1 **Quantitative evaluation of multi-wall carbon nanotube uptake by terrestrial plants**

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ABSTRACT: *The extent and mechanisms by which multi-wall carbon nanotubes (MWCNTs) are accumulated by terrestrial plant species is currently unknown. In this study, we successfully measured the MWCNTs content in different plant (Arabidopsis thaliana, rice, maize and soybean) using ¹⁴C labeled MWCNTs. Quantitative relationships among the uptake amount of MWCNTs in different Arabidopsis tissues, the physiological (water loss, pigment content, and total protein content) and biochemical parameters (antioxidant enzyme activities) were further evaluated. Our results indicated that changes in biochemical parameters were much more sensitive than physiological parameters. The lowest CAT activities in the treatment with (1,3-dinitrobenzene + MWCNTs) were only 0.13- fold of that in the 1,3-dinitrobenzene alone treatment, suggesting MWCNTs could alleviate the toxicity to Arabidopsis. Due to the toxicity caused by small polar aromatic organic molecules (SPAOMs), the MWCNTs uptake in leaf in the treatment with (2,4-dichlorophenol + MWCNTs) was only 0.07-fold relative to the MWCNTs alone treatment. Hydrodynamic diameter of MWCNTs in solution did not significantly affect MWCNTs uptake. Uptake models for MWCNTs into root and leaf tissue were constructed via stepwise multiple linear regression analysis. This study will provide important information for the accurate determination of risk associated with MWCNT exposure.*

1. Introduction

Carbon nanotubes (CNTs) are allotropes of carbon with a cylindrical nanostructure [1] that can form both naturally [2-4] and artificially. Due to their unique electronic, optical, thermal, and photoactive properties, carbon nanotubes (CNTs) have been applied widely [5]. Current global multi-wall CNTs (MWCNTs) production capacity is estimated to be 13,996 tons [1]. With a sharply increasing demand and use for CNTs [1], these materials will inevitably be released into the environment during production, transport, handling, use and disposal, with soil being a primary source and sink. Gottschalk *et al.* forecasted that the annual input of CNTs into agricultural soils treated with sewage sludge could be increased by 74 ng/kg based on CNTs input data from 2008 [6]. Owing to their inherent hydrophobicity, the concentration of CNTs in soil could be approximately 20-fold higher than in water [7]. As such, CNT exposure to terrestrial plants is likely. CNTs accumulated by crops could bioaccumulate and biomagnify along the food chain, eventually leading to human exposure [8-15]. Since CNTs may pose a significant risk to ecological and human health, it is essential to understand their toxicity, uptake, and translocation of CNTs in plant [8, 16-19].

CNT accumulation has been reported to vary considerably among different plant species [8, 12, 20-24]. No accumulation was reported in the roots of cabbage [20], carrot [20], cucumber [20], lettuce [20], onion [20], tomato [20], Alfalfa [21] and wheat [21]. However, CNTs uptake by maize [15], soybean [15], pea [11], sunflower [11], tomato [11], wheat [11, 12], rapeseed [12] and different plant cells [8-10, 13, 14] was observed. Notably, most of these studies only used qualitative methods such as Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and Raman spectroscopy [8-10, 13-15, 20, 22-25]. There are some distinct disadvantages when using qualitative methods to confirm the presence of CNTs in biological samples. For example, CNTs detection in plant tissues by SEM/TEM requires significant sample pretreatment and fixation, and minor variations in sample dissection can create significant experimental artifacts. Raman spectroscopic analysis can demonstrate CNTs accumulation in plants by detecting the relative CNT signal, but the method sensitivity is quite low. Irin *et al.* did not detect CNT Raman signals in alfalfa grown for 14 days in 1000 mg/kg MWCNT soil, although the presence of MWCNT was confirmed by thermal gravimetric analysis and microwave approaches [26]. Clearly, robust quantitative methods are needed in order to accurately characterize the toxicity, uptake, and translocation of CNTs in plants.

Several quantitative approaches, including isotopic labeling [12], near infrared fluorescence [27], photoluminescence [28], thermogravimetry [29, 30] have been used with CNTs recently [12, 31, 32]. However, to date there has not been sufficient CNT accumulation data obtained to accurately describe the relationship between CNT content in plant tissues, CNT properties, and resulting biochemical/physiological effects. Once these relationships are defined, a mechanistic understanding of the governing processes will be more likely.

Small polar aromatic organic molecules (SPAOMs) occur either naturally (e.g. L-phenylalanine, 4-aminobenzoic acid) or from anthropogenic activity (e.g. nitrobenzene, phenols) and are ubiquitous in the environment. However, little is known about the effect of SPAOMs on CNTs fate in planted systems. There are limited but related studies focusing on accumulation of pesticides by plants in the presence of CNTs [33-35]. The results showed that pesticide uptake was generally reduced by CNTs [33-35]. Given the evidence for the potential toxicity of CNTs to animals [8, 16] and plants [19, 20, 22, 36-40], the effects of SPAOMs on CNT fate should be carefully studied. Our previous study showed that SPAOMs can promote CNTs dispersion and enhance material stability in solution as a function of organic co-solute water solubility [41]. In addition, due to their phytotoxicity [42, 43], SPAOMs could also indirectly impact CNTs accumulation in plants by affecting plant biochemical/physiological properties.

In this study, one model plant *Arabidopsis thaliana*, three crops, including *Oryza sativa* (rice), *Zea mays* (maize), and *Glycine max* (soybean), were selected for MWCNTs quantitative uptake study. Additionally, we further examined the physiological and biochemical responses of *Arabidopsis* under the co-contaminated environment. The representative SPAOMs included 1,4-dinitrobenzene (1,4DNB, water solubility Cs 69 mg/L), 1,2-dinitrobenzene (1,2DNB, 133 mg/L), 1,3-dinitrobenzene (1,3DNB 574.9 mg/L), nitrobenzene (NB, 1936 mg/L), 2,4-dichlorophenol (2,4DCP, 4600 mg/L, pKa=7.90), 2-chlorophenol (2CP, 28500 mg/L, pKa=8.44) and 4-chlorophenol (4CP, 26300 mg/L, pKa=9.38) [44]. C-14 labeled MWCNTs were used to quantify the uptake and distribution of MWCNTs in plants. The specific objectives of the current study were to 1) quantitatively evaluate the distribution of MWCNTs in different plant tissues and 2) characterize the relationship between CNTs uptake, plant biochemical/physiological properties, and CNT properties in the presence of SPAOMs. The results of this study will provide important information for the accurate determination of risk associated with CNT exposure.

2. Materials and Methods

2.1 Chemicals and Reagents

¹⁴C-labeled MWCNTs were synthesized by a modified chemical vapor deposition (CVD) and were treated with a 3:1 mixture of sulfuric to nitric acid as described in previous studies [45-48]. The MWCNTs had a specific surface area of 111 m²/g and a specific radioactivity of 0.1 mCi/g. The surface oxygen content was 8.6% as measured by X-ray photoelectron spectroscopy analysis [47]. A MWCNTs suspension was obtained by mixing 100 mg MWCNTs into 1 L of de-ionized water via ultrasonication (200 W; Cole-Parmer CV33) for 2 h; the solution was then kept at 25±1 °C for 10 h before use. The initial concentration of MWCNTs was 74.1 mg/L by using liquid scintillation counting (LSC; Benchman [Fullerton, CA] LS6500). Thermal gravimetric and scanning electron microscopic analyses are provided in the Supporting Information (Figure S1). The average MWCNT diameter was 36.5 ± 12.7 nm (n = 80, the uncertainty indicates the standard deviation), and the average length was 350 nm (n = 836; Figure S1c).

Nitrobenzene was purchased from Acros Organics Co. Dinitrobenzenes (1,2DNB, 1,3DNB and 1,4DNB) and chlorophenols (2,4DCP, 2CP and 4CP) were obtained from Sigma-Aldrich Co. Murashige and Skoog (MS) basal medium with vitamins and sucrose were both from PhytoTechnology Lab (Shawnee Mission, Kansas).

2.2 Hydroponic Experiment

Prior to the hydroponic experiment, *Arabidopsis* was grown in different concentrations (0, 0.45, 0.9, 2.25 and 4.5 mg/L) of MWCNTs amended half-strength (1/2X) MS semisolid medium (2.22 g MS basal medium with vitamins, 10 g sucrose in 1 L deionized water, pH 5.7) for 25 days under an environmentally controlled growth chamber (light period: 16/8 hrs; temperature: 22/20 °C, day/night) to determine the preferred concentration (based on fresh biomass) (Figure 1a). Briefly, *Arabidopsis* seeds were sterilized with ethanol (70%, v/v) for 10 min and then twice soaked in Clorox (25%, v/v) for 15 min. The seeds were washed five times with autoclaved deionized water before use [49]. A series of MWCNTs concentrations (0, 0.45, 0.9, 2.25 and 4.5 mg/L) were prepared in 1/2X MS semisolid medium supplemented with 8 g phytoblend agar (Caisson Laboratories, UT). Twenty-five sterilized seeds were placed on each dish and were stratified at 4 °C for 24 h before transferred to the growth chamber (Percival Scientific, Perry, IA). After seed germination, the Petri dishes were placed vertically in order to facilitate shoot and root growth during an additional 25 days. At harvest, the total biomass in each treatment was

used to select the MWCNTs concentration in the following experiment. The results showed that 2.25 mg/L MWCNT had no impact on *Arabidopsis* growth, whereas significantly lower biomass was evident upon exposure to 4.50 mg/L (Figure S2). Hence, 2.25 mg/L MWCNT was chosen for the subsequent hydroponic experiment. Given that our primary goal was to quantify MWCNT uptake by and distribution in plants, although the MWCNT concentrations were high, they were fit for purpose [50]. The hydroponic experimental design is shown in Figure 1b. *Arabidopsis* was incubated in Petri dishes for 14 days and was then transferred to Hoagland's solution (PhytoTechnology Laboratories, KS) in 250 mL beaker for 7 days to acclimatize the new environment. *Arabidopsis* was then moved into 90 mL fresh Hoagland's solution amended with 2.25 mg/L MWCNT suspension and 0.325 mM SPAOMs for 24 hours exposure. The reason we chose 0.325 mM for SPAOMs is that they significantly affect MWCNTs dispersibility [41]. At harvest, parameters (described below) including MWCNT uptake, pigment analysis, and antioxidant enzyme activities were measured in each treatment. Each treatment was repeated at least four times to ensure the reproducibility of the results.

Fig. 1- Impact of MWCNTs on *Arabidopsis* germination and seedling growth. (a) Images of *Arabidopsis* treated with different concentrations of MWCNTs (b) Hydroponic experimental design.

For three crop plants, the seeds were germinated in vermiculite for 14 days. The seedlings were then transferred to 1/2X Hoagland's solution amended test tube and allowed to acclimatize for 5 days. The identical concentration of MWCNTs (2.25 mg/L) was used in the uptake study.

All three plants were exposed to the identical concentration of MWCNTs (2.25 mg/L) as used in *Arabidopsis* exposure experiment for 1 day. At harvest, roots and shoots were separately rinsed with deionized water three times, then fresh tissues were used for quantitative analysis of the contents of MWCNTs using LSC.

2.3 Quantitative Evaluation of MWCNTs in Plant Tissues

Approximately 50 mg plant tissues (root, sheath/stem and leaf) were used to measure MWCNTs content. Roots were carefully washed by deionized water to remove the adsorbed MWCNTs on root surface. A control experiment was applied to determine the adsorbed MWCNTs on root surface by immersing the root into 2.25 mg/L MWCNTs solution for 1 min. The identical washing procedure was used to remove the adsorbed MWCNTs. The results showed that no detectable MWCNTs were adsorbed on root surface. The plant tissue was then grinded on an agate mortar and carefully transferred into 3 mL scintillation cocktail (Ultima gold XR, Fisher Scientific, PA) for LSC. Preliminary experiments used a fixed amount of MWCNTs with and without 50 mg grinded plant tissues were analyzed by LSC to test for matrix effects, but no significant difference was found (Figure S3). The background signal for LSC analysis is 29.3 ± 2.0 DPM. The detection limit for LSC analysis of C-14 MWCNTs is 0.05 $\mu\text{g/mL}$.

2.4 Stability of MWCNTs Suspension

A particle size analysis (Nanosizer, 90Plus, Brookhaven) was performed to determine and compare the stabilization of aqueous phase MWCNTs (2.25 mg/L) in the presence of 0.325 mM SPAOMs. The morphology of suspended MWCNTs was investigated using transmission electron microscopy (TEM, H-7650, Hitachi, Japan). MWCNTs suspensions with and without SPAOMs were diluted in order to minimize background solution influence and were fixed on a nickel grid for observation under TEM. The pH value in MWCNTs suspension with the presence of SPAOMs was measured by a pH meter (XL 200, Fisher Scientific accumet).

2.5 Physiological and Biochemical Assays

2.5.1 Transpiration and Pigment Measurement

The transpiration of each *Arabidopsis* treatment was calculated by the mass difference between the initial and final beaker system after 24 hours. *Arabidopsis* shoots were harvested and grounded in liquid nitrogen for anthocyanin extraction. Fifty mg of grounded tissues were mixed with 1 mL methanol amended with 1% (v/v) HCl and were then incubated in the dark at 4 °C overnight. A mixture containing 500 μL of chloroform and 500 μL of DI H₂O was used to

separate anthocyanin from chlorophyll. The supernatant absorbance was measured at 530 nm and 657 nm by UV-Vis spectrophotometer (Agilent 8453). The final concentration of anthocyanin was determined by using $A_{530} - 1/4 A_{657}$. Chlorophyll was measured as described by Lichtenthaler et al. with revision [51]. Briefly, 50 mg of fresh leaves were collected and cut into pieces (less than 1 cm). A volume of 10 mL 95% ethanol was used to extract the total chlorophyll. All samples were kept in the dark for 3 days to avoid chlorophyll degradation. The absorbance was measured at 664.2 nm and 648.6 nm by UV-Vis spectrophotometer. Chlorophyll a, chlorophyll b and total chlorophyll were calculated by the following equations: $Chla = 13.36A_{664.2} - 5.19A_{648.6}$, $Chlb = 27.43A_{648.6} - 8.12A_{664.2}$ and Total chlorophyll = Chla + Chlb.

2.5.2 Total Protein Measurement

The Bradford reagent (purchased from Sigma Aldrich) was used to measure the total protein content in plant tissues [52]. A sample of 50 mg of *Arabidopsis* seedlings (shoots and roots) was extracted in 2 mL of 10 mM Tris-HCl (pH 7.2) solution. The mixture was then centrifuged at 4000 rpm for 20 min at 4 °C. One hundred μ L of supernatant was used to react with 1000 μ L of Bradford reagent for 15 min at ambient temperature, and then the absorbance was measured at 595 nm by UV-Vis spectrophotometer.

2.5.3 Antioxidative Enzyme Measurement

Catalase (CAT) in *Arabidopsis* seedlings (shoots and roots) was extracted in 25 mM KH_2PO_4 with pH 7.4. Decreased absorbance in the reaction that contained 100 μ L of supernatant and 1900 μ L of reaction buffer (10 mM H_2O_2) was recorded at 240 nm for 3 min. The H_2O_2 extinction coefficient was $23.148 \text{ mM}^{-1} \text{ cm}^{-1}$ [53]. Ascorbate peroxidase (APX) was extracted in 100 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 0.1 mM ascorbate and 2% β -mercaptoethanol. The reaction buffer contained 50 mM phosphate buffer (pH 7.0) and 0.6 mM ascorbic acid. The total 2 mL reaction system contained 100 μ L of enzyme extract and 1900 μ L of reaction buffer. Decreased absorbance was monitored at 290 nm for 3 min after initiating the reaction with 10% H_2O_2 [54]. Superoxide dismutase (SOD) was extracted in 50 mM phosphate (pH 7.8) containing 0.1% (w/v) ascorbate, 0.1 % (w/v) bovine serum albumin (BSA), and 0.05% (w/v) β -mercaptoethanol. Nitroblue tetrazolium (NBT) was used to indirectly determine SOD activities. Briefly, 100 μ L of enzyme extract and 1900 μ L of 50 mM phosphate buffer (pH 7.8) containing 9.9 mM L-methionine, 57 μ M NBT, 0.0044% (w/v) riboflavin and 0.025% (w/v)

Triton X-100 were mixed in cuvette and placed at 37 °C for 20 min. Reduction of NBT was recorded at 560 nm [54].

2.6 Statistical Analysis

Replicate *Arabidopsis* root, stem and leaf tissues were separated and analyzed in quadruplicate for each treatment. Data were statistically analyzed using SPSS 18.0. The least-significant differences (LSDs) were used to determine difference of statistical significance among the different assays. Stepwise multiple linear regression analysis was conducted using SPSS. Q-Q plots and Kolmogorov-Smirnov test were applied to evaluate data normality [44, 48]. The applicability domain of stepwise multiple linear regression results were verified by the leverage approach using the plot of standardized residuals versus leverages (hat diagonals), i.e. the Williams plot [44, 48]. The robustness of stepwise multiple linear regression equations are estimated through an initial two step development: 1) splitting the data into training and validation sets and 2) validating the data via internal and external certification (Supporting Information Stepwise multiple linear regression part) [44, 48].

3. Results and Discussion

3.1 MWCNTs Uptake by Plants

The ¹⁴C-labeled MWCNTs content in different plant tissues ranged from 0.53 ± 0.31 (in maize sheath) to 76.6 ± 6.1 (in soybean root) mg/kg, thus demonstrating that MWCNTs could not only enter the roots, but it could also effectively translocate to the aboveground tissues (Figure 2). The hydrodynamic radius of 2.25 mg/L MWCNTs in Hoagland's solution was measured at 248.12 ± 14.24 nm; given that the pore diameters of plant cell walls range from 5 to 20 nm, the uptake of MWCNTs likely depends on the enlargement of pores or the induction of physical damage resulting in compromised cell wall integrity [55]. The MWCNTs content in rice root was approximately 22.5 mg/kg, which was more than 2-fold that of the sheath (Figure 2F). MWCNTs content in rice leaf decreased to one tenth of the root. Both maize and soybean accumulated high amounts of MWCNTs in their roots as compared with the aboveground tissues (Figure 2G and 2H), but no significant differences were evident between the stem/sheath and leaf tissues. The MWCNT content in *Arabidopsis* leaf (13.0 ± 0.9 mg/kg) was the highest among four plants, which was more than 10-fold that of the stem (1.0 ± 0.3 mg/kg). Comparing the four scenarios of uptake in Figure 2, the MWCNTs content in soybean root was 1.4, 3.4 and 2.4-fold higher than *Arabidopsis*, rice and maize, respectively. However, the MWCNTs translocation

factor (TF: ratio of average MWCNTs content in leaves and stems versus in roots) indicated that rice could translocate more MWCNTs (TF=0.582) to the aboveground tissues (leaf and sheath) as compared to *Arabidopsis*, maize and soybean, whose TF was 0.2620, 0.054 and 0.141, respectively (Table S4). Our TF values ranging from 0.054-0.582 in four plants, which are higher than the values in wheat and rapeseed as reported by Larue *et al.* [12]. This difference might be attributed to the different plant species used and the different sizes of MWCNTs. In our study, the length of MWCNTs was 350 nm compared to $2.65 \pm 1.55 \mu\text{m}$ and $2.90 \pm 1.55 \mu\text{m}$ in the study by Larue *et al.* [12].

Fig. 2- MWCNTs distribution in *Arabidopsis*, rice, maize and soybean. (A-D) Phenotypic images of each plant species; (E-H) MWCNTs content different plant tissues. Values in the same plant followed by the same letter (a–c) were not significantly different at $p < 0.05$. Error bar represents standard error of four replicates.

3.2 MWCNTs Uptake in the Presence of SPAOMs by *Arabidopsis*

The ^{14}C -labeled MWCNTs content in *Arabidopsis* tissues across all SPAOMs treatments ranged from 0.22 to 79.68 mg/kg (Figure 3). The accumulation of MWCNTs varied from *Arabidopsis* roots to shoots, with the pattern of distributions in all treatments following the sequence: root > leaf > stem (Figure 2). These findings agree with previous studies on CNTs accumulation in maize [15], soybean [15], wheat [12] and rapeseed [12]. In our work (Figure 1b), the roots were the only tissue in direct contact with the MWCNTs suspension, resulting in high nanotube concentrations (CK treatment of Figure 3a). The stems accumulated the lowest

amount of MWCNTs (CK treatment of Figure 3c), indicating that passage through the stems to the leaves is likely a rapid progress [15]. Importantly, the uptake efficiency was significantly and distinctly altered by co-exposure to different SPAOMs (Figure 3). 2,4DCP reduced MWCNTs uptake by stems and leaves to the greatest extent, whereas 1,4DNB inhibited root accumulation most significantly (Figure 3). The TF value showed that the MWCNT uptake amount in the presence of 2,4DCP is the lowest (Figure 3), which might be attributed to the plant biochemical/physiological properties, and CNT properties changes induced by SPAOMs.

Fig. 3- MWCNT distribution in *Arabidopsis* in the presence of SPAOMs. a) MWCNT content in roots; b) MWCNT content in leaves; c) MWCNT content in stems. Error bars represent standard error of four replicates. Values among different SPAOM treatments followed by the same letter (A–D) were not significantly different at $p < 0.05$. d) Translocation factor (MWCNT content in leaves and stems versus MWCNT in roots) of MWCNT in *Arabidopsis*. Error bars represent standard error of four replicates.

3.2 Toxicity Effects on MWCNTs Accumulation

Current reports on the phytotoxicity of MWCNTs are somewhat contradictory [1, 56]. CNTs induced positive effects on tomato flowering [57] fruiting [57] and water channel protein production [58], spinach photosynthetic activity [59] as well as wheat and maize root growth [21, 39, 60]. Conversely, others have observed inhibited root and shoot growth [20, 36, 39], increased reactive oxygen species [37, 38], and chromosomal aberrations [19] upon CNT exposure. Many

factors can affect plant growth in the presence of MWCNTs, including nanotube physical/chemical properties, plant species, and experimental conditions (exposure time, substrate, growth conditions). In our study, the presence of MWCNTs (2.25 mg/L) had no impact on measured physiological parameters, including transpiration, anthocyanin contents and chlorophyll contents (CK treatment of Figures 4a-d). In the co-exposure treatments with SPAOMs, MWCNTs either increased or had no impact on the anthocyanin content as compared with the respective controls (SPAOMs alone treatment) (Figure 4b). For example, the anthocyanin contents in both co-exposure treatments of 1,2 DNB+MWCNTs and 4CP+MWCNTs were 2-fold greater than the respective controls. No differences were evident in most of the remaining treatments, with the exception of 1,4 DNB+MWCNTs, where significantly lower anthocyanin content was found relative to the 1,4 DNB alone treatment (Figure 4b). During the 24 hours exposure, the total chlorophyll content did not change upon exposure to MWCNTs, SPAOMs, and SPAOMs + MWCNTs (Figure 4c and d). Notably, SPAOMs exposure reduced the total protein content in *Arabidopsis* seedlings by approximately 50% as compared to controls. In the co-exposure treatments, MWCNTs presence restored the protein content back to the level of the MWCNTs control group, suggesting that nanotubes could alleviate some of the SPAOMs-induced toxicity and help to stabilize plant protein content, although the total protein content was also reduced in the MWCNT alone treatment as compared with the unexposed control. The possible explanation might be that the CNTs can decrease bioavailability of the organic compounds, due to its high adsorption affinities for polar and hydrophobic compounds. Previous studies also agreed that MWCNT could generally reduce pesticides' uptake by plants [33-35].

Antioxidant enzyme activities, including APX, CAT, and SOD, were significantly induced, particularly in the SPAOMs alone treatments (Figures 4e, f and g). For example, the presence of 1,3 DNB, NB, and 2,4 DCP elevated APX activities by approximately 7, 6, and 7-fold, respectively. Similarly, CAT and SOD activities were also significantly higher among these three exposures as compared to the controls and the remainder of the treatments (Figure 4f). Interestingly, the addition of MWCNTs decreased (in some cases e.g. 1,3DNB, NB, 2,4DCP significantly decreased) the antioxidant enzyme activities relative to its respective SPAOMs alone treatment. The results were consistent with the physiological data, where MWCNTs significantly reduced the phytotoxicity of SPAOMs. In a previous study, significant elevation of

antioxidant enzyme activity indicated that CNTs could cause ROS overproduction and subsequently result in damages to biological molecules such as lipids, proteins and DNA [49]. Conversely, our results suggested that the presence of MWCNTs could significantly alleviate SPAOMs toxicity to *Arabidopsis* seedlings. In addition, the concentration of nutrients in 1/2X Hoagland's solution with/without MWCNTs was measured (Table S2). The levels of several nutrients (B, Ca, K, Mg, Mn, S) were significantly higher in the pure Hoagland's solution than in the MWCNTs amended solution; the exception being Na and P, both of which did not show difference between the two treatments. This is probably ascribed to the sorption of nutrients by MWCNTs. Across all eight parameters in Figure 4, changes in antioxidant enzyme activities are more sensitive than the physiological parameters across all treatments after 24 hours exposure. Reductions in antioxidant enzyme activities in co-exposure treatments suggested that the presences of MWCNTs could significantly counteract SPAOMs toxicity to *Arabidopsis* seedlings, subsequently restoring antioxidant enzyme activity back to the nanotube control levels. The mechanisms driving these nanotube-contaminant-plant interactions are unknown but are the topic of ongoing investigation. The activities of antioxidant enzymes, including APX, CAT, and SOD, can scavenge ROS in form of H_2O_2 and $O_2^{\cdot-}$ and break these molecules down to H_2O or O_2 . Elevated activities of three antioxidant enzymes indicated that the presence of SPAOMs could increase the ROS levels in plants. For example, APX activity in 2,4-DCP treated *Arabidopsis* was 6.9-fold higher relative to the control group (Figure 4f); similarly, CAT and SOD activities were 17.5-fold and 9.8-fold that of the control, respectively (Figures 4g and h). Although the other SPAOMs could also increase the activities of three antioxidant enzymes in *Arabidopsis*, 2,4-DCP seems to be the most phytotoxic in terms of antioxidant response. When comparing the TF of MWCNT in *Arabidopsis*, the presence of 2,4-DCP significantly decreased MWCNT translocation (Figure 3d), suggesting that plant may down regulate the activity of some specific transporters in the cell membrane in response to organic contaminant exposure. The presence of MWCNTs has been shown to have the opposite effect, inducing up regulation of a water channel protein (*LeAqp2*) in tomato roots, and subsequently enhance the seed germination and plant growth [25]. Under the stress conditions, SPAOMs might modulate the stress-related genes (aquaporin genes), effectively decreasing the likelihood of plants to accumulate MWCNTs.

Fig. 4- Inhibition of *Arabidopsis* by SPAOMs with (black bar) and without (white bar) MWCNTs addition at both physiological and biochemical levels after 24 hrs exposure. a) Transpiration; b) Anthocyanin; c) Chlorophyll a; d) chlorophyll b; e) Total Protein; f) Ascorbate peroxidases; g) Catalase; h) Superoxide dismutases. Error bars represent standard error of four replicates. Values among different SPAOM treatments followed by the same letter (A–D) were not significantly different at $p < 0.05$. Values in the same SPAOM treatments with and without MWCNT followed by the same letter (a–b) were not significantly different at $p < 0.05$.

3.3 Stability Effects on MWCNTs Accumulation.

Particle and aggregate sizes have been shown to influence the uptake of MWCNTs by plants [13, 15]. Our previous study demonstrated that SPAOMs can disperse MWCNTs and that this dispersibility correlated well with water solubility [41]. Here, TEM images confirmed that dispersibility of MWCNTs increased with increasing SPAOM water solubility (Figure 5a).

Fig. 5- Effect of MWCNTs stability on their accumulation. a) TEM images of MWCNTs suspensions in the presence of different water solubility SPAOMs; b) suspension pH value and particle size in the presence of different water solubility SPAOMs; c) Quantitative correlation between MWCNT uptake and hydrodynamic diameter of MWCNTs in different plant tissues. Error bar represents standard error of four replicates.

Specifically, increasing SPAOMs solubility resulted in a decrease of solution pH and of MWCNTs hydrodynamic diameter (Figure 5b). In the experiment pH range (5.4-5.7), all applied chemicals are in their molecular forms. Thus, the decrease of solution pH and MWCNTs hydrodynamic diameter with increasing SPAOMs solubility probably suggests a possible dispersion mechanism. The adsorbed SPAOMs likely facilitated hydrophilic moieties' (e.g. -OH, -COOH), which were initially obstructed in MWCNTs bundles and aggregates, interactions with the aqueous phase. The exposed hydrophilic moieties could then release H⁺ and decrease the pH of the solution. The correlation between MWCNTs hydrodynamic diameter and uptake by the plants was evaluated (Figure 5c). Although the accumulation in root and leaf showed negative relationship (-0.0726 and -0.0289, respectively) between MWCNTs hydrodynamic diameter and nanotube uptake, the correlation was not statistically significant. Thus, our results imply that MWCNTs hydrodynamic diameter does not significantly affect MWCNTs uptake by *Arabidopsis*.

3.4 Stepwise Multiple Linear Regression Study

It was difficult to find a relationship between a single plant biochemical/physiological property or CNT property and the MWCNTs uptake in different tissues, as indicated by low correlation coefficients R² (Figure 5c and Figure S4). Thus, the correlation of MWCNTs uptake amount in root, stem and leaf tissue with plant biochemical/physiological properties and CNT properties was further analyzed by stepwise multiple linear regression analysis of the [U, WL, Ant, Chla, Chlb, TP, APX, CAT, SOD, HD] matrix. This yielded the following equations:

$$U_{\text{root}} = (-1776.922 \pm 706.266) \text{ CAT} + (69.944 \pm 14.134) \quad R^2 = 0.513 \quad (1)$$

$$U_{\text{leaf}} = (-0.441 \pm 0.085) \text{ APX} + (-1.292 \pm 0.373) \text{ SOD} + (28.097 \pm 3.890) \quad R^2 = 0.877 \quad (2)$$

where U represents the uptake amount in plant tissues (mg/kg); WL is the amount of water loss caused by transpiration (g); Ant indicates anthocyanin absorbance at 530 nm; Chla and Chlb are chlorophyll a and chlorophyll b content, respectively (mg/g); TP is the total protein content (mg/mL); HD represents CNT hydrodynamic diameter (nm). Analysis of variance results suggests that the regression equation has statistical significance (Table S4). The predicted versus measured U values of the training set and validation set 1 and 2 are close to the 1:1 line, indicating the robustness of Eqns. (1) and (2) (Figure 6a). The applicability domain of the regression models was verified using a William plot (Figure 6b). All training and validation sets

are within the domain, demonstrating that there are no outliers and that the predictive capacity of the models is reliable. The negative correlations between U_{root} and CAT, between U_{leaf} and APX and between U_{leaf} and SOD further confirmed that the presence of SPAOMs could severely inhibit MWCNTs uptake in *Arabidopsis*. Unfortunately, we were unable to describe CNTs accumulation in the stems, which is likely the result of the rapid movement of MWCNTs through the stems to the leaves [15]. Our regression model of CNT accumulation in root and leaf directly points out that the phytotoxicity of SPAOMs could significantly impact CNTs accumulation in plants by affecting plant biochemical/physiological properties.

Fig. 6- (a) Predicted versus measured uptake amount of MWCNTs in roots and leaves. The dash line represents the 1:1 line. (b) The Williams plot for verifying the applicability domain of the model. This figure shows no outliers of training and validation data and that the predictability of the model is reliable.

4. Summary

The successful evaluation of nanotube uptake by *Arabidopsis*, rice, maize and soybean demonstrates that radioactivity measurement is a feasible method for quantitative detection of MWCNTs in plant tissues. Additionally, this study revealed the potential and quantitative

relationships between the stability and uptake of MWCNTs and the toxicity of SPAOMs using stepwise multiple linear regression model. Further study in the field of plant and carbon nanotube interactions is clearly necessary to solve the questions regarding the underlying uptake mechanisms from the molecular aspect, whether the differences of the uptake patterns are plant species-specific, whether MWCNT properties significantly influence uptake. Given the results from our present study, MWCNT interactions with biota and co-existing contaminants are strikingly complicated and have significant implications for the prediction of exposure to and risk from carbon-based nanomaterials in the environment.

Acknowledgements

We thank Dr. Elijah J. Petersen from National Institute of Standards and Technology for providing ^{14}C -labeled MWCNTs used in this study. This work was in part supported by Hundreds Talents Program of Chinese Academy of Sciences awarded to Q.Z. (2014-2019), CAS Pioneer Hundred Talents Program awarded to X.-J.Z. (2015-2020), Youth Innovation Promotion Association CAS awarded to S.-Y.Z. (2017-2020), Open Foundation of State Key Laboratory of Environmental Criteria and Risk Assessment, Chinese Research Academy of Environmental Sciences (SKLECRA2015OFP10), USDA-NIFA (2011-67006-30181) and USDA-NIFA Hatch program (MAS 00475).

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Quantitative evaluation of multi-wall carbon nanotube uptake by terrestrial plants

Graphical Abstract: