g. Ni, are much more stable than those with carboxylic acids.[72] Cu in the roots of Cu-tolerant *A. maritima* exists as a Cu−proline complex.[73] EXAFS spectra demonstrated Cu(II) complexation with the nonproteogenic amino acid nicotianamine, which shows very high affinity for Cu(II).[74] Kupper et al. assumed that plants keep dissolved metals out of the cytoplasm and sequester/complex them into the vacuole or cell wall.[75,76] The up-regulated amino acids may also reflect an attempt by the cucumber plants to sequester Cu in stems. Although stem metabolites were not analyzed, it is possible that amino acids are also secreted in the xylem sap to bind with Cu^{2+} in the transpiration stream. It is reported that more than 99% Cu in tomato and chicory xylem sap was in a bound form with histidine (His) and nicotianamine (NA).[52] Amino acids in root exudate not only bind metals, but also serve as signaling molecules and have an antioxidant defense function.[71]

If the upregulation of these amino acids is an active defense of cucumber plant to excess nano-Cu/Cu ions, the increased amino acids possibly will decrease the uptake of Cu by cucumber plants. In order to verify this hypothesis, two-week-old cucumber plants were cultivated in 20 mg/L nano-Cu nutrient solution with different levels of Ser for 48 h. Interestingly, Cu accumulation in roots decreased with increasing Ser concentration, even though the free Cu ions in nutrient solution were much higher in the presence of Ser

DOI: 10.1021/acs.est.5b05011
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

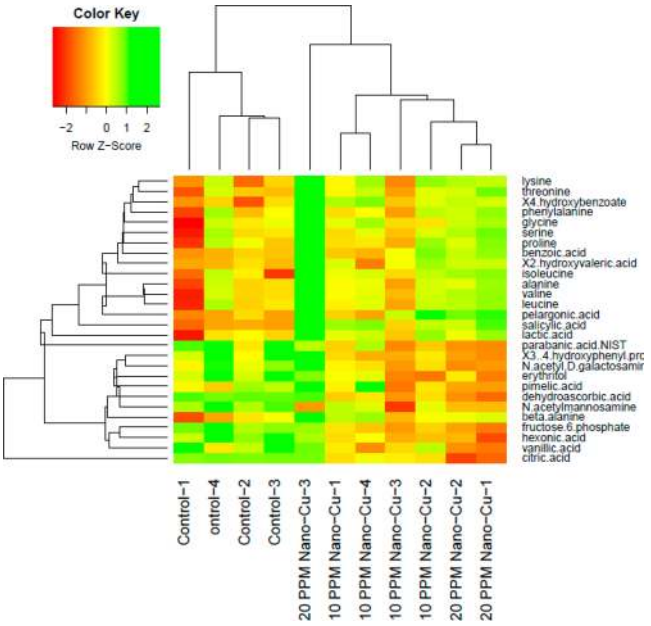


Figure 3. Heat map generated by hierarchical cluster analysis of GC-MS data of root exudate. Centroid method and Euclidean distance were used for clustering analysis. GC-MS data was log2 transformed and row scaled prior to cluster analysis. Red rectangles indicate a significant decrease in metabolite content, and green rectangles represent a significant increase in metabolite content. The dendrograms reveal the relationships between the different treatments (control, 10 ppm nano-Cu, 20 ppm nano-Cu) based on their metabolites abundance levels. The four control samples were clearly separated from nano-Cu treatments. Two nano-Cu treatments were mixed together indicating similar changes of metabolites after treatment. Two nano-Cu samples at 20 ppm were clustered together and showed larger changes for those metabolites that responded to the nano-Cu treatment. Metabolites with similar abundance patterns are also clustered together horizontally and practically formed two big clusters coresponding to up-regulated and down-regulated metabolites.

(Figure 4). After the plants were removed, the pH in nutrient solutions was determined. As shown in Figure 5, the pH decreased from 6.37 to 6.01 as the Ser concentration increased

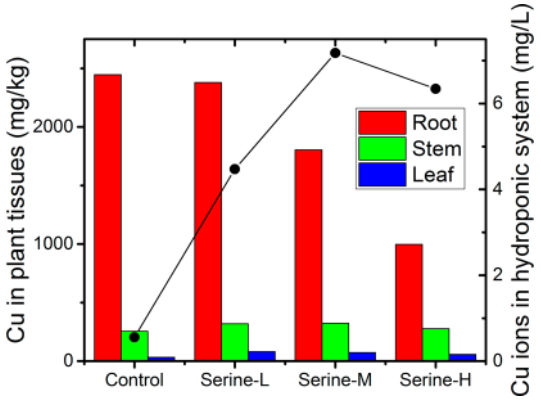


Figure 4. Cu uptake in cucumber tissues and nutrient solution. The black dots represent the Cu concentrations in the nutrient solution at harvest. Two-week-old cucumber seelings were cultivated in half strengh Hogland nutrient solution containing 20 mg/L nano-Cu with different levels of serine (0, 6.25, 12.5, and 25 mM) for 48 h. At harvest, the Cu concentrations in plant tissues and remaining hydroponic solution were analyzed by ICP-MS.

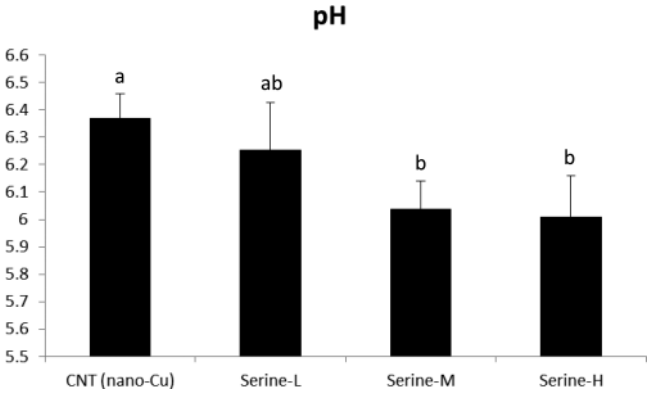


Figure 5. pH in hydroponic solutions in which two-week-old cucumber plants were exposed to 20 mg/L nano-Cu (CNT = control) and different levels of serine (CNT = 0, L = 6.25, M = 12.5, and H = 25 mM).

from 0 to 25 mM, which may explain why more Cu ions were released in the presence of Ser (nano-Cu with different concentrations of Ser) compared to the control (only nano-Cu). Those results strongly indicate that amino acids are possibly released to detoxify nano-Cu by binding with Cu ions. However, we cannot rule out the possibility that up-regulation of some amino acids was due to membrane damage, which caused the leakage.

Organic Acids. Organic acids are strong cation chelators, which play an important role in facilitating mineral element uptake and sequester or exclude toxic metals. Several studies have shown that citric, oxalic, and succinic acids are involved in the detoxification of various dissolved metals.[77−80] GC-MS results showed citric, succinic, malic, and fumaric acids were the most abundant low molecular weight organic acids in cucumber root exudates, which is consistent with previous reports on organic acid composition in cucumber root exudates.[81,82] Surprisingly, patterns of succinic, malic, and fumaric acids were not changed by nano-Cu (data not shown), which indicates those organic acids did not respond to Cu stress. In contrast, citric acid, the most abundant organic acid, was down-regulated by nano-Cu (Figure 3). Citric acid in root exudate decreased 5× at 10 ppm nano-Cu and 60× at 20 ppm in treated cucumber plants compared to the control. It is known that organic acids play an important role in restricting the passage of metals across the root, due to their strong affinity to form stable extracellular complexes with Cu, Al, and Cd.[78,80] Other reports showed organic acids in the rhizosphere help solubilize minerals and facilitate their uptake by the plant.[23] Citric acid has been shown to assist copper uptake in a moderate-accumulator plant (*Brassica napus L.*).[83] The down-regulation of citric acid is possibly an active process to decrease the dissolution, uptake, and translocation of Cu into cucumber tissues, or it could represent a shift in metabolism in the tricarboxylic acid cycle (TCA cycle).

As mentioned before, citric acid can play a dual role: it can either mobilize metals to accelerate uptake in plants that are deficient in some elements,[23] or it can complex with metals to hinder their translocation.[78,80] In this study, we hypothesized that citric acid played a role in mobilizing Cu ions release from nano-Cu, increasing Cu accumulation in cucumber plants. To confirm this hypothesis, we conducted an additional experiment in which we exposed cucumber plants to 20 mg/L nano-Cu at different concentrations of citric acid (details shown in

DOI: 10.1021/acs.est.5b05011
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

Supporting Information). We found that the pH of the hydroponic system decreased from 6.37 (20 mg/L nano-Cu without citric acid) to 5.28 (20 mg/L nano-Cu with 6.25 mM citric acid). This decrease in pH led to increased dissolved Cu ions to concentrations that were 8 times higher than that in the control (20 mg/L nano-Cu without citric acid) (Figure S7A and B). This result indicates that citric acid has a strong ability to dissolve nano-Cu by decreasing the system pH. Therefore, it is not surprising that Cu levels in cucumber plants grown in 20 mg/L nano-Cu in the presence of 6.25 mM citric acid were 4 times higher than that in plants grown in 20 ppm nano-Cu without additional citric acid (Figure S7C). However, it is noteworthy that citric acid at this concentration may damage biological membranes and lead to passive uptake of Cu through leaky membranes. At least, these additional experiments demonstrate that citric acid increased nano-Cu dissolution in cucumber plants. Taken together, the up-regulation of amino acids and down-regulation of citric acid are likely plant strategies to hinder the uptake of Cu and detoxify the Cu toxicity.

Fatty Acids. The release of fatty acids from the membrane is involved in plant tolerance to biotic and abiotic stresses.[84−87] Pelargonic acid was up-regulated in root exudates after exposure to nano-Cu (Figure 3). Pelargonic acid is a natural nonselective herbicide, which can disrupt intercellular pH and membrane integrity causing rapid cell death.[88] Evidence has shown that pelargonic acid participates in stress response and can be an indicator of root membrane damage.[89,90] The increased pelargonic acids in nano-Cu treated root exudates may be an indicator of membrane damage. Copper also interferes with fatty acid metabolism.[91]

Phenolic Compounds. Phenolics are secondary metabolites with an important role in stress response.[92] The concentrations of salicylic and benzoic acids were up-regulated in the presence of nano-Cu/Cu, especially salicylic acid (Figure 3). Both compounds are reported to have antioxidant and antifungal activities and play a crucial role in plant defense against a variety of biotic and abiotic stressors.[93,94] The concentrations of salicylic acid in root exudate of 10 and 20 ppm nano-Cu treated plants were 13 and 26 times higher than that in the control. It is reported that salicylic acid serves as an internal signaling molecule in the activation of plant defense after pathogen attack.[95] Previous studies also suggested salicylic acid treatment significantly reduced malondialdehyde (MDA) and H_2O_2 concentrations in the roots and leaves of rice (*Oryza sativa L.*) under Cu stress, therefore alleviating Cu toxicity.[96] In addition, 4-hydroxybenzoate, a phenolic derivative of benzoic acid, which is a secondary metabolite and also plays a key role in stress response, was up-regulated by exposure to nano-Cu. The up-regulation of these three phenolics is possibly a self-protection mechanism of cucumber to nano-Cu exposure at these levels.

Other Down-Regulated Metabolites. As shown in Figure 3, most of the down-regulated compounds are sugar-related metabolites, which might explain why citric acid exudation decreased as it was preserved for the TCA cycle. It is interesting to find that the level of dehydroascorbic acid (DHA) in nano-Cu treated root exudates is apparently down-regulated (Figure 3). DHA is known to participate in the plant defense against oxidative stress,[97] and it is also an oxidized form of ascorbic acid. Dehydroascorbate is an intermediate product of the reaction between ascorbic acid and ROS, and can be oxidized to threonic acid. Thus, the down-regulation of DHA is an indicator that ROS was triggered by nano-Cu in the rhizosphere and a response to oxidative stress occurred. It is possible that the membrane damage occurred due to lipid peroxidation induced by ROS.

Environmental Implications. Because the nutritional sources of microbes in the rhizosphere come from root exudates,[98] the altered pattern of amino acids, carboxylic acids, phenolics, and sugar metabolites may possibly affect microbial activities and communities in the rhizosphere. Yuan et al.[99] revealed that n-CeO_2 did not affect soil bacterial communities in the absence of soybean plants, but did affect them in the presence of soybean plants, likely through the change of quantity and composition of root exudates. Our results indicate that nano-Cu altered the metabolite profile in cucumber root exudates, and this change may alter the mutually beneficial feedback interactions between plant and microbes in the rhizosphere.

Root exudate as a hidden part of plant defense system, has been neglected or underestimated in previous studies investigating plant-related nanotoxicity. In this study, the profiling analysis of root exudate metabolites revealed important detoxification mechanisms of cucumber plants to nano-Cu induced stress. In addition, ^{1}H NMR and GC-MS based metabolomics provided a comprehensive understanding of the changes in metabolites induced by exposure to nano-Cu, and this powerful combination of analytical techniques may be very useful in revealing effects of other NPs on different plant tissues, even at sublethal NP concentrations. The findings reflect the situation in hydroponic media, which is quite relevant for cucumber production in many parts of the world. However, the results may be different in soil media. Further studies are clearly needed to elucidate the contribution of membrane damage to up-regulated metabolites in root exudate.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b05011.

Biomass data, Cu distribution in different tissue, PCA scores plot from ^{1}H NMR spectra of leaf extracts and root exudates, one-dimensional ^{1}H NMR spectra of cucumber root extracts, pH of nutrient solution, Cu concentrations in plant tissues and nutrient solution (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +1 805 893 7548; fax: +1 805 893 7612; e-mail: keller@bren.ucsb.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the National Science Foundation (NSF) and the U.S. Environmental Protection Agency (EPA) under NSF-EF0830117. Any opinions, findings, conclusions, or recommendations expressed in this material are those of the authors do not necessarily reflect the views of the funding agencies. The MRL shared Experimental Facilities are supported by the MRSEC Program of the NSF under Award DMR 1121053; a member of the NSF-funded Materials Research Facilities Network. We also appreciate Agilent

DOI: 10.1021/acs.est.5b05011
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

Technologies for the Agilent Thought Leader Award given to A.A.K.

REFERENCES

(1) Faúndez, G.; Troncoso, M.; Navarrete, P.; Figueroa, G. Antimicrobial activity of copper surfaces against suspensions of Salmonella enterica and Campylobacter jejuni. *BMC Microbiol.* **2004**, *4* (1), 19.

(2) Garcia, P. C.; Rivero, R. M.; Ruiz, J. M.; Romero, L. The role of fungicides in the physiology of higher plants: Implications for defense responses. *Bot. Rev.* **2003**, *69* (2), 162−172.

(3) de Oliveira-Filho, E. C.; Lopes, R. M.; Paumgartten, F. J. R. Comparative study on the susceptibility of freshwater species to copper-based pesticides. *Chemosphere* **2004**, *56* (4), 369−374.

(4) California Department of Pesticide Regulation. *CDPR Database* (2009a); 2009.

(5) Mastin, B.; Rodgers, J. H., Jr Toxicity and bioavailability of copper herbicides (Clearigate, Cutrine-Plus, and copper sulfate) to freshwater animals. *Arch. Environ. Contam. Toxicol.* **2000**, *39* (4), 445−451.

(6) Kanhed, P.; Birla, S.; Gaikwad, S.; Gade, A.; Seabra, A. B.; Rubilar, O.; Duran, N.; Rai, M. In vitro antifungal efficacy of copper nanoparticles against selected crop pathogenic fungi. *Mater. Lett.* **2014**, *115*, 13−17.

(7) Lei, R.; Wu, C.; Yang, B.; Ma, H.; Shi, C.; Wang, Q.; Wang, Q.; Yuan, Y.; Liao, M. Integrated metabolomic analysis of the nano-sized copper particle-induced hepatotoxicity and nephrotoxicity in rats: A rapid invivo screening method for nanotoxicity. *Toxicol. Appl. Pharmacol.* **2008**, *232* (2), 292−301.

(8) Chang, Y. N.; Zhang, M. Y.; Xia, L.; Zhang, J.; Xing, G. M. The Toxic Effects and Mechanisms of CuO and ZnO Nanoparticles. *Materials* **2012**, *5* (12), 2850−2871.

(9) Lin, S.; Taylor, A. A.; Ji, Z.; Chang, C. H.; Kinsinger, N. M.; Ueng, W.; Walker, S. L.; Nel, A. E. Understanding the Transformation, Speciation, and Hazard Potential of Copper Particles in a Model Septic Tank System Using Zebrafish to Monitor the Effluent. *ACS Nano* **2015**, *9* (2), 2038−2048.

(10) Lee, W. M.; An, Y. J.; Yoon, H.; Kweon, H. S. Toxicity and bioavailability of copper nanoparticles to the terrestrial plants mung bean (Phaseolus radiatus) and wheat (Triticum aestivum): Plant agar test for water-insoluble nanoparticles. *Environ. Toxicol. Chem.* **2008**, *27* (9), 1915−1921.

(11) Hong, J.; Rico, C. M.; Zhao, L.; Adeleye, A. S.; Keller, A. A.; Peralta-Videa, J. R.; Gardea-Torresdey, J. L. Toxic effects of copper-based nanoparticles or compounds to lettuce (Lactuca sativa) and alfalfa (Medicago sativa). *Environmental Science: Processes & Impacts* **2015**, *17* (1), 177−185.

(12) Zhao, L. J.; Sun, Y. P.; Hernandez-Viezcas, J. A.; Servin, A. D.; Hong, J.; Niu, G. H.; Peralta-Videa, J. R.; Duarte-Gardea, M.; Gardea-Torresdey, J. L. Influence of CeO2 and ZnO Nanoparticles on Cucumber Physiological Markers and Bioaccumulation of Ce and Zn: A Life Cycle Study. *J. Agric. Food Chem.* **2013**, *61* (49), 11945−11951.

(13) Zhao, L. J.; Sun, Y. P.; Hernandez-Viezcas, J. A.; Hong, J.; Majumdar, S.; Niu, G. H.; Duarte-Gardea, M.; Peralta-Videa, J. R.; Gardea-Torresdey, J. L. Monitoring the Environmental Effects of CeO2 and ZnO Nanoparticles Through the Life Cycle of Corn (Zea mays) Plants and in Situ mu-XRF Mapping of Nutrients in Kernels. *Environ. Sci. Technol.* **2015**, *49* (5), 2921−2928.

(14) Viant, M. R.; Rosenblum, E. S.; Tjeerdema, R. S. NMR-based metabolomics: A powerful approach for characterizing the effects of environmental stressors on organism health. *Environ. Sci. Technol.* **2003**, *37* (21), 4982−4989.

(15) Lankadurai, B. P.; Nagato, E. G.; Simpson, M. J. Environmental metabolomics: an emerging approach to study organism responses to environmental stressors. *Environ. Rev.* **2013**, *21* (3), 180−205.

(16) Whitfield Åslund, M. L.; McShane, H.; Simpson, M. J.; Simpson, A. J.; Whalen, J. K.; Hendershot, W. H.; Sunahara, G. I. Earthworm sublethal responses to titanium dioxide nanomaterial in soil detected by 1H NMR metabolomics. *Environ. Sci. Technol.* **2012**, *46* (2), 1111−1118.

(17) Deng, Y.; Zhang, Y.; Zhang, R.; Wu, B.; Ding, L.; Xu, K.; Ren, H. Mice in vivo toxicity studies for monohaloacetamides emerging disinfection byproducts based on metabolomic methods. *Environ. Sci. Technol.* **2014**, *48* (14), 8212−8218.

(18) Santos, E. M.; Ball, J. S.; Williams, T. D.; Wu, H.; Ortega, F.; Van Aerle, R.; Katsiadaki, I.; Falciani, F.; Viant, M. R.; Chipman, J. K.; Tyler, C. R. Identifying health impacts of exposure to copper using transcriptomics and metabolomics in a fish model. *Environ. Sci. Technol.* **2010**, *44* (2), 820−826.

(19) Samuelsson, L. M.; Björlenius, B.; Förlin, L.; Larsson, D. J. Reproducible 1H NMR-based metabolomic responses in fish exposed to different sewage effluents in two separate studies. *Environ. Sci. Technol.* **2011**, *45* (4), 1703−1710.

(20) Davis, J. M.; Collette, T. W.; Villeneuve, D. L.; Cavallin, J. E.; Teng, Q.; Jensen, K. M.; Kahl, M. D.; Mayasich, J. M.; Ankley, G. T.; Ekman, D. R. Field-Based Approach for Assessing the Impact of Treated Pulp and Paper Mill Effluent on Endogenous Metabolites of Fathead Minnows (Pimephales promelas). *Environ. Sci. Technol.* **2013**, *47* (18), 10628−10636.

(21) Southam, A. D.; Lange, A.; Hines, A.; Hill, E. M.; Katsu, Y.; Iguchi, T.; Tyler, C. R.; Viant, M. R. Metabolomics Reveals Target and Off-Target Toxicities of a Model Organophosphate Pesticide to Roach (Rutilus rutilus): Implications for Biomonitoring. *Environ. Sci. Technol.* **2011**, *45* (8), 3759−3767.

(22) Bu, Q.; Yan, G. Y.; Deng, P. C.; Peng, F.; Lin, H. J.; Xu, Y. Z.; Cao, Z. X.; Zhou, T.; Xue, A. Q.; Wang, Y. L.; Cen, X. B.; Zhao, Y. L. NMR-based metabonomic study of the sub-acute toxicity of titanium dioxide nanoparticles in rats after oral administration. *Nanotechnology* **2010**, *21* (12), 125105.

(23) Badri, D. V.; Vivanco, J. M. Regulation and function of root exudates. *Plant, Cell Environ.* **2009**, *32* (6), 666−681.

(24) Marschner, H. *Marschner's Mineral Nutrition of Higher Plants*; Academic Press, 2011.

(25) Walker, T. S.; Bais, H. P.; Grotewold, E.; Vivanco, J. M. Root exudation and rhizosphere biology. *Plant Physiol.* **2003**, *132* (1), 44−51.

(26) Römheld, V. The role of phytosiderophores in acquisition of iron and other micronutrients in graminaceous species: an ecological approach. *Plant Soil* **1991**, *130* (1−2), 127−134.

(27) Hinsinger, P. How do plant roots acquire mineral nutrients? Chemical processes involved in the rhizosphere. *Adv. Agron.* **1998**, *64*, 225−265.

(28) Xu, J.; Yang, L.; Wang, Y.; Wang, Z. Advances in the study uptake and accumulation of heavy metal in rice (Oryza sativa) and its mechanisms. *Chin. Bull. Botany* **2004**, *22* (5), 614−622.

(29) Ma, J. F.; Ryan, P. R.; Delhaize, E. Aluminium tolerance in plants and the complexing role of organic acids. *Trends Plant Sci.* **2001**, *6* (6), 273−278.

(30) Dong, J.; Mao, W. H.; Zhang, G. P.; Wu, F. B.; Cai, Y. Root excretion and plant tolerance to cadmium toxicity - a review. *Plant Soil Environ.* **2007**, *53* (5), 193−200.

(31) Murphy, A. S.; Eisinger, W. R.; Shaff, J. E.; Kochian, L. V.; Taiz, L. Early copper-induced leakage of K+ from Arabidopsis seedlings is mediated by ion channels and coupled to citrate efflux. *Plant Physiol* **1999**, *121* (4), 1375−1382.

(32) Adeleye, A. S.; Conway, J. R.; Perez, T.; Rutten, P.; Keller, A. A. Influence of extracellular polymeric substances on the long-term fate, dissolution, and speciation of copper-based nanoparticles. *Environ. Sci. Technol.* **2014**, *48* (21), 12561−12568.

(33) Hong, J.; Peralta-Videa, J. R.; Rico, C.; Sahi, S.; Viveros, M. N.; Bartonjo, J.; Zhao, L.; Gardea-Torresdey, J. L. Evidence of translocation and physiological impacts of foliar applied CeO2 nanoparticles on cucumber (Cucumis sativus) plants. *Environ. Sci. Technol.* **2014**, *48* (8), 4376−4385.

(34) Lazareva, A.; Keller, A. A. Estimating Potential Life Cycle Releases of Engineered Nanomaterials from Wastewater Treatment Plants. *ACS Sustainable Chem. Eng.* **2014**, *2* (7), 1656−1665.

DOI: 10.1021/acs.est.5b05011
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

(35) Holden, P. A.; Klaessig, F.; Turco, R. F.; Priester, J. H.; Rico, C. M.; Avila-Arias, H.; Mortimer, M.; Pacpaco, K.; Gardea-Torresdey, J. L. Evaluation of Exposure Concentrations Used in Assessing Manufactured Nanomaterial Environmental Hazards: Are They Relevant? *Environ. Sci. Technol.* **2014**, *48* (18), 10541−10551.
(36) Zhao, F.; Hamon, R.; McLaughlin, M. J. Root exudates of the hyperaccumulator Thlaspi caerulescens do not enhance metal mobilization. *New Phytol.* **2001**, *151* (3), 613−620.
(37) Kim, H. K.; Choi, Y. H.; Verpoorte, R. NMR-based metabolomic analysis of plants. *Nat. Protoc.* **2010**, *5* (3), 536−549.
(38) Badri, D. V.; Chaparro, J. M.; Zhang, R. F.; Shen, Q. R.; Vivanco, J. M. Application of Natural Blends of Phytochemicals Derived from the Root Exudates of Arabidopsis to the Soil Reveal That Phenolic-related Compounds Predominantly Modulate the Soil Microbiome. *J. Biol. Chem.* **2013**, *288* (7), 4502−4512.
(39) Fiehn, O.; Wohlgemuth, G.; Scholz, M.; Kind, T.; Lee, D. Y.; Lu, Y.; Moon, S.; Nikolau, B. Quality control for plant metabolomics: reporting MSI-compliant studies. *Plant J.* **2008**, *53* (4), 691−704.
(40) Stampoulis, D.; Sinha, S. K.; White, J. C. Assay-Dependent Phytotoxicity of Nanoparticles to Plants. *Environ. Sci. Technol.* **2009**, *43* (24), 9473−9479.
(41) Musante, C.; White, J. C. Toxicity of silver and copper to Cucurbita pepo: differential effects of nano and bulk-size particles. *Environ. Toxicol.* **2012**, *27* (9), 510−517.
(42) Reichman, S.; Minerals, A. *The Responses of Plants to Metal Toxicity: A Review Focusing on Copper, Manganese and Zinc*; Australian Minerals & Energy Environment Foundation: Melbourne, 2002.
(43) Kennedy, C. D.; Gonsalves, F. A. N. The Action of Divalent Zn, Cd, Hg, Cu and Pb Ions on the Atpase Activity of a Plasma-Membrane Fraction Isolated from Roots of Zea-Mays. *Plant Soil* **1989**, *117* (2), 167−175.
(44) Luna, C. M.; Gonzalez, C. A.; Trippi, V. S. Oxidative Damage Caused by an Excess of Copper in Oat Leaves. *Plant Cell Physiol.* **1994**, *35* (1), 11−15.
(45) Weckx, J. E.; Clijsters, H. M. Oxidative damage and defense mechanisms in primary leaves of Phaseolus vulgaris as a result of root assimilation of toxic amounts of copper. *Physiol. Plant.* **1996**, *96* (3), 506−512.
(46) Savoure, A.; Thorin, D.; Davey, M.; Hua, X. J.; Mauro, S.; Van Montagu, M.; Inze, D.; Verbruggen, N. NaCl and CuSO4 treatments trigger distinct oxidative defence mechanisms in Nicotiana plumbaginifolia L. *Plant, Cell Environ.* **1999**, *22* (4), 387−396.
(47) Chen, L.-M.; Lin, C. C.; Kao, C. H., Copper toxicity in rice seedlings: changes in antioxidative enzyme activities, H2O2 level, and cell wall peroxidase activity in roots. *Botanical Bull. Academia Sinica* **2000**, *41*.
(48) Fernandes, J.; Henriques, F. Biochemical, physiological, and structural effects of excess copper in plants. *Bot. Rev.* **1991**, *57* (3), 246−273.
(49) Graham, R. D. Transport of copper and manganese to the xylem exudate of sunflower. *Plant, Cell Environ.* **1979**, *2* (2), 139−143.
(50) White, M. C.; Chaney, R. L.; Decker, A. M. Metal complexation in xylem fluid III. Electrophoretic evidence. *Plant Physiol.* **1981**, *67* (2), 311−315.
(51) Pich, A.; Scholz, G. Translocation of copper and other micronutrients in tomato plants (Lycopersicon esculentum Mill.): nicotianamine-stimulated copper transport in the xylem. *J. Exp. Bot.* **1996**, *47* (1), 41−47.
(52) Liao, M.; Hedley, M.; Woolley, D.; Brooks, R.; Nichols, M. Copper uptake and translocation in chicory (Cichorium intybus L. cv Grasslands Puna) and tomato (Lycopersicon esculentum Mill. cv Rondy) plants grown in NFT system. II. The role of nicotianamine and histidine in xylem sap copper transport. *Plant Soil* **2000**, *223* (1−2), 245−254.
(53) Hossain, M. A.; Piyatida, P.; da Silva, J. A. T.; Fujita, M. Molecular mechanism of heavy metal toxicity and tolerance in plants: central role of glutathione in detoxification of reactive oxygen species and methylglyoxal and in heavy metal chelation. *J. Bot.* **2012**, *2012*, 1.
(54) Cotruvo, J. A., Jr; Aron, A. T.; Ramos-Torres, K. M.; Chang, C. J. Synthetic fluorescent probes for studying copper in biological systems. *Chem. Soc. Rev.* **2015**, *44*, 4400.
(55) Lexmond, T. M.; Van der Vorm, P. The effect of pH on copper toxicity to hydroponically grown maize. *Netherlands J. Agric. Sci. (Netherlands)* **1981**, *29*, 217−238.
(56) Yau, P.; Loh, C.; Azmil, I. Copper toxicity of clove [Syzygium aromaticum (L.) Merr. and Perry] seedlings. *MARDI Res. J.* **1991**, *19*, 49−53.
(57) Ouzounidou, G.; Čiamporová, M.; Moustakas, M.; Karataglis, S. Responses of maize (Zea mays L.) plants to copper stress—I. Growth, mineral content and ultrastructure of roots. *Environ. Exp. Bot.* **1995**, *35* (2), 167−176.
(58) Waters, B. M.; Armbrust, L. C. Optimal copper supply is required for normal plant iron deficiency responses. *Plant Signaling Behav.* **2013**, *8* (12), e26611.
(59) Ziemniak, S. E.; Jones, M. E.; Combs, K. E. S. Magnetite Solubility and Phase-Stability in Alkaline Media at Elevated-Temperatures. *J. Solution Chem.* **1995**, *24* (9), 837−877.
(60) Rouached, H.; Secco, D.; Arpat, A. B. Getting the most sulfate from soil: Regulation of sulfate uptake transporters in Arabidopsis. *J. Plant Physiol.* **2009**, *166* (9), 893−902.
(61) Pätsikkä, E.; Kairavuo, M.; Šeršen, F.; Aro, E.-M.; Tyystjärvi, E. Excess copper predisposes photosystem II to photoinhibition in vivo by outcompeting iron and causing decrease in leaf chlorophyll. *Plant Physiol.* **2002**, *129* (3), 1359−1367.
(62) Shelp, B. J.; Bown, A. W.; McLean, M. D. Metabolism and functions of gamma-aminobutyric acid. *Trends Plant Sci.* **1999**, *4* (11), 446−452.
(63) Bouche, N.; Fromm, H. GABA in plants: just a metabolite? *Trends Plant Sci.* **2004**, *9* (3), 110−115.
(64) Roberts, M. R. Does GABA Act as a Signal in Plants? Hints from Molecular Studies: Hints from Molecular Studies. *Plant Signaling Behav.* **2007**, *2* (5), 408−409.
(65) Zulak, K. G.; Weljie, A. M.; Vogel, H. J.; Facchini, P. J. Quantitative 1H NMR metabolomics reveals extensive metabolic reprogramming of primary and secondary metabolism in elicitor-treated opium poppy cell cultures. *BMC Plant Biol.* **2008**, *8* (1), 5.
(66) Sohanpal, B. K.; El-Labany, S.; Lahooti, M.; Plumbridge, J. A.; Blomfield, I. C. Integrated regulatory responses of fimB to N-acetylneuraminic (sialic) acid and GlcNAc in Escherichia coli K-12. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101* (46), 16322−16327.
(67) Beloborodova, N.; Bairamov, I.; Olenin, A.; Shubina, V.; Teplova, V.; Fedotcheva, N. Effect of phenolic acids of microbial origin on production of reactive oxygen species in mitochondria and neutrophils. *J. Biomed. Sci.* **2012**, *19*, 89.
(68) Akula, R.; Ravishankar, G. A. Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling Behav.* **2011**, *6* (11), 1720−1731.
(69) *MetaboAnalyst 3.0: A Comprehensive Tool Suite for Metabolomic Data Analysis*. http://www.metaboanalyst.ca/.
(70) Xia, J.; Wishart, D. S. MSEA: a web-based tool to identify biologically meaningful patterns in quantitative metabolomic data. *Nucleic Acids Res.* **2010**, *38*, W71−W77.
(71) Sharma, S. S.; Dietz, K. J. The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. *J. Exp. Bot.* **2006**, *57* (4), 711−726.
(72) Homer, F.; Reeves, R.; Brooks, R. The possible involvement of aminoacids in nickel chelation in some nickel-accumulating plants. *Curr. Top. Phytochem* **1997**, *14*, 31−33.
(73) Farago, M.; Mullen, W. Plants which accumulate metals. Part IV. A possible copper-proline complex from the roots of Armeria maritima. *Inorg. Chim. Acta* **1979**, *32*, L93−L94.
(74) Mijovilovich, A.; Leitenmaier, B.; Meyer-Klaucke, W.; Kroneck, P. M.; Götz, B.; Küpper, H. Complexation and toxicity of copper in higher plants. II. Different mechanisms for copper versus cadmium detoxification in the copper-sensitive cadmium/zinc hyperaccumulator Thlaspi caerulescens (Ganges ecotype). *Plant Physiol.* **2009**, *151* (2), 715−731.

DOI: 10.1021/acs.est.5b05011
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

(75) Küpper, H.; Götz, B.; Mijovilovich, A.; Küpper, F. C.; Meyer-Klaucke, W. Complexation and toxicity of copper in higher plants. I. Characterization of copper accumulation, speciation, and toxicity in Crassula helmsii as a new copper accumulator. *Plant Physiol.* **2009**, *151* (2), 702−714.
(76) Rauser, W. E. Structure and function of metal chelators produced by plants - The case for organic acids, amino acids, phytin, and metallothioneins. *Cell Biochem. Biophys.* **1999**, *31* (1), 19−48.
(77) Ma, J. F. Role of organic acids in detoxification of aluminum in higher plants. *Plant Cell Physiol.* **2000**, *41* (4), 383−390.
(78) Meier, S.; Alvear, M.; Borie, F.; Aguilera, P.; Ginocchio, R.; Cornejo, P. Influence of copper on root exudate patterns in some metallophytes and agricultural plants. *Ecotoxicol. Environ. Saf.* **2012**, *75*, 8−15.
(79) Nigam, R.; Srivastava, S.; Prakash, S.; Srivastava, M. Cadmium mobilisation and plant availability−the impact of organic acids commonly exuded from roots. *Plant Soil* **2001**, *230* (1), 107−113.
(80) Duressa, D.; Soliman, K.; Taylor, R.; Senwo, Z. Proteomic analysis of soybean roots under aluminum stress. *Int. J. Plant Genomics* **2011**, *2011*, 1.
(81) Kamilova, F.; Kravchenko, L. V.; Shaposhnikov, A. I.; Azarova, T.; Makarova, N.; Lugtenberg, B. Organic acids, sugars, and L-tryptophane in exudates of vegetables growing on stonewool and their effects on activities of rhizosphere bacteria. *Mol. Plant-Microbe Interact.* **2006**, *19* (3), 250−256.
(82) Liu, S. Y.; Hu, X. J.; Lohrke, S. M.; Baker, C. J.; Buyer, J. S.; de Souza, J. T.; Roberts, D. P. Role of sdhA and pfkA and catabolism of reduced carbon during colonization of cucumber roots by Enterobacter cloacae. *Microbiology* **2007**, *153*, 3196−3209.
(83) Zaheer, I. E.; Ali, S.; Rizwan, M.; Farid, M.; Shakoor, M. B.; Gill, R. A.; Najeeb, U.; Iqbal, N.; Ahmad, R. Citric acid assisted phytoremediation of copper by Brassica napus L. *Ecotoxicol. Environ. Saf.* **2015**, *120*, 310−317.
(84) Upchurch, R. G. Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. *Biotechnol. Lett.* **2008**, *30* (6), 967−977.
(85) Allakhverdiev, S. I.; Kinoshita, M.; Inaba, M.; Suzuki, I.; Murata, N. Unsaturated Fatty Acids in Membrane Lipids Protect the Photosynthetic Machinery against Salt-Induced Damage inSynechococcus. *Plant Physiol.* **2001**, *125* (4), 1842−1853.
(86) Xu, L. X.; Han, L. B.; Huang, B. R. Membrane Fatty Acid Composition and Saturation Levels Associated with Leaf Dehydration Tolerance and Post-Drought Rehydration in Kentucky Bluegrass. *Crop Sci.* **2011**, *51* (1), 273−281.
(87) Singh, S. C.; Sinha, R. P.; Hader, D. P. Role of lipids and fatty acids in stress tolerance in cyanobacteria. *Acta Protozool.* **2002**, *41* (4), 297−308.
(88) Dombrowski, J. E.; Martin, R. C. Green leaf volatiles, fire and nonanoic acid activate MAPkinases in the model grass species Lolium temulentum. *BMC Res. Notes* **2014**, *7* (1), 807.
(89) Kranner, I.; Colville, L. Metals and seeds: biochemical and molecular implications and their significance for seed germination. *Environ. Exp. Bot.* **2011**, *72* (1), 93−105.
(90) Rico, C. M.; Morales, M. I.; McCreary, R.; Castillo-Michel, H.; Barrios, A. C.; Hong, J.; Tafoya, A.; Lee, W. Y.; Varela-Ramirez, A.; Peralta-Videa, J. R.; Gardea-Torresdey, J. L. Cerium Oxide Nanoparticles Modify the Antioxidative Stress Enzyme Activities and Macromolecule Composition in Rice Seedlings. *Environ. Sci. Technol.* **2013**, *47* (24), 14110−14118.
(91) Smith, K. L.; Bryan, G. W.; Harwood, J. L. Changes in Endogenous Fatty-Acids and Lipid-Synthesis Associated with Copper Pollution in Fucus Spp. *J. Exp. Bot.* **1985**, *36* (165), 663−669.
(92) Taiz, L.; Zeiger, E. *Plant Physiology*; Sinauer Associates, 2010.
(93) War, A. R.; Paulraj, M. G.; War, M. Y.; Ignacimuthu, S. Role of salicylic acid in induction of plant defense system in chickpea (Cicer arietinum L.). *Plant Signaling Behav.* **2011**, *6* (11), 1787−1792.
(94) Shabana, Y.; Abdel-Fattah, G.; Ismail, A.; Rashad, Y. Control of brown spot pathogen of rice (Bipolaris oryzae) using some phenolic antioxidants. *Braz. J. Microbiol.* **2008**, *39* (3), 438−444.
(95) Klessig, D. F.; Durner, J.; Noad, R.; Navarre, D. A.; Wendehenne, D.; Kumar, D.; Zhou, J. M.; Shah, J.; Zhang, S.; Kachroo, P.; et al. Nitric oxide and salicylic acid signaling in plant defense. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97* (16), 8849−8855.
(96) Mostofa, M. G.; Fujita, M. Salicylic acid alleviates copper toxicity in rice (Oryza sativa L.) seedlings by up-regulating antioxidative and glyoxalase systems. *Ecotoxicology* **2013**, *22* (6), 959−973.
(97) Potters, G.; Horemans, N.; Bellone, S.; Caubergs, R. J.; Trost, P.; Guisez, Y.; Asard, H. Dehydroascorbate influences the plant cell cycle through a glutathione-independent reduction mechanism. *Plant Physiol.* **2004**, *134* (4), 1479−1487.
(98) Pinton, R.; Varanini, Z.; Nannipieri, P. *The Rhizosphere: Biochemistry and Organic Substances at the Soil-Plant Interface*; CRC Press, 2007.
(99) Ge, Y.; Priester, J. H.; Van De Werfhorst, L. C.; Schimel, J. P.; Holden, P. A. Potential Mechanisms and Environmental Controls of TiO2 Nanoparticle Effects on Soil Bacterial Communities. *Environ. Sci. Technol.* **2013**, *47* (24), 14411−14417.

DOI: 10.1021/acs.est.5b05011
Environ. Sci. Technol. XXXX, XXX, XXX−XXX