Article (refereed) - postprint

Liu, Yang; Baas, Jan; Peijnenburg, Willie J.G.M.; Vijver, Martina G. 2016.
Evaluating the combined toxicity of Cu and ZnO nanoparticles: utility of the concept of additivity and a nested experimental design.
Environmental Science & Technology, 50 (10). 5328-5337.
10.1021/acs.est.6b00614

Evaluating the combined toxicity of Cu and ZnO nanoparticles: utility of the concept of additivity and a nested experimental design

Yang Liu [†, ‡, *], Jan Baas [‡, §], Willie J.G.M. Peijnenburg [‡, ||], Martina G. Vijver [‡]

[†] Faculty of Environmental Science and Engineering, Kunming University of Science and Technology, 650500 Kunming, Yunnan Province, China

[‡] Institute of Environmental Sciences (CML), Leiden University, 2300 RA Leiden, The Netherlands

[§] Centre for Ecology & Hydrology (CEH), MacLean Building, Benson Lane, OX10 8BB Wallingford, Oxfordshire, United Kingdom

[||] National Institute of Public Health and the Environment (RIVM), Center for Safety of Substances and Products, 3720 BA Bilthoven, The Netherlands

* Corresponding author. Tel.: +31(0)71 527 7477; fax: +31 (0)71 527 7434; e-mail address: minipig6@163.com (Yang Liu)

Text words excluding references: 5359

Small tables: 3 (900 words)

Small figures: 2 (600 words); Large figures: 1 (600 words)

Supporting information: 3 figures and 2 tables

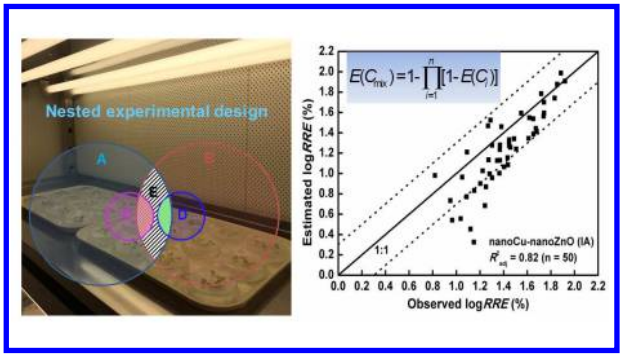
Total words: 7459

Abstract

Little is understood regarding the effects of mixtures of different metal-based nanoparticles (NPs). Using concentration addition (CA) and independent action (IA) models, we evaluated the combined toxicity of Cu and ZnO NPs based on five nested combinations, i.e. $Cu(NO_3)_2$-CuNPs, $Zn(NO_3)_2$-ZnONPs, $Cu(NO_3)_2$-ZnONPs, $Zn(NO_3)_2$-CuNPs, and CuNPs-ZnONPs on root elongation of *Lactuca sativa* L. The CA and IA models performed equally well in estimating the toxicity of mixtures of $Cu(NO_3)_2$-CuNPs, $Zn(NO_3)_2$-ZnONPs and $Zn(NO_3)_2$-CuNPs, whereas the IA model was significantly better to fit the data of $Cu(NO_3)_2$-ZnONPs and CuNPs-ZnONPs mixtures. Dissolved Cu proved to be the most toxic metal species to lettuce roots in the tests, followed by Cu NPs, dissolved Zn, and ZnO NPs respectively. An antagonistic effect was observed for ZnO NPs on the toxicity of Cu NPs. This antagonistic effect is expected to be the result of interactions between dissolved Cu and dissolved Zn, particulate Zn and dissolved Zn, particulate Cu and dissolved Zn, and between particulate Zn and dissolved Cu. In general terms, assuming additivity gives a first indication of the combined toxicity with soluble and insoluble metal particles both being important in driving toxicity of metal-based NPs to higher plants.

Keywords: Cu, Zn, nanoparticles, mixture, toxicity

Abstract graphic image



1. Introduction

Nanotechnology has been applied to create novel materials with unique characteristics in a large variety of consumer and household products. For example, engineered zinc oxide nanoparticles (NPs) are added into personal care products and coatings, which benefit from their ability to efficiently absorb UV-light and their increased transparency to visible light[1]. Nano-Cu powders can be dispersed into catalysts, conductive pastes, sintering additives, anti-bacteria products, and lubricant additives owing to their potential catalytic, dielectric, and biomedical properties[2].

Dissolution and aggregation/agglomeration are the two main processes that can strongly influence the state of metal-based NPs present in suspensions, and consequently impact the bioavailability, uptake and toxicity of NPs[3]. It has been reported that various characteristics of the exposure media can affect dissolution and aggregation of metal-based NPs, e.g. pH, ionic strength and the presence of naturally occurring organic matter[4]. Dissolution of NPs is a dynamic process in which constituent molecules of the dissolving solid migrate from the surface to the bulk solution through a diffusion layer[5]. The adsorption of molecules and ions from solution can promote or delay the dissolution process by modifying the characteristics of the diffusion layer[6]. Apart from heteroaggregation, particles can also be bound together (homoaggregation) when their equilibrium solubility is above saturation concentrations[7], which can increase the overall diffusion layer thickness and hinder dissolution of NPs.

Increasing numbers of applications may lead to direct or indirect releases of engineered metal-based NPs into the environment. This may pose effects on a variety of organisms in aquatic and terrestrial ecosystems, and in turn requires more attention on their eco-toxicological effects. Metal-based NPs are commonly predicted to occur in the environment as colloids [8]. Metal ions or small inorganic complexes produced by engineered metal-based NPs consisting of highly toxic elements inevitably drive the partial toxicity of metal-based NPs to organisms[3]. However, it is still a challenge to clarify which metal species contribute most to the nano-toxicity. Some studies suggested that the toxic effects of ZnO NPs and Cu

NPs on organisms were most likely due to the dissolved metal species rather than being particle-dependent [9-11]. Other researchers argued that the particulate forms of ZnO NPs and Cu NPs contributed substantially to the cytotoxic effects on mammalian and piscine cell lines [12, 13]. The translation from an effect on a cell line to a whole organism is not straightforward and depends on numerous factors such as the type of cell lines used and the physical and chemical composition of the NPs under consideration. Karlsson et al. [14] found for instance that the oxidative stress of mouse embryonic stem (mEs) cells was induced by the released Cu ions of CuO NPs whereas the stress was particle related for NiO NPs.

The lowest median $L(E)C_{50}$ value for nano-ZnO to aquatic organisms was observed to be <0.1 mg/L. This value classifies nano-ZnO as being 'extremely toxic', whereas the lowest values of $L(E)C_{50}$ (0.1~1 mg/L) classify nano-Cu as 'very toxic' [15]. These NPs were found to be simultaneously present in wastewater effluents [16-18]. They thus can jointly enter the terrestrial system by the application of bio-solids from sewage systems as a fertilizer [19]. Plants such as *Lactuca sativa* and *Medicago sativa* [20] have been reported to be able to take up and store metal-based NPs in their tissues [21]. To date, the knowledge of the eco-toxicity of Cu NPs and ZnO NPs is far from being adequate as compared to their large-scale application [22, 23], especially under conditions of co-exposure.

This study aims at improving the understanding of the effects of Cu NPs, ZnO NPs and their mixtures on *L. sativa* L. by unravelling the following two research questions: (1) Will the dissolved metals and the particulate metals of each type of metal-based NPs act jointly following the common rules of additivity? (2) Will Cu NPs interact with ZnO NPs and influence the toxicity of each other? Theoretically, if Cu NPs and ZnO NPs would act comparable to their metal salts, the existing models for predicting the combined effects of metals can be applied to predict the toxicity of mixtures of metal-based NPs. As shown in our previous studies [24, 25], Cu^{2+} competed with Zn^{2+} for binding to the biotic ligand of lettuce. However, this research is more complex than in case of mixtures of metal salts because suspensions of each type of metal-based NPs consist of a mixture mainly containing

dissolved metal species and undissolved particles. Suspensions of Cu NPs and ZnO NPs will therefore contain four metal species that are relevant to the toxicity of mixtures, i.e. dissolved Cu, dissolved Zn, particulate Cu and particulate ZnO. Cedergreen et al.[26] have shown that the joint effect of ternary mixtures can be predicted from binary mixture toxicity results. Thus, an elaborate nested experiment (binary, ternary, quaternary mixtures) was designed to include all possible combinations in order to trace down the potential 'interactions' between Cu NPs and ZnO NPs and where these 'interactions' (if any) take place. The two classic concepts of additivity i.e. concentration addition (CA) and independent action (IA) were both used for mixture toxicity predictions. A $Zn(NO_3)_2$ or $Cu(NO_3)_2$ solution was applied as a reference to assess the behavior of the dissolved Zn or Cu from the NPs in solution. The combined effects on root growth by nanoCu-nanoZnO mixtures were then compared with the overall effects of $Cu(NO_3)_2$ and $Zn(NO_3)_2$ reported in previous studies. The following combinations were studied:

- $Cu(NO_3)_2$ and Cu NPs (dissolved Cu and particulate Cu, Cu-nanoCu)
- $Zn(NO_3)_2$ and ZnO NPs (dissolved Zn and particulate ZnO, Zn-nanoZnO)
- $Zn(NO_3)_2$ and Cu NPs (dissolved Zn, dissolved Cu and particulate Cu, Zn-nanoCu)
- $Cu(NO_3)_2$ and ZnO NPs (dissolved Cu, dissolved Zn and particulate ZnO, Cu-nanoZnO)
- Cu NPs and ZnO NPs (dissolved Cu, dissolved Zn, particulate Cu and particulate ZnO, nanoCu-nanoZnO)

2. Methods

2.1 Test compounds and nutrient solution

Uncoated Cu NPs (nano-spheres, nominal particle size 50 nm, coded NM-0014, purity 99.8%) and uncoated ZnO NPs (nano-sticks, nominal particle size 150 nm, coded NM-110) were purchased from the io-li-tec company (Heilbronn, Germany). $Cu(NO_3)_2 \cdot 3H_2O$ (purity 99.5%), $Zn(NO_3)_2 \cdot 6H_2O$ (purity 99.5%) and other salts used in preparing the nutrient solution were all purchased from the Merck KGaA company (Darmstadt, Germany). The nutrient

solution was composed of $Ca(NO_3)_2 \cdot 4H_2O$ (236.1 mg/L), $MgSO_4 \cdot 7H_2O$ (60 mg/L), $NaHCO_3$ (50 mg/L), and $KHCO_3$ (10 mg/L) totally dissolved in demi-water (pH 7.8) and was applied for culturing plants and preparing the exposure media.

2.2 Experimental design

To allow the data to be interpreted over a wide range of concentrations of particulate and dissolved species of Cu and Zn, the composition of the five mixtures was chosen according to a full factorial experimental design. The nominal concentrations of $Cu(NO_3)_2$ ranged from 0.06 to 0.30 mg/L, the range of $Zn(NO_3)_2$ was from 2.90 to 17.39 mg/L, the range of Cu NPs was from 0.10 to 0.80 mg/L, and the range of ZnO NPs was from 0.50 to 50.00 mg/L. A detailed description of the experimental setup after pre-screening tests is provided in Table S1 on the basis of actual concentrations. Mixture treatments of each combination were repeated twice with negative controls (nutrient solution) and positive controls (single compounds, i.e. $Cu(NO_3)_2$, $Zn(NO_3)_2$, Cu NPs, ZnO NPs individually) for further analysis (section 2.6) and to reduce the variation of non-simultaneous toxicity tests. Hydroponic exposure was used as a starting point to study the mutual impacts between metal-based NPs and to avoid interference from complex interactions with soil particles. To keep the concentrations of compounds in solution constant, the test media were replaced every day. Stock suspensions of Cu NPs and ZnO NPs were daily prepared in nutrient solution and sonicated in an S 40 H Elmasonic water bath sonicator (Elma, Germany) for 10 min. All the stock solutions including the nitrate salts were further diluted 10 times with nutrient solution to obtain the nominal concentrations for each treatment.

2.3 Exposure of lettuce and toxicity determination

Lactuca sativa L. was selected as the test organism because its bioassay has been recommended to be a relatively easy and quick way of evaluating potential environmental risks by the Organization for Economic Cooperation and Development (OECD) [27]. The toxicity tests were performed according to guidelines of the US Environmental Protection Agency (EPA) [28]. As compared to the germination rate of seeds, the relative root elongation

rate (*RRE*, %) of lettuce seedlings is more accurate and more effective in reflecting the impact of external stressors [29], and therefore *RRE* was exploited as the toxicological endpoint in this study. Lettuce seeds were purchased from the Horti Tops company (Amsterdam, the Netherlands) and germinated on expanded perlite in a climate room (18°C, 80% humidity, and a 16:8 h light: dark cycle) for 96 h. After germination, seedlings with taproot lengths more than 3 cm were chosen to be fixed on parafilm strips floating on the surface of glass petri dishes containing 30 ml test medium. In each petri dish, 4 seedlings were introduced. Before and after 96 h exposure, the length of lettuce taproot was measured from the transition point between the hypocotyls and the root to the root tip. The root growth of each treatment was defined as the mean value of differences in root length of 4 seedlings before and after exposure. Then *RRE* was determined according to equation 1:

$$RRE = \frac{RG_S}{RG_C} \times 100\% \quad (1)$$

RG_s: the root growth of plants in the sample solution, cm;

RG_c: the root growth of plants in the control solution, cm.

2.4 Characterization of nanoparticles

The primary morphology and particle size of metal-based NPs prepared in lettuce culture medium were characterized using a JEOL 1010 Transmission Electron Microscope (TEM, JEOL, Japan). The particle size of Cu NPs and ZnO NPs was analyzed using a Nano Measurer 1.2 (Fudan University, China). The distribution of hydrodynamic diameter and the zeta-potential of NPs in seven types of test media (i.e. nano-Cu, nano-ZnO, Cu-nanoCu, Zn-nanoZnO, Cu-nanoZnO, Zn-nanoCu, nanoCu-nanoZnO mixtures prepared in lettuce culture medium) were measured after 1 h and 24 h of preparation by Dynamic Light Scattering (DLS) on a Zetasizer Nano-ZS instrument (Malvern, United Kingdom).

2.5 Chemical analysis

A Cu-ion selective electrode (Cu-ISE, Metrohm, Switzerland) was used as a direct way to measure the free Cu-ion activity in solution after 1 h and 24 h. A Zn-ion selective electrode

was not used in this study because the detection limit was not sufficient for the test. To check whether particles will reduce the sensitivity of the Cu-electrode membrane, plain polystyrene fluorescent microspheres # 103125-05 (nominal particle size 70 nm, Microspheres-Nanospheres, American) were added to compare the activities of Cu^{2+} with those in solutions of $Cu(NO_3)_2$ alone. The actual total concentrations of Ca, Mg, Na, K, Cu, Zn, and the dissolved concentrations of Cu and ZnO NPs after 1 h and 24 h of equilibration were analyzed using Flame Atomic Absorption Spectroscopy (FAAS, Perkin Elmer AAnalyst 100, American). Centrifugation of samples removed particles from suspensions. The supernatants were obtained and used for testing after 20 min of centrifugation in a Centrifuge 5415D (Eppendorf, Germany) at 13 300 g [12]. The particle suspensions, the supernatants and the liquids with nitrate salts were digested using HNO_3 and sampled for FAAS analysis.

2.6 Data analysis

To check the potential chemical-chemical interactions before entering the organism, relationships between the free Cu^{2+} activities in the solution (or the dissolved metal species of Cu NPs or ZnO NPs) and the added amount of one compound in mixtures of Cu-nanoCu, Zn-nanoZnO, Cu-nanoZnO, Zn-nanoCu, and nanoCu-nanoZnO after 1 h and 24 h were all analyzed using the linear regression method in the GraphPad Prism 5 software (GraphPad, American). For the Cu-nanoCu mixtures, the activities of Cu^{2+} released from Cu NPs were calculated by subtracting the Cu^{2+} activities of $Cu(NO_3)_2$ from the totally measured activities of Cu^{2+} in mixture solutions. The actually total or dissolved concentrations of Cu NPs or ZnO NPs in Cu-nanoCu and Zn-nanoZnO mixtures were calculated in a similar way.

The independent action (IA) model and the concentration addition (CA) model based on the rules of 'additivity' [30] were both used to predict the combined toxicity of mixtures of Cu-nanoCu, Zn-nanoZnO, Cu-nanoZnO, Zn-nanoCu and nanoCu-nanoZnO. The observed effects were then compared with the estimated values of CA or IA models. By definition, the basic assumption of additivity is that one compound is non-interactive with the other

compounds in a mixture [31]. The CA model is thought to be valid for mixtures where the components have similar target sites and a similar mode of action (MoA) [32]. The concept of IA originates from statistical considerations of independent responses and is supposed to be satisfactory for modeling effects of mixtures where the components differ in uptake pathways or MoA [30]. However, information on detailed MoA is still not available for the majority of chemicals[33]. Thus, the additive effects of mixtures were predicted by both multiplying the responses of mixture components (for IA), and by summing the scaled exposure levels (for CA) in this study. The IA model is mathematically presented by equation 2:

$$E(C_{\text{mix}}) = 1 - \prod_{i=1}^{n} [1 - E(C_i)] \quad (2)$$

$E(C_{\text{mix}})$: the estimated effect of an n-compound mixture;

$E(C_i)$: the effect of the *i*th compound applied singly at a fixed concentration, relative to the *RG*c of untreated controls.

To facilitate the estimation of toxic effects of mixtures on the basis of concentration addition, the sum of toxic units (TU_{mix}, a dimensionless ratio) was introduced to represent the toxic strength of a mixture [34]. Strict concentration addition occurs when the value of TU_{mix} equals one according to equation 3:

$$TU_{\text{mix}} = \sum_{i=1}^{n} \frac{c_i}{EC_{xi}} \quad (3)$$

c_i: the concentration of individual compound *i* in the mixture with *n* compounds;

EC*xi*: the effect concentration of individual compound *i* that results in the same effect (x%) as the mixture. The concentration-response relationship of a mixture was obtained using the logistic fuction [35] presented in equation 4 programmed in OriginPro 8 (Origin Lab, United Kingdom):

$$RRE = \frac{100}{1 + \left(\frac{TU_{\text{mix}}}{TU_{\text{mix}}^{50\%}}\right)^{\beta}} \quad (4)$$

RRE: RG_s of mixture treatments relative to RG_c of negative controls; β: the shape parameter that determines the steepness of the response curves; $TU_{mix}^{50\%}$: the sum of toxic units of a mixture inducing 50% inhibition of root elongation.

If compounds in a mixture do not follow the rules of 'additivity', 'interactions' between compounds may either increase or decrease the toxicity of mixtures relative to the model predictions [36]. Finding interactions in mixtures is always a challenge, especially when a mixture contains more than two components. Since the suspensions of mixtures of Cu-nanoZnO, Zn-nanoCu, and nanoCu-nanoZnO contain more than two metal species, searching interactions between these different metal species cannot be done using the existing models for binary mixtures. Therefore, a different approach was used in this study, which will be explained by the following example of Cu-nanoZnO mixtures.

To examine the influence of $Cu(NO_3)_2$ on the toxicity of ZnO NPs, the median effective concentrations (EC_{50}) of ZnO NPs in the co-exposure with $Cu(NO_3)_2$ were compared with the EC_{50} value of ZnO NPs in single exposure. The *RRE*s induced by ZnO NPs in co-exposure with $Cu(NO_3)_2$ can be calculated using the root growth of positive controls (with $Cu(NO_3)_2$ alone) as RG_c in equation (1). The *RRE*s induced by ZnO NPs alone can be calculated by using the root growth of negative controls (nutrient solution only) as RG_c in equation (1). The EC_{50}s of ZnO NPs in single-exposure or co-exposure with $Cu(NO_3)_2$ were calculated using the log (inhibitor) vs. normalized response-variable slope function in GraphPad Prism 5. It was assumed that if the EC_{50}s of ZnO NPs in the co-exposure with $Cu(NO_3)_2$ were significantly different from the value of ZnO NPs in single exposure, then the influence of $Cu(NO_3)$ on the toxicity of ZnO NPs was statistically significant. The EC_{50} values of ZnO NPs were plotted as a function of increasing concentrations of $Cu(NO_3)_2$ following linear regression in OriginPro 8. As an initial attempt, the slope of the obtained straight lines was compared with zero to indicate the overall antagonism or synergism. A non-significant slope is indicative of no substantial interactive effects of $Cu(NO_3)_2$ on the toxicity of ZnO NPs, a

265 significant positive ($p < 0.05$) slope implies the decreased toxicity of ZnO NPs by $Cu(NO_3)_2$
266 or the occurrence of antagonistic effects of $Cu(NO_3)_2$ on ZnO NPs, and a significant negative
267 slope indicates the increased toxicity of ZnO NPs by $Cu(NO_3)_2$ or synergistic effects. Similar
268 methods can be used to find out the influence of ZnO NPs (in both dissolved and particulate
269 forms) on the toxicity of $Cu(NO_3)_2$ and for other mixtures investigated in this study.

270 **3. Results**

271 ***3.1 Characterization of nanoparticles***

272 The TEM images of Cu NPs, ZnO NPs and their mixtures are shown in Figure 1. The
273 primary sizes and shapes of the particles were estimated based on the TEM images. The Cu
274 NPs were shown to be of spherical shape, 127 nm in size (size variation of 119-137 nm).
275 The ZnO NPs crystals displayed an approximatively tetragonal morphology (width: 55 nm,
276 size variation of 24-110 nm; length: 144 nm, size variation of 95-224 nm). The size
277 distribution of the hydrodynamic diameter of NPs in lettuce culture solution and in
278 suspensions of the five combinations investigated was determined using DLS and are shown
279 in Table S2. Initial particle sizes changed quickly after the NPs were submerged in lettuce
280 culture solution. Both NPs were present as aggregates (370 nm - 1531 nm) in lettuce culture
281 solution and in mixture suspensions. The hydrodynamic particle sizes of Cu NPs and ZnO
282 NPs increased by a factor of 1.5 to 2 after being submerged for 24 h in lettuce culture
283 solution and in suspensions of mixtures of Zn-nanoCu, nanoCu-nanoZnO, Zn-nanoZnO.

284 ***3.2 Fate analysis***

285 Relationships between the free activities of Cu^{2+} in solution and added ZnO NPs, $Zn(NO_3)_2$,
286 Cu NPs, $Cu(NO_3)_2$ after 1 h and 24 h are plotted in Figure S1. After 24 h, the activities of
287 Cu^{2+} were generally increased in mixtures of Cu-nanoCu, Zn-nanoCu, Cu-nanoZnO, and
288 nanoCu-nanoZnO as compared to the values after 1 h. However, no consistently significant
289 effects of increasing concentrations of $Cu(NO_3)_2$, Cu NPs, $Zn(NO_3)_2$, ZnO NPs were
290 observed on the activities of Cu^{2+} in solution after 1h and 24 h of equilibration using the Cu-

ISE. This indicated that the amount of free Cu^{2+} released from either Cu NPs or $Cu(NO_3)_2$ was not substantially affected by other compounds of Cu or Zn added to the solution. It is shown in Figure S2 that the trend of increasing Cu^{2+} activities in solution with polystyrene fluorescent microspheres remained constant when more $Cu(NO_3)_2$ was added and the slope of linear curves remained positive.

The background concentrations of Na, K, Ca, Mg in nutrient solution were measured to be respectively 11.9 ± 0.3 mg/L, 3.68 ± 0.07 mg/L, 31.24 ± 0.5 mg/L, and 5.49 ± 0.08 mg/L. The impacts of other compounds on the dissolved concentrations of Cu NPs and ZnO NPs after 1h and 24 h of equilibration in mixtures of Cu-nanoCu, Zn-nanoZnO, Cu-nanoZnO, Zn-nanoCu, nanoCu-nanoZnO are represented in Figure S3. Generally, the more NPs were added to the solution, the lower the percentage of dissolved metal-based NPs was measured after 1 h and 24 h. The dissolved concentrations of Cu NPs at the same concentration levels were found to be higher after 24 h in all combinations, which coincided with increases of the free Cu^{2+} activities. No statistically significant and consistent impacts were observed from addition of $Zn(NO_3)_2$, $Cu(NO_3)_2$, and Cu NPs on the dissolution of ZnO NPs. Although the dissolved concentrations of Cu NPs at lower concentrations were significantly increased by the added $Cu(NO_3)_2$ after 1 h, the influence was not constant across the whole range of concentrations. Only for Zn-nanoCu mixtures, it was found that after 24 h the dissolved concentrations of Cu NPs were significantly reduced by the addition of $Zn(NO_3)_2$.

3.3 Toxicity of individual compounds

Following the full factorial experimental design, a complete concentration-response curve was obtained for each compound investigated in this study, which was used to calculate the EC_{50} to *L. sativa* L. As shown in Table 1, ZnO NPs had the lowest acute toxicity to lettuce roots. By contrast, $Cu(NO_3)_2$ showed the highest toxic effects on root growth, which was followed by Cu NPs and $Zn(NO_3)_2$. The EC_{50} values of Cu NPs and $Cu(NO_3)_2$ were similar, but the EC_{50} value of ZnO NPs was twice as high as for $Zn(NO_3)_2$.

3.4 Toxicity of mixtures

The accuracy of model predictions is shown in Figure 2 and Table 2. Generally, 62%~100% of the estimated values of *RRE* were within a factor of 2 of the observed values. On the basis of 'additivity' or no interactions, the IA model performed better than the CA model in estimating toxicity of Cu-nanoCu, Zn-nanoZnO, Cu-nanoZnO, and nanoCu-nanoZnO mixtures at different exposure levels, as evidenced by a 0.04~24 percentage of increase in R^2. Apart from the combination of Cu-nanoCu, the combined effects of Zn-nanoZnO, Zn-nanoCu, Cu-nanoZnO, and nanoCu-nanoZnO mixtures were mostly underestimated using the IA model (Figure 2 F-J). This indicated that the predictive ability of IA and CA models was also combination-specific for mixtures with metal-based NPs.

To examine whether 'interactions' were the cause of remaining deviations from the model, the effective concentrations causing a 50% reduction in root elongation of Cu NPs, ZnO NPs, $Cu(NO_3)_2$, $Zn(NO_3)_2$ in single-exposure and in co-exposures of Cu-nanoCu, Zn-nanoZnO, Cu-nanoZnO, Zn-nanoCu, and nanoCu-nanoZnO were plotted as a function of various concentration levels of Cu or Zn in solution (Figure 3). Significant linear fits (p<0.05) with a positive slope were generally found in four out of five combinations, which indicated overall antagonistic effects between mixture components. In Figure 3 A-D, the EC_{50} values of nano-Cu were shown not to be statistically significantly increased upon increasing concentrations of $Cu(NO_3)_2$ and significant impacts of Cu NPs on the EC_{50}s of $Cu(NO_3)_2$ were neither observed. This implied that the dissolved Cu and the particulate Cu did not affect the toxicity of each other to lettuce (Table 3). For the combination of Cu-nanoZnO, the EC_{50} values of nano-Zn and $Cu(NO_3)_2$ cannot be accurately calculated when concentrations were beyond 40 mg/L and 0.06 mg/L respectively due to the small difference in root length as compared to positive controls. Without these data points, the EC_{50} values of $Cu(NO_3)_2$ were significantly increased upon increasing amounts of ZnO NPs in the solution (F-H, Figure 3), and the EC_{50}s of nano-Zn were significantly increased, up to a factor of 5.5 at 0.05 mg/L of $Cu(NO_3)_2$ (small graph inside E, Figure 3). For the combination of Zn-nanoCu, the EC_{50}s of $Zn(NO_3)_2$

were found to be sharply increased by the added Cu regardless of the metal species in solution, and a similar result was observed in turn at low concentrations (small graph inside I, Figure 3). The EC_{50} values of nano-ZnO significantly increased upon increasing concentrations of $Zn(NO_3)_2$ in solution. At lower concentrations of ZnO NPs, the EC_{50}s of $Zn(NO_3)_2$ were also increased with increasing concentrations of Zn NPs (small graphs inside N-P, Figure 3). This finding indicated that the dissolved Zn may compete against the particulate Zn for inducing toxicity to lettuce at lower concentrations of Zn NPs (< 20 mg/L). For nanoCu-nanoZnO mixtures, without the EC_{50}s of ZnO NPs at higher concentrations of Cu NPs, non-significant impacts of Cu NPs (<0.05 mg/L) were observed on the toxicity of ZnO NPs. The EC_{50}s of Cu NPs were observed to be significantly increased with an increased amount of ZnO NPs in solution (< 5mg/L).

4. Discussion

4.1 Fate of nanoparticles

The absolute values of the zeta-potential were < 14 mV, which implied that suspensions of NPs were unstable and general aggregation of the particles was observed because of the Van Der Waals inter-particle forces. This explained why the hydrodynamic size of Cu NPs and ZnO NPs in culture media of lettuce was not observed to be strongly affected by addition of $Cu(NO_3)_2$ and $Zn(NO_3)_2$ after 1 h and 24 h of equilibration. The high concentrations of salts in the nutrient solution can be an additional reason for the rapid aggregation observed[4]. The dissolution or ion release of Cu NPs or ZnO NPs was insignificantly and inconsistently hindered or stimulated by the increasing concentrations of Cu or Zn in the exposure media except in Zn-nanoCu mixtures after 24 h. The observation of significant impacts of Zn on the dissolution of Cu NPs may be related to the $Zn(NO_3)_2$ concentrations used which were in between 3.23 and 100 times higher than the concentrations of Cu NPs. The increasing amount of Zn^{2+} in water may bind to the negatively charged surface of Cu NPs, and thus hinder the dissolution of Cu NPs by increasing thickness of the diffusion layer of NPs or by introducing kinetic hindrance to the Cu^{2+}

372 diffusion process[5]. Nevertheless, a similar observation was not made for nanoCu-nanoZnO
373 mixtures, which implied that the impacts of nanoZnO on dissolution of nanoCu were not
374 concentration-dependent only. Alternatively, unlike the other properties of water chemistry
375 such as pH, HPO_4^{2-} and DOM [37], the increased background concentrations of Cu and Zn
376 may not strongly influence the dissolution of Cu NPs and ZnO NPs. Further research is still
377 needed on how to accurately quantify these two competing processes (i.e. dissolution and
378 aggregation) and their mutual impacts in water system for metal-based NPs.

379 ***4.2 Toxicity of individual compounds***

380 In this study, we found that both Cu NPs and ZnO NPs reduced the root size of lettuce.
381 Although copper and zinc are essential for plant growth, they are also toxic to plants at
382 concentrations that exceed critical levels [38]. The decrease of nutrients such as P and Fe, the
383 alteration of enzyme activity [20], and the higher production of reactive oxygen species (ROS)[39]
384 within the plants may be explanations for the impacts of metal-based NPs on lettuce growth.
385 In addition, the portion of aqueous Cu species dissolved from Cu NPs may also damage the
386 plasmalemma of root cells and result in loss of K, N, and other solutes [40]. Results of the
387 present study and of the previous studies showed that Cu is more toxic to lettuce seedlings
388 than Zn regardless of the metal being in the form of a cation or nanoparticles. This suggests
389 metal-specific responses of lettuce or different toxicokinetics of excess Cu and Zn in
390 terrestrial plants. However, the EC_{50} of dissolved Zn calculated in this study was significantly
391 lower than the value predicted for Zn^{2+} in the research of Le et al. [24]. This difference can be
392 the result of differences in the chemical composition of the background medium used and
393 the subsequent variation in speciation as calculated by the Windermere Humic-Aqueous
394 Model VI. Additionally, the effects of Cu or Zn seemed to be associated with the form of the
395 metal species that the relatively lower EC_{50} values of nitrate salts were calculated in this
396 study as compared to the corresponding nanoparticles. This finding was consistent with the
397 research of Hong et al. [20], in which the size of particles was shown to play an important role
398 in Cu uptake.

4.3 Combined toxicity of Cu NPs and ZnO NPs

4.3.1 Comparison of CA and IA models

The present study used the CA as well as the IA model to assess the combined toxicity of mixtures and their departures from additivity. The CA concept is usually proposed as a default conservative option as compared to the IA concept, which was also the case in our study. However, the fitting performance of the IA model was significantly better than the fitting performance of the CA model based on the value of R^2 for combinations of Cu-nanoZnO and nanoCu-nanoZnO. This is different from the findings of our previous studies [41, 42] showing that the combined effects of binary metal mixtures with Cu were equally well explained by these two classic mixture models. The mechanisms of toxicity of metal-based NPs in co-exposure were so complex that they cannot be directly extrapolated from normal metal mixtures. Therefore, it is suggested that both models should remain as statistical statements of joint effects, especially for mixtures of metal-based NPs.

4.3.2 Comparison with previous research

The good fittings provided by IA and CA (R^2_{adj}=0.90~0.94) models and the non-significant 'interactions' observed between dissolved and particulate Cu simultaneously verified the assumption of Song et al. [13] that addition models can be used to estimate the relative contributions of ionic and particulate forms to the cytotoxicity of Cu NPs. Both models also showed reasonable predictive power in estimating toxicity of Zn-nanoZnO mixtures (R^2_{adj}=0.79~0.83). Antagonistic interactions were identified between dissolved and particulate Zn which helped explain variations in modelling. Antagonistic effects were also observed for dissolved Zn or Cu resulting from NPs on the toxicity of their nitrate salts (Figure 3 G, K). This was consistent with the competition between Cu^{2+} and Zn^{2+} at the organism level reported in previous studies [24, 25, 36]. The feed-back mechanism [43] explained that an increase of copper in plant cell decreases the quantity of zinc importer proteins and blocks channels for zinc. In turn, the presence of low amounts of zinc may exert a positive effect on cell homeostasis and on the tolerance of cells to copper [18]. Until now, only Li et al.

[18] reported (1) the potentiation effects on the human hepatoma cell line HepG2 co-exposed to Cu NPs and ZnO NPs and (2) suggested that the nano-particulate ZnO NPs were attributable to the enhancement of Cu NPs toxicity. By contrast, ZnO NPs were observed to have an antagonistic effect on the impact of Cu NPs on root growth of lettuce at low concentrations, whereas the toxicity of ZnO NPs was insignificantly affected by Cu NPs. This difference may be caused by different features between animal cells and plant cells which lead to a diverse bioavailability or toxicity across species. Additionally, the one-sided antagonistic effects may be because the Zn concentrations were many times higher than the concentrations of Cu in the mixtures [36]. In compliance with the second finding of Li et al. [18], the particulate NPs were also observed to correlate with the 'interactions' and the overall toxicity of Cu NPs and ZnO NPs, which may be due to their physical effects [8] produced on the plant surface.

In summary, the concentration addition and independent action models performed equally well in assessing the combined toxicity of Cu-nanoCu, Zn-nanoZnO, and Zn-nanoCu mixtures on lettuce roots. However, for the combinations of Cu-nanoZnO and nanoCu-nanoZnO, the IA model significantly accounted for more variation in root growth. Based on the results of the Cu-nanoCu, Zn-nanoZnO, Zn-nanoCu, Cu-nanoZnO mixtures, the one-sided antagonistic effects observed for nanoCu-nanoZnO mixtures may be attributed to 'interactions' occurring between dissolved Cu and dissolved Zn, particulate Zn and dissolved Zn, particulate Cu and dissolved Zn, particulate Zn and dissolved Cu. It was thus demonstrated that considering various species of Cu NPs and ZnO NPs in water (dissolved and particulate) is of great importance for assessing their toxicity to terrestrial plants. The combined effects of dissolved species from NPs were similar to the effects produced by common metal mixtures. To our knowledge, exposure methods have been mostly used for assessing the ecological effects of single type NPs. Considering naturally occurring conditions, our experiments constitute the first study of mixture effects of NPs to higher plants. Although detailed information of interactive mechanisms of metal-based NPs with

453 their environment remains to be obtained, there is no doubt that this research will enrich the
454 rapid evolving field of nano-toxicology and help scientists develop approaches to evaluate
455 the potential impacts of metal-based NPs and their mixtures on ecosystems.

456 **Acknowledgments**

457 Supports provided to MG Vijver by the NWO VIDI project (No. 864.13.010), to J Baas by the
458 EU Marie Curie BIOME project (No. 328931), and to WJGM Peijnenburg by the EU-
459 sponsored FP7 project 'FutureNanoNeeds' (No. 604602) are gratefully acknowledged. The
460 authors thank Marcel Biermans for assisting in experiments.

461 **Declaration of interest**

462 The authors declare no competing financial interest.

463 **Supporting Information.** Figure S1 of relationships between free Cu^{2+} activities and the
464 added amount of ZnO NPs, $Zn(NO_3)_2$, Cu NPs, $Cu(NO_3)_2$, Figure S2 of relationships
465 between free Cu^{2+} activities and the added amount of $Cu(NO_3)_2$ in the presence of plain
466 polystyrene fluorescent microspheres, Figure S3 of relationships between the percentage of
467 dissolved amount of one compound and the added amount of other compounds in mixtures,
468 Table S1 of the experimental set up for mixtures, Table S2 of particle characterization by
469 dynamic light scattering. This information is available free of charge via the Internet at
470 http://pubs.acs.org/.

471 **References**

472 (1) Rousk, J.; Ackermann, K.; Curling, S. F.; Jones, D. L. Comparative toxicity of
473 nanoparticulate CuO and ZnO to soil bacterial communities. *PloS one* **2012**, *7*, e34197.
474 (2) Mortimer, M.; Kasemets, K.; Kahru, A. Toxicity of ZnO and CuO nanoparticles to ciliated
475 protozoa *Tetrahymena thermophila*. *Toxicology* **2010**, *269* (2), 182-189.

(3) Misra, S. K.; Dybowska, A.; Berhanu, D.; Luoma, S. N.; Valsami-Jones, E. The complexity of nanoparticle dissolution and its importance in nanotoxicological studies. *Sci. Total Environ*. **2012**, *438*, 225-232.

(4) Franklin, N. M.; Rogers, N. J.; Apte, S. C.; Batley, G. E.; Gadd, G. E.; Casey, P. S. Comparative toxicity of nanoparticulate ZnO, bulk ZnO, and $ZnCl_2$ to a freshwater microalga (*Pseudokirchneriella subcapitata*): the importance of particle solubility. *Environ. Sci. Technol.* **2007**, *41* (24), 8484-8490.

(5) Borm, P.; Klaessig, F. C.; Landry, T. D.; Moudgil, B.; Pauluhn, J.; Thomas, K.; Trottier, R.; Wood, S. Research strategies for safety evaluation of nanomaterials, part V: role of dissolution in biological fate and effects of nanoscale particles. *Toxicol. Sci.* **2006**, *90* (1), 23-32.

(6) Adamson, A. W.; Gast, A. P. *Physical chemistry of surfaces*; Wiley Press: New York, U.S., 1997.

(7) Holsapple, M. P.; Farland, W. H.; Landry, T. D.; Monteiro-Riviere, N. A.; Carter, J. M.; Walker, N. J.; Thomas, K. V. Research strategies for safety evaluation of nanomaterials, part II: toxicological and safety evaluation of nanomaterials, current challenges and data needs. *Toxicol. Sci.* **2005**, *88* (1), 12-17.

(8) Ribeiro, F.; Gallego-Urrea, J. A.; Goodhead, R. M.; Van Gestel, C. A.; Moger, J.; Soares, A. M.; Loureiro, S. Uptake and elimination kinetics of silver nanoparticles and silver nitrate by *Raphidocelis subcapitata*: The influence of silver behaviour in solution. *Nanotoxicology* **2014**, *9* (6), 686-695.

(9) Blinova, I.; Ivask, A.; Heinlaan, M.; Mortimer, M.; Kahru, A. Ecotoxicity of nanoparticles of CuO and ZnO in natural water. *Environ. Pollut.* **2010**, *158* (1), 41-47.

(10) Bondarenko, O.; Ivask, A.; Käkinen, A.; Kahru, A. Sub-toxic effects of CuO nanoparticles on bacteria: Kinetics, role of Cu ions and possible mechanisms of action. *Environ. Pollut*. **2012**, *169*, 81-89.

(11) Ivask, A.; Juganson, K.; Bondarenko, O.; Mortimer, M.; Aruoja, V.; Kasemets, K.; Blinova, I.; Heinlaan, M.; Slaveykova, V.; Kahru, A. Mechanisms of toxic action of Ag, ZnO

and CuO nanoparticles to selected ecotoxicological test organisms and mammalian cells in vitro: A comparative review. *Nanotoxicology* **2013**, *8* (sup1), 57-71.

(12) Fernández-Cruz, M. L.; Lammel, T.; Connolly, M.; Conde, E.; Barrado, A. I.; Derick, S.; Perez, Y.; Fernandez, M.; Furger, C.; Navas, J. M. Comparative cytotoxicity induced by bulk and nanoparticulated ZnO in the fish and human hepatoma cell lines PLHC-1 and Hep G2. *Nanotoxicology* **2013**, *7* (5), 935-952.

(13) Song, L.; Connolly, M.; Fernández-Cruz, M. L.; Vijver, M. G.; Fernández, M.; Conde, E.; de Snoo, G.; Peijnenburg, W. J., Navas, J. M. Species-specific toxicity of copper nanoparticles among mammalian and piscine cell lines. *Nanotoxicology* **2014**, *8* (4), 383-393.

(14) Karlsson, H. L.; Gliga, A. R.; Calléja, F. M.; Gonçalves, C. S.; Wallinder, I. O.; Vrieling, H.; Fadeel, B.; Hendriks, G. Mechanism-based genotoxicity screening of metal oxide nanoparticles using the ToxTracker panel of reporter cell lines. *Part. Fibre. Toxicol.* **2014**, *11* (1), 1-14.

(15) Kahru, A.; Dubourguier, H. C. From ecotoxicology to nanoecotoxicology. *Toxicology* **2010**, *269* (2), 105-119.

(16) Bystrzejewska-Piotrowska, G.; Golimowski, J.; Urban, P. L. Nanoparticles: their potential toxicity, waste and environmental management. *Waste Manage*. **2009**, *29* (9), 2587-2595.

(17) Bolyard, S. C.; Reinhart, D. R.; Santra, S. Behavior of engineered nanoparticles in landfill leachate. *Environ. Sci. Technol.* **2013**, *47* (15), 8114-8122.

(18) Li, L.; Fernández-Cruz, M. L.; Connolly, M.; Conde, E.; Fernández, M.; Schuster, M.; Navas, J. M. The potentiation effect makes the difference: Non-toxic concentrations of ZnO nanoparticles enhance Cu nanoparticle toxicity in vitro. *Sci. Total Environ.* **2015**, *505*, 253-260.

(19) Batley, G. E.; Kirby, J. K.; McLaughlin, M. J. Fate and risks of nanomaterials in aquatic and terrestrial environments. *Accounts Chem. Res.* **2012**, *46* (3), 854-862.

(20) 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

532 lettuce (*Lactuca sativa*) and alfalfa (*Medicago sativa*). *Environ. Sci. Processes Impacts* **2015**,
533 *17*, 177-185.
534 (21) Rico, C.M.; Majumdar, S.; Duarte-Gardea, M.; Peralta-Videa, J. R.; Gardea-Torresdey,
535 J. L. Interaction of nanoparticles with edible plants and their possible implications in the food
536 chain. *J. Agr. Food. Chem*. **2011***, 59*, 3485-3498.
537 (22) Hu, C. W.; Li, M.; Cui, Y. B.; Li, D. S.; Chen, J.; Yang, L. Y. Toxicological effects of TiO_2
538 and ZnO nanoparticles in soil on earthworm *Eisenia fetida*. *Soil Biol. Biochem.* **2010**, *42* (2),
539 586-591.
540 (23) Song, W.; Zhang, J.; Guo, J.; Zhang, J.; Ding, F.; Li, L.; Sun, Z. Role of the dissolved
541 zinc ion and reactive oxygen species in cytotoxicity of ZnO nanoparticles. *Toxicol. Lett.* **2010**,
542 *199* (3), 389-397.
543 (24) Le, T. T. Y.; Vijver, M. G.; Jan Hendriks, A.; Peijnenburg, W. J. Modeling toxicity of
544 binary metal mixtures (Cu^{2+}-Ag^{+}, Cu^{2}+-Zn^{2+}) to lettuce, *Lactuca sativa*, with the biotic ligand
545 model. *Environ. Toxicol. Chem*. **2013**, *32* (1), 137-143.
546 (25) Liu, Y.; Vijver, M. G.; Peijnenburg, W. J. Comparing three approaches in extending
547 biotic ligand models to predict the toxicity of binary metal mixtures (Cu–Ni, Cu–Zn and Cu–
548 Ag) to lettuce (*Lactuca sativa* L.). *Chemosphere* **2014**, *112*, 282-288.
549 (26) Cedergreen, N.; Sørensen, H.; Svendsen, C. Can the joint effect of ternary mixtures be
550 predicted from binary mixture toxicity results? *Sci. Total. Environ*. **2012**, *427*, 229-237.
551 (27) *OECD Test Guidelines 208: Terrestrial plant test-seedlings emergence and seedling*
552 *growth test*; OECD Guidelines for the Testing of Chemicals; Organization for Economic Co-
553 operation and Development: Paris, 2006.
554 (28) *Protocols for short term toxicity screening of hazardous waste sites*; EPA 600/3-88/029;
555 United States Environmental Protection Agency: Washington, DC, 1988.
556 (29) Pfleeger, T.; Mc Farlane, C.; Sherman, R.; Volk, G. Short-term bioassay for whole plant
557 toxicity. In *Plants for toxicity assessment*; Gorsuch, J. W., Lower, W. R., Lewis, M. A., Wang,
558 W. C., Eds.; American Society for Testing and Materials Press: Philadelphia 1991; 355-364.
559 (30) Bliss, C. I. The toxicity of poisons applied jointly. *Ann. Appl. Biol*. **1939**, *26* (3), 585-615.

(31) Berenbaum, M. C. The expected effect of a combination of agents: the general solution. *J. Theor. Biol*. **1985**, *114*, 413-431.

(32) Loewe, S.; Muischnek, H. Uber die Kombinationswirkungen. 1. Mitteilung: Hilfsmittel der Fragestellung. *Naunyn. Schmiedebergs Arch. Exp. Pathol. Pharmakol*. **1926**, *114*, 313-326. (German)

(33) *Harmonisation of human and ecological risk assessment of combined exposure to multiple chemicals*; EFSA Supporting publication EN-784; European Food Safety Authority: Edinburgh, 2015.

(34) Sprague, J. B. Measurement of pollutant toxicity to fish. II. Utilizing and applying bioassay results. *Water Research* **1970**, *4*, 3-32.

(35) Thakali, S.; Allen, H. E.; Di Toro, D. M.; Ponizovsky, A. A.; Rooney, C. P.; Zhao, F. J.; McGrath, S. P. A terrestrial biotic ligand model. 1. Development and application to Cu and Ni toxicities to barley root elongation in soils. *Environ. Sci. Technol*. **2006**, *40*, 7085-7093.

(36) Sharma, S. S.; Schat, H.; Vooijs, R.; Van Heerwaarden, L. M. Combination toxicology of copper, zinc, and cadmium in binary mixtures: concentration-dependent antagonistic, nonadditive, and synergistic effects on root growth in *Silene Vulgaris*. *Environ. Toxicol. Chem.* **1999**, *18* (2), 348-355.

(37) Li, M.; Lin, D.; Zhu, L. Effects of water chemistry on the dissolution of ZnO nanoparticles and their toxicity to *Escherichia coli*. *Environ. Pollut*. **2013**, *173*, 97-102.

(38) 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.

(39) Remans, T.; Thijs, S.; Truyens, S.; Weyens, N.; Schellingen, K.; Keunen, E.; Gielen, H.; Cuypers, A.; Vangronsveld, J. Understanding the development of roots exposed to contaminants and the potential of plant-associated bacteria for optimization of growth. *Ann. Bot*. **2012**, *110*, 239-252.

(40) Ali, N. A.; Bernal, M. P.; Ater, M. Tolerance and bioaccumulation of copper in *Phragmites australis* and *Zea mays*. *Plant Soil* **2002**, *239*, 103-111.

(41) Le, T. T. Y.; Vijver, M. G.; Kinraide, T. B.; Peijnenburg W. J. G. M. Modelling metal-metal interactions and metal toxicity to lettuce *Lactuca sativa* following mixture exposure (Cu^{2+}-Zn^{2+} and Cu^{2+}-Ag^{+}). *Environ. Pollut*. **2013**, *176*, 185-192.

(42) Liu, Y.; Vijver, M. G.; Qiu, H.; Baas, J.; Peijnenburg, W. J. G. M. Statistically significant deviations from additivity in metal mixtures, what do they mean? *Ecotox. Environ. Safe.* **2015**, *122*, 37-44.

(43) Qiu, A.; Hogstrand, C. Functional expression of a low-affinity zinc uptake transporter (FrZIP2) from pufferfish (*Takifugu rubripes*) in MDCK cells. *Biochem. J.* **2005**, *390* (Pt 3), 777-786.

615

616

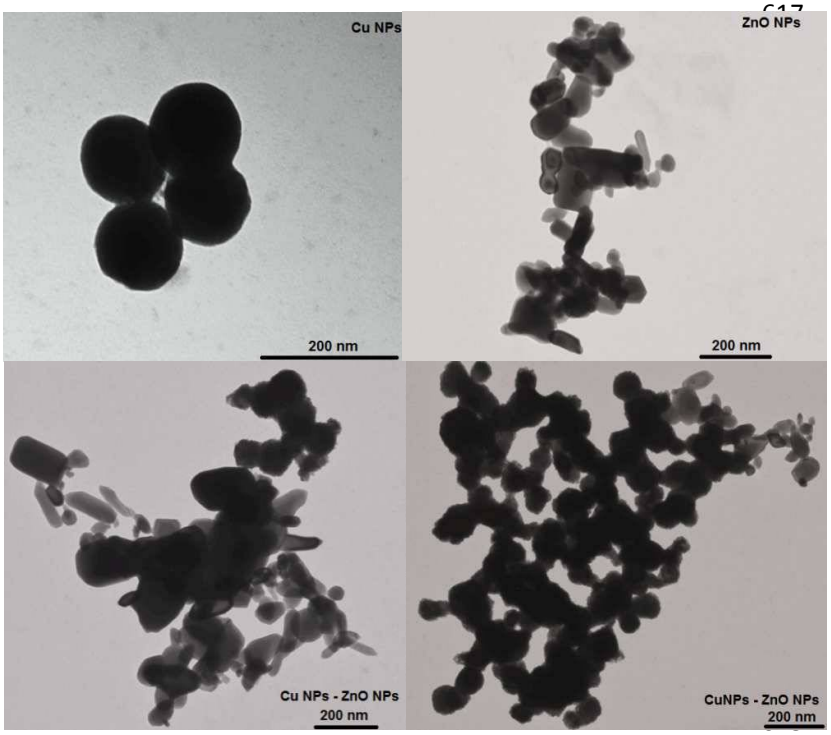


Figure 1. TEM images of Cu NPs, ZnO NPs and their mixtures (prepared in lettuce culture medium). Scale bars indicate size (nm).

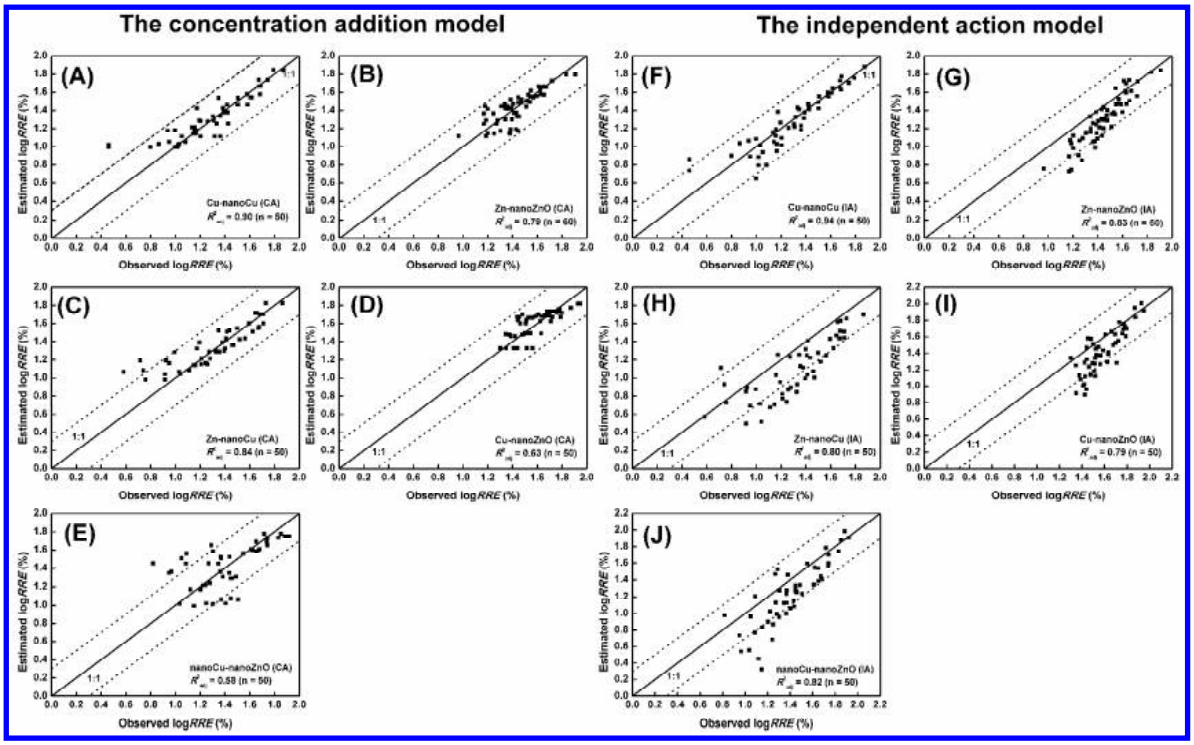
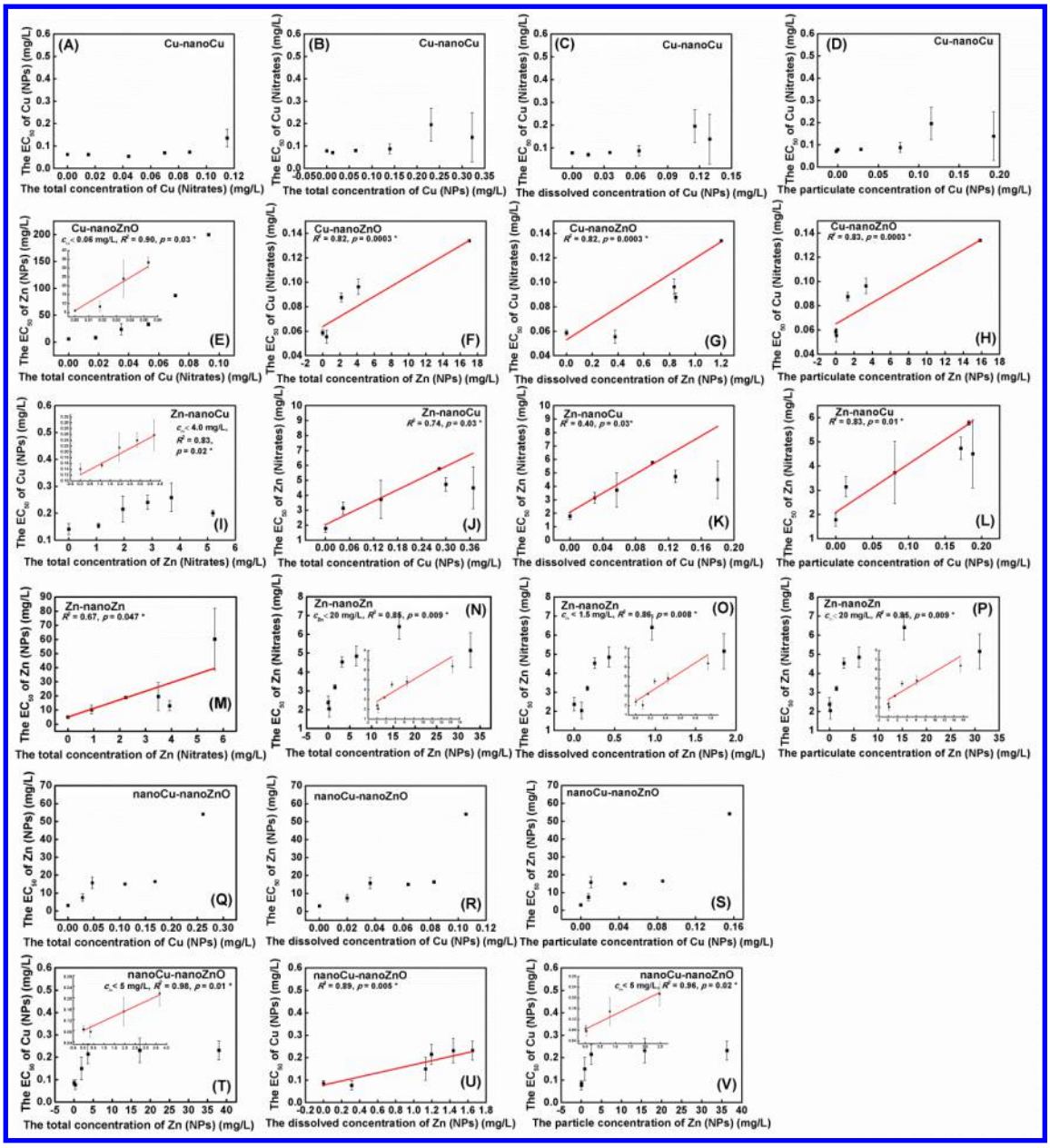


Figure 2. Relationships between the observed and estimated log 4-d relative root elongation (*RRE*, %) of Cu-nanoCu, Zn-nanoZnO, Cu-nanoZnO, Zn-nanoCu and nanoCu-nanoZnO mixtures for lettuce *Lactuca sativa* L. using the CA model (A-E) and the IA model (F-J). R^2_{adj} indicates the adjusted determination coefficient. n indicates the number of data points. The solid line represents the perfect fit (1:1 line) and the dotted lines represent a difference of a factor of 2 between the observed and estimated values.



643

644 Figure 3. Relationships between the median effective concentrations (EC_{50}s) of one
645 component for *L. sativa* L. after 4-d exposure and the concentration (total, dissolved,
646 particulate) of the other components in mixtures of Cu-nanoCu, Zn-nanoZnO, Cu-nanoZnO,
647 Zn-nanoCu, nanoCu-nanoZnO. Data are presented as mean ± standard error of the mean.
648 Solid lines represent the statistically significant linear fits (a positive slope indicates an
649 overall antagonistic effects; a negative slope indicates an overall synergistic effects). The
650 smaller graphs inside figures depict significant effects occurring at low concentration levels.

651 The dissolved concentrations of metal-based NPs were expressed as the average value
652 after 1 h and 24 h of equilibration. R^2 indicates the determination coefficient adjusted for the
653 degrees of freedom. *p* indicates the statistical significance level. * indicates that the slope of
654 linear curve is significantly different from zero at the 5% significance level.

655

656

657

658

659

660

661

662

663

664

665

666

667

668

669

670

671

672 Table 1. The median effective concentrations (EC_{50}, mg/L) or median effective activities
673 (EA_{50}, μmol/L) with the standard error of the mean (SEM) or the 95% confidence interval (CI)
674 of Cu NPs, ZnO NPs, $Cu(NO_3)_2$ and $Zn(NO_3)_2$ individually on 4-d root elongation of lettuce (*L.*
675 *sativa* L.) in the present study and in the previous study.

Compounds	Metal species	EC_{50} (mg/L) or EA_{50} (μmol/L)	n	Sources
Cu NPs	Total Cu	0.10 (±0.01)	7	This study
ZnO NPs	Total Zn	4.47 (±0.62)	7	This study
$Cu(NO_3)_2$	Dissolved Cu	0.07 (±0.006)	4	This study
$Zn(NO_3)_2$	Dissolved Zn	2.08 (±0.25)	4	This study
$Cu(NO_3)_2$	Cu^{2+}	0.03 (0.02 ~ 0.04)	-	24
$Zn(NO_3)_2$	Zn^{2+}	106 (91.1 ~ 124)	-	24

676 n: the number of replicates; -: not determined.
677
678
679
680
681
682
683
684
685
686
687

688 Table 2. Fitting results of the toxicity of Cu-Zn, Cu-nanoCu, Zn-nanoZnO, Cu-nanoZnO, Zn-nanoCu, and nanoCu-nanoZnO mixtures by the
689 concentration addition (CA) model and the independent action (IA) model.

Mixtures	The IA model		The CA model				Sources
	R^2_{adj}	*p* value	R^2_{adj}	*p* value	$TU^{50\%}_{mix}$ (±SE)	β (±SE)	
Cu-Zn (n=122)	0.92	＜0.0001*	0.92	＜0.0001*	-	-	41
Cu-nanoCu (n=50)	0.94	＜0.0001*	0.90	＜0.0001*	0.79 (±0.04)	1.34 (±0.07)	This study
Zn-nanoZnO (n=60)	0.83	＜0.0001*	0.79	＜0.0001*	0.92 (±0.08)	0.79 (±0.06)	This study
Cu-nanoZnO (n=50)	0.79	＜0.0001*	0.63	＜0.0001*	1.16 (±0.13)	0.62 (±0.07)	This study
Zn-nanoCu (n=50)	0.80	＜0.0001*	0.84	＜0.0001*	1.44 (±0.07)	1.65 (±0.12)	This study
nanoCu-nanoZnO (n=50)	0.82	＜0.0001*	0.58	＜0.0001*	0.81 (±0.11)	0.88 (±0.12)	This study

690 R^2_{adj}: the adjusted coefficient of determination; *p*: the outcome of the likelihood ratio test; *: significant at the 5% significance level; β: the shape
691 parameter that determines the steepness of the response curves; $TU^{50\%}_{mix}$: the sum of toxic units of a mixture inducing 50% inhibition of root
692 elongation; SE: standard error; -: not determined; n: the number of data points.
693

694 Table 3. Mutual impacts found between mixture components in combinations of Cu-Zn, Cu-nanoCu, Zn-nanoZnO, Cu-nanoZnO, Zn-nanoCu,
695 and nanoCu-nanoZnO in the exposure of 4-d lettuce seedlings.

Substance 1 / Substance2	$Zn(NO_3)_2$	$Cu(NO_3)_2$	Cu NPs	Dissolved Cu	Particulate Cu
$Cu(NO_3)_2$	Anta\Anta[24,25]	n.d.	No\No	No\n.d.	No\n.d.
$Zn(NO_3)_2$	n.d.	Anta\Anta[24,25]	Anta\No	Anta\n.d.	Anta\n.d.
ZnO NPs	Anta\Anta	Anta\Anta	No\Anta	No\n.d.	No\n.d.
Dissolved Zn	n.d.\Anta	n.d.\Anta	n.d.\Anta	n.d.	n.d.
Particulate ZnO	n.d.\Anta	n.d.\Anta	n.d.\Anta	n.d.	n.d.

696 No significant effect (No): the slope of the linear fits in Figure 3 is not significantly different from zero, which indicates that no significant effects
697 of one component were observed on the EC_{50} values of another component; Antagonism (Anta): the positive slope of linear fits in Figure 3
698 significantly deviates from zero ($p < 0.05$), which indicates an antagonistic effect of one component on another component in the mixture; n.d.:
699 not detected in this study; \: the left side indicates the effects of Substance 1 on Substance 2 and the right side indicates the effects of
700 Substance 2 on Substance 1.