Diuron Sorbed to Carbon Nanotubes Exhibits Enhanced Toxicity to *Chlorella vulgaris*

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Supporting Information

**ABSTRACT:** Carbon nanotubes (CNT) are more and more likely to be present in the environment, where they will associate with organic micropolllutants due to strong sorption. The toxic effects of these CNT-micropolllutant mixtures on aquatic organisms are poorly characterized. Here, we systematically quantified the effects of the herbicide diuron on the photosynthetic activity of the green alga *Chlorella vulgaris* in presence of different multiwalled CNT (industrial, purified, pristine, and oxidized) or soot. The presence of carbonaceous nanoparticles reduced the adverse effect of diuron maximally by <78% (industrial CNT) and <34% (soot) at 10.0 mg CNT/L, 5.0 mg soot/L, and diuron concentrations in the range 0.73–2990 μg/L. However, taking into account the measured dissolved instead of the nominal diuron concentration, the toxic effect of diuron was equal to or stronger in the presence of CNT by a factor of up to 5. Sorbed diuron consequently remained partially bioavailable. The most pronounced increase in toxicity occurred after a 24 h exposure of algae and CNT. All results point to locally elevated exposure concentration (LEEC) in the proximity of algal cells associated with CNT as the cause for the increase in diuron toxicity.

**INTRODUCTION**

The current production volume of carbon nanotubes (CNT) has reached about ∼1000 t/a, and due to their increasing use, they possess a growing potential for environmental exposure.1–3 First ecotoxicological studies showed that CNT seem to be relatively nontoxic for aquatic organisms (i.e., end point concentrations typically in the mg/L range).4−7 However, these studies did not discuss their strong affinity for organic pollutants.8,9 In the environment, CNT will always occur together with a great number of micropolllutants that strongly sorb to carbonaceous sorbents such as black carbon.10,11

Numerous recent studies report reduced bioavailability of organic compounds in the presence of carbonaceous nanomaterials under laboratory conditions.8–10,12–19 CNT addition to pyrene contaminated soils reduced pyrene bioaccumulation in earthworms substantially at concentrations of 3 mg CNT/kg soil.14 Other studies found reduced polycyclic aromatic hydrocarbon extractability and bioaccessibility due to sorption to fullerene soot, single-walled, and multivalled CNT,15 and reduced bioavailability of adsorbed phenanthrene on multivalled CNT.13 In benthic systems, data are rare,17 and in aquatic systems, no data are available for CNT, but Knauer et al. demonstrated that the toxicity of the herbicide diuron to green algae was reduced in the presence of black carbon.10 Similar observations were reported for 17α-ethinylestradiol sorbed to buckminsterfullerene (C60) nanoparticles and adult male zebra fish (*Danio rerio*), resulting in a 100% reduced toxicity of the organic compound.16

It is generally assumed that the dissolved concentration of organic pollutants reflects the bioavailable fraction for algae.10,11,13–16,20 This is a common but maybe oversimplified view,9,21 and more recent studies call this assumption into question. It was postulated that uptake of particles loaded with organic compounds can lead to an augmented bioaccumulation depending on the uptake pathway into the organism. If CNT are adsorbed and/or accumulated by an organism, the organic compound could be released in high quantity.8,9 Such a “facilitated transport”,22,23 also called “carrier effect”18 or “carrier mediated transport”,24 may substantially increase the

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toxicity of environmental micropollutants, which are collected especially on supersorbents such as CNT. This phenomenon was observed by Baun et al. for C60 loaded with different contaminants and the alga Pseudokirchneriella subcapitata and the crustacean *Daphnia magna*. They reported toxicities of C60-phenanthrene mixtures that were 1.6 times higher than for postulated in earlier studies for sorption to colloids and was as to our best knowledge not yet known for CNT but was observed, is enhanced uptake through the aqueous boundary layer of the organisms. This diusive flux concept is as to our best knowledge not yet known for CNT but was postulated in earlier studies for sorption to colloids and fullerenes.

Obviously, most of the studies on combined effects of nanoparticle-pollutant mixtures investigated the toxic effects or the pollutant concentrations separately. Therefore, little is known on the exact distribution of the organic compounds in the biota—nanoparticle—pollutant system (e.g., the nominal and the dissolved concentrations).

The aim of this work was therefore to (a) measure bioavailability (fraction of the total concentration that is made available at the site of physiological activity) of the herbicide diuron, reflected by the toxicological end point photosynthetic activity, in a biota-nanoparticle-pollutant model system using the green alga *Chlorella vulgaris* and the nanoparticle CNT, (b) relate the observed effects resulting from total diuron exposure to the analytically determined diuron concentration and sorption data in the aqueous phase, (c) follow the temporal development of effects as the system approaches equilibrium, and as a function of exposure time, (d) compare the behavior of pristine, oxidized, purified, and industrial grade CNT and diesel soot as a reference sorbent over time, and (e) provide a mechanistic explanation for the observed processes taking into account CNT agglomeration and algae-CNT interactions.

### EXPERIMENTAL SECTION

**Chemicals.** 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (diuron) and 3-(3,4-dichlorophenyl)-1,1-dimethyl(6H)urea (D6-diuron), both >99% pure, were purchased from Labor Dr. Ehrenstorfer (Germany). Diuron and D6-diuron primary, working, and internal standard stock solutions were prepared gravimetrically in OECD algal growth medium with 1 vol % acetonitrile. The concentration of acetonitrile in the aqueous solution/suspension was controlled to be below 0.1 vol % to avoid cosolvent effects and toxicity to algae.

**Nanomaterials.** Purified pristine and oxidized CNT were synthesized by catalytic chemical vapor deposition (CVD) by EPFL Lausanne, Switzerland. Industrial pristine CNT were purchased from Cheap Tubes Inc., Brattleboro, VT 05301, U.S.A. as native black carbon reference sorbent with spherical primary particles in the range of 35 nm, a forklift diesel soot standard (soot) was used (Standard Reference Material 2975, National Institute of Standards and Technology (NIST), U.S.A.). High resolution transmission electron microscopy (HR-TEM) images and the detailed properties of all four materials are given in Table 1 and Figure S1, further details are presented in Schwab et al.

### Table 1. Properties of the Investigated Carbonaceous Nanomaterials

<table>
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<tr>
<th>particle type</th>
<th>purified pristine CNT</th>
<th>purified oxidized CNT</th>
<th>industrial pristine CNT</th>
<th>soot</th>
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<td>Cheap Tubes Inc.</td>
<td>NIST</td>
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<td>production</td>
<td>CVD</td>
<td>CVD</td>
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<td>oxidized</td>
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<tr>
<td>length range</td>
<td>(μm) 2–5</td>
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<td>0.5–2.0</td>
<td>0.035</td>
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<tr>
<td>diam. range</td>
<td>(nm) 5–15</td>
<td>5–15</td>
<td>≤5</td>
<td>35</td>
</tr>
<tr>
<td>length avg.</td>
<td>(μm) 3.5</td>
<td>2.9</td>
<td>1.25</td>
<td>0.035</td>
</tr>
<tr>
<td>diam. avg.</td>
<td>(nm) 11.6</td>
<td>11.7</td>
<td>5.7</td>
<td>35</td>
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<tr>
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<td>(length/diam.)</td>
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<td>248</td>
<td>219</td>
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<tr>
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<td>95.18</td>
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<td>87.2</td>
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<tr>
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<td>(wt %) 80.3</td>
<td>97.1</td>
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<td>metals</td>
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</table>

5 main metal impurities (descending order): Fe, Co, Si, Ca, Al Fe, Co, Si, Al, Ni Co, Cd, Fe, Cr, Ca Zn, Fe, Cu, Ti=Cr=Ni

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Preparation of CNT Suspensions. CNT stock suspensions prepared for all following experiments were characterized and prepared according to standardized procedures described in detail elsewhere.4 In short, dry CNT (5.00 ± 0.05 mg) and 100 mL of algal growth medium were sonicated in 100 mL flasks 8 × 15 min (agitation in between) in an ultrasonication bath (Bandelin Electronic, Germany, 35 kHz). The pH of the suspensions was reset after sonication to 7.00 ± 0.05 using a 1.00 M hydrochloric acid and a 1.00 M sodium hydroxide solution. The size distribution and polydispersity of the suspensions was quantified using dynamic light scattering (DLS). The suspensions were used within 24 h.

Cultivation of Algae. Chlorella vulgaris 211-11b was purchased from the Culture Collection of Algae (SAG, University of Göttingen, Germany). Algal cultures were grown in OECD algal test medium33 buffered with 1.00 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) to keep the pH constant at 7.0 ± 0.3 during the whole duration of the experiments. The algal cultures were grown in an incubation shaker (Multitron, Infors, Switzerland) at 24.00 ± 0.50 °C and 100 rpm under sterile conditions. An illumination of the incubation shaker of 80 ± 5 μEm²⁻¹ s⁻¹ was provided by daylight lamps (Sylvania Gro-Lux F15W/Gro T8, Infors, Switzerland) under a light/dark regime of 16:8 h. All experiments were conducted using axenic, exponentially growing algal strains.

Photosystem II (PSII) Inhibition by Diuron. C. vulgaris, 6.7–8.4 × 10⁴ cells/mL were incubated in glass vials at constant illumination of 15 ± 5 μEm²⁻¹ s⁻¹ with diuron concentrations of 10, 20, 40, 80, and 160 μg/L. Algal cells were adapted prior to exposure for at least 1 h to the above incubation conditions. The photosynthetic activity of the algal cells as a measure of bioavailable diuron was quantified by in vivo fluorescence using a pulse amplitude modulated fluorometer (ToxY-PAM Dual Channel Yield Analyzer, Heinz Walz GmbH, Effeltrich, Germany) as described in the Supporting Information.

PSII Inhibition in Ternary Systems. Particle–diuron mixtures were prepared with concentrations of 10.0 mg CNT/L and 5.00 mg soot/L, respectively, and 9–10 different initial diuron concentrations cᵢ in the range 0.73–2 990 μg/L covering 3 orders of magnitude to account for the expected nonlinear sorption behavior of CNT.32 To investigate the effect of the particles alone on the photosynthetic activity of C. vulgaris, the PSII inhibition of the CNT (10.0–40.0 mg CNT/L) and soot (5.0 mg soot/L) without diuron was quantified in control replicates in parallel to each trial. All concentrations were tested at least in duplicate, and control treatments at least in triplicate. In addition, tests with a constant diuron concentration and variable CNT concentrations were accomplished to investigate the reproducibility of the experiments. The maximal particle concentration was controlled to yield fluorescence signals of the algae in the recommended range of the ToxY-PAM fluorometer.10 Reproducibility of the experiments was further tested by independent repetition of a trial with purified pristine CNT and by accomplishing the ternary trials under different incubation conditions (in 50.0 mL Erlenmeyer flasks at 100 rpm and 80 ± 15 μEm²⁻¹ s⁻¹).

Previous studies showed that sorption of diuron to the purified pristine and oxidized CNT used in this work reached equilibrium after a few hours.32,37 Thus, the CNT or soot–diuron mixtures were pre-equilibrated, after preparation of the suspensions as described above, in sealed screw cap vials (22 mL) with polytetrafluoroethylene fittings, additionally covered with Parafilm, in the dark at 24 ± 2 °C and 175 rpm on an orbital laboratory shaker for 20 h, and under equal conditions for 28 d to study bioavailability of diuron under nonequilibrium and equilibrium conditions, respectively. All test suspensions were prepared under sterile conditions. Test suspensions were microscopically screened for microbial contamination. Contaminated replicates were excluded from evaluation. Especially the purified pristine and the purified oxidized CNT, but not the industrial CNT suspensions agglomerated extensively, during pre-equilibration as well as during incubation with algae (also reflected by high polydispersity and DLS size distribution).3,32

The mixed toxicity of the particle–diuron suspension was measured after 20 h or 28 d of pre-equilibration. Therefore, 7–37 × 10⁴ cells/mL of C. vulgaris from a stock culture in the exponential growth phase were added to the pre-equilibrated CNT– or soot–diuron mixtures, and the algae–CNT or soot–diuron mixture was incubated at the same incubation conditions as described for the diuron treatment only. Photosynthetic activity was measured after 3, 6, 15, and 24 h of incubation to be comparable to the diuron treatment. Control experiments with CNT or soot, and diuron were measured in parallel to exclude a potential effect of particles to

Figure 1. Dose–response (PSII inhibition) curves of diuron to C. vulgaris after 20 h incubation in the absence and presence of CNT or soot (28 d pre-equilibrated). (A) In absence of CNT or soot. The curve was generated using pooled data of eight independent diuron dose–response curves (including 95% confidence interval). (B) Dose–response curves as a function of initially added diuron concentration cᵢ. The dashed line represents the dose–response curve in the absence of particles. (C) Same data as in panel B but as function of the measured dissolved diuron concentration cᵢ.
the photosynthetic activity, and to determine the toxicity of diuron alone. An algae control in pure growth medium was used as a reference to calculate the PSII inhibition (eq 1).

To evaluate the under- or overestimation of diuron toxicity in the presence of different particles at different dissolved diuron concentration c, the ratio of measured versus predicted PSII inhibition (I'/I) was calculated. The predicted PSII inhibition I (%) was calculated using eq 1 for each replicate

\[ I'(\%) = 100\% - \frac{100\%}{1 + \left(\frac{c}{EC_{50,dieron}}\right)^{\text{dose}}} \]  

Equation (1) taking into account c in the replicate and the dose–response curve of diuron alone (Figure 1A). The measured PSII inhibition I (%) of each replicate was then divided by I'.

**Diuon Quantification.** The dissolved diuron concentrations c in the particle–diuron–alga mixtures were determined in 2 mL aliquots taken from each vial <1 min after the photosynthetic activity was measured (after 3, 6, 15, and 24 h incubation with algae). More details and quality control are described in the Supporting Information.

**Brightfield Microscopy and Hyperspectral Imaging (HSI).** Conventional microscopy and HSI were used to visualize the CNT– or soot–diuron exposed algal cells. The measurement of HSI enables to map specific materials in a highly resolved microscopic photograph based on a parallel visible/near-infrared (Vis/NIR) spectrum scan of each of the pixels in the image. If the spectrum of a pixel matches the reference spectrum of the specific material, the pixel is highlighted and the specific material is consequently located.38 For HSI, high resolution was achieved by installing an annular condenser (CytoViva, Auburn, AL, U.S.A.) as described by Vainrub et al.39 on a conventional dark-field light microscope, using 1000X magnification and immersion oil.

**RESULTS**

**Effects of Diuron on the Photosynthetic Activity.** The dose–response curve of diuron to *C. vulgaris* yielded an effect concentration at which 50% inhibition occurred (EC\(_{50}\)) of 80.8 [74.8, 87.3] \(\mu\text{g/L}\) diuron (N = 94, values in square brackets: 95% confidence interval) (Figure 1A). The curve was based on data from eight individual dose–response curves (Figure S2, Supporting Information). The EC\(_{50}\) values were roughly comparable to those in the literature, 40,41 but note that comparison of the EC\(_{50}\) with published values is limited by the difference of the experimental setups, which greatly affects the PSII inhibition.42 The variability was accounted for by control treatments with sole diuron and sole growth medium in every individual assay and is thus taken into account in the interassay comparisons. Since PSII inhibition stayed constant after 60 min, all in vivo PSII measurements were performed after this incubation time.43

**Effects of CNT and Soot on the Photosynthetic Activity.** The PSII quantum yield \(\text{Y}\) in CNT or soot controls and controls without particulate matter (0.57 ± 0.02, N = 50) (Supporting Information Figure S3) was not significantly different from growth medium control replicates, neither for suspensions pre-equilibrated for 20 h nor for control replicates equilibrated for 24 h (Supporting Information Figure S3). These results are in agreement with previous studies on the same or similar particles (CNT or soot with low amount of impurities), in which up to 50 mg CNT/L did not change the photosynthetic activity significantly (detailed discussion in refs 4, 10, and 44).

**Toxicity of Diuron in Equilibrium with CNT or Soot.** Figure 1B shows the PSII inhibition after 24 h as a function of initially established diuron c\(_{\text{initial}}\). Taking into account only c\(_{\text{initial}}\) industrial pristine CNT and soot reduced the effect of diuron by <78%, and <34%, respectively, as compared to the diuron control dose–response curve (Figure 1A, dotted line Figure 1A–C). The sorption experiments run in parallel confirmed that diuron strongly sorbed to all CNT and soot. Freundlich coefficients, K\(_F\), for the different CNT and soot were 10\(^{31.51}\)–10\(^{39.28}\) \(\mu\text{g/kg}_{\text{CNT}}\) (\(\mu\text{g/L}\))\(^{-1}\) and 10\(^{32.12±0.13}\) \(\mu\text{g/kg}_{\text{soot}}\) (\(\mu\text{g/L}\))\(^{-1}\), respectively.34 The EC\(_{50}\) based on c\(_{\text{initial}}\) of industrial CNT— or soot–diuron mixtures shown in Supporting Information Table S1 demonstrate that the toxicity of diuron decreased for most
incubation times compared to diuron alone (strongest in the presence of industrial pristine CNT where EC_{50} was 698 [629, 767] \mu g/L after 3 h of incubation with algae only). Diuron in the presence of soot inhibited the photosynthesis in all experiments consistently less than diuron alone (Figure 1 and Supporting Information Figure S4 and Table S2). For soot, the effect of diuron was similar to that described in Knauer et al., where 5 \mu g/L diuron in presence of 50 mg soot/L inhibited the PSII activity of P. subcapitata by 34.0 \pm 1.8%.30 Fully pre-equilibrated industrial CNT and soot reduced diuron toxicity almost identically, except after a 24 h exposure of algae where the presence of soot led to a slight reduction of PSII inhibition (Figure 2).

In contrast to the industrial CNT and soot, c_{w,init} in presence of purified CNT, both pristine and oxidized, was in the majority of the experiments as toxic as if no CNT would have been added (Figure 1B). All the data points of purified CNT lay on the dotted line presenting the diuron toxicity without CNT. Little or no toxicity reduction due to sorption was observed, although purified pristine CNT clearly adsorbed most of the diuron (evident from the high K_p for CNT). Taking into account c_{w} instead of c_{w,init}, diuron in the presence of CNT had an up to five times enhanced toxicity compared to what was expected from c_{w} alone (Figures 1C and 2).32

This increased toxicity was confirmed in experiments pre-equilibrated for different times and incubated with C. vulgaris for different hours (Figure 2, details in the Supporting Information, Figures S5, S6, and S7 and Table S1). Both experimental setups (constant diuron concentration and variable CNT concentrations, or vice versa) led to the same sorption coefficients32 and similar reduction of the response of the green algae to diuron (constant CNT concentration, Figure 1C and Supporting Information Figure S5; constant c_{w}, Supporting Information Figure S6). Internal diuron controls in all trials (* symbols in Figure 1A–C and Supporting Information Figure S4) confirmed that interassay variability of diuron toxicity was small and cannot have affected the PSII inhibition significantly. The photosynthetic inhibition decreased at high CNT concentrations, further suggesting that CNT inhibit PSII (Supporting Information Figure S6). Only for CNT concentrations >10 mg CNT/L, the presence of CNT led to the expected reduction of toxicity, which was strongest (~99%) at 40 mg CNT/L for 28 d pre-equilibration and 3 h incubation with algae and lowest (~82%) for 20 h pre-equilibration and 24 h incubation with algae (Supporting Information Figure S6).

The dissolved diuron concentration c_{w} increased in trials with fully pre-equilibrated purified CNT by ~15–20% during the 24 h of incubation with algae.32 Equally treated control experiments with CNT and diuron without algae showed no increase of c_{w}. Redissolved diuron contributed to the increase in toxicity if only c_{w,init} was considered (Figure 1B) but cannot explain the increased toxicity if c_{w} was taken into account only (Figures 1C and 2).

**Diuron Toxicity in Nonequilibrium Conditions.** Sorption of diuron to the industrial pristine CNT (equilibrium achieved within days) was slower than to purified CNT (equilibrium achieved within hours).32 For these nonequilibrated sorbent–sorbate systems, the toxicity of c_{w} was slightly lower or not significantly different from the control dose–response curve of diuron (Supporting Information Figure S4). Increased toxicity became more apparent at 15 and 24 h after algal addition, when the sorbent–sorbate systems were approaching equilibrium (Figure 2).

**Measured/Predicted PSII Inhibition.** Figure 2 illustrates that, for purified pristine and oxidized CNT, the PSII inhibition was almost always higher than predicted using eq 1, irrespective if the CNT were equilibrated for 20 h or for 28 d. After 24 h of incubation with algae, the underestimated of PSII inhibition was even more pronounced. Except for a few data points, the ratio I/I' was always >1 (see also Supporting Information Figure S8). For soot, the ratio I/I' ranged from ~0.7 to 1 (Supporting Information Figure S7) for all tested concentration and incubation times (Supporting Information Figure S8). Industrial CNT, again, behaved sometimes more like purified CNT and sometimes more like soot. Overall, an exponential increase of underestimation of PSII inhibition was observed with decreasing c_{w} (Supporting Information Figures S8 and S7). At high c_{w}, the toxicity of the dissolved diuron dominated, so that an additional toxicity increase due to the presence of CNT became negligible. The toxicity at low c_{w} increased by a factor of 4–5 for purified CNT (pristine and oxidized), 2–3 for industrial pristine CNT, and remained unchanged (~1) for soot. At high c_{w}, the ratio converged to 1 for all materials (Supporting Information Figures S8 and S7).

**Brightfield Microscopy and HSI of Exposed Algal Cells.** Neither the size nor the morphology of algal cells in the presence of CNT or soot appeared differently compared to control cells. No broken cell walls were observed. Brightfield microscopy demonstrated that algal cells agglomerated with CNT during incubation. The presence of diuron did not affect the size or the shape of the agglomerates. Algal cells did attach...
only little to soot. Earlier, more quantitative work confirms that, in presence of 10 mg CNT/L, 20–40% of algal cells were located inside of CNT agglomerates.32

Hyperspectral imaging demonstrates that CNT attached to the cell surface, but most likely, CNT did not enter the cells (Figure 3). In cross sections of the algal cell interior, no pixels matched to the library of Vis/NIR spectra collected for CNT suspended in algal growth medium (Supporting Information Figure S9). Thus, internalization of CNT by algal cells is unlikely. Similar observations were made for another algal species (Supporting Information Figure S9).

**DISCUSSION**

The present work demonstrates that diuron sorbed to CNT, but not to soot, was partially toxic for green algae if the CNT were associated with algal cells. Microscopic investigations showed, in agreement with the literature,4,45 that CNT, but not soot, increasingly attached to the surface of the algal cells at long incubation times. As demonstrated by HSI, and considering the intact photosynthetic activity of the algae, CNT were most probably not internalized. Knowing the strong sorption properties of the CNT under the same experimental conditions,32 high amounts of diuron sorbed to these CNT agglomerates came in close vicinity to the algal cells. Soot, in contrast, attached only very little to algal cells4 and was much better dispersed, as was the industrial CNT.32 Diuron sorbed to soot or any well dispersed carbonaceous material is therefore spatially more uniformly distributed. The absence of CNT internalized by algal cells in hyperspectral images suggest that toxicity of diuron due to internalized CNT loaded with diuron is not likely. This was confirmed by a back-of-the-envelope calculation of the intracellular CNT mass that would sorb enough diuron to increase the PSII inhibition as observed. The calculated CNT mass would correspond to >100 times the dry weight of *C. vulgaris* (8.98 × 10−9 mg/cell, own data). Such an amount of CNT in an algal cell should be visible even by light microscopy. Rather than via uptake of CNT, it is much more likely that (analytically not accessible) locally elevated exposure concentration (LEEC) in the aqueous boundary layer of green algae led to the observed increase of diuron toxicity.

Enhanced toxicity of diuron in the presence of CNT due to photosynthetic inhibition caused by the nanoparticles themselves is not likely at the concentrations used in the tests. The control experiments of CNT or soot alone run in parallel to all ternary trials, demonstrated that the photosynthetic yield of *C. vulgaris* remained unchanged, even at concentrations up to 40 mg CNT/L. These findings are in agreement with previous studies, in which CNT or soot alone did not inhibit the photosynthesis of *C. vulgaris* and other green algae.4,10 The absent photosynthetic inhibition of CNT or soot shows that photosynthesis of *C. vulgaris* was not affected neither by long-term shaking of CNT, CNT agglomeration, toxic metal catalysts, or sorption of nutrients to CNT. All tested CNT and soot contained less than 0.89 wt % metal impurities. No toxicity of the filtrate of the CNT possessing the highest metal impurity content was observed.4 The increase in toxicity at low diuron concentrations demonstrates that the presence of diuron and/or algae cannot have led to increased photosynthetic inhibition (e.g., due to increased dispersion of CNT). In this case, the toxicity increase would have been highest at high diuron concentrations. Finally, if CNT alone would have inhibited the photosynthetic activity to the algae, the dose–response curves of the ternary trials would not start with 0% inhibition. They would start with the baseline inhibition of 10 mg CNT/L, which is clearly not the case.

By calculating the predicted/measured diuron toxicity, or the factor of toxicity increase \((c_w/I)/(c_w^0/I^0)\), it was possible to quantify the enhanced toxicity. Using these ratios, higher bioavailability of diuron in the complex algae–CNT mixture can be demonstrated (more details are in the Supporting Information, sections “Factor of Toxicity Increase” and “Hill Slope”). The toxicity of \(c_w^0\) in nonequilibrated CNT-diuron mixtures increased with incubation time and coincided with the amount of sorbed diuron.32 This relationship was not observed for soot, which confirms that, for soot, \(c_w^0\) corresponded to the bioavailable diuron fraction. Thus, the process of sorption went along with diuron bioavailability in all experiments, which is further support for LEEC.

Purified CNT and industrial CNT had similar sorption strengths of \(K_f^0 = 10^{0.24 ± 0.32} \mu g/\text{kgCNT}(\mu g/L)^{-1}\) and \(K_f = 10^{6.24 ± 0.10} \mu g/\text{kgCNT}(\mu g/L)^{-1}\), respectively.32 However, the diuron toxicity in presence of purified CNT was generally not reduced, while this was the case for industrial CNT. This further supports that diuron sorbed to the purified CNT was more bioavailable for the green algae. It also shows that diuron sorbed to CNT in well dispersed suspensions, such as those of the industrial CNTs, was less available for the green algae than diuron sorbed to CNT that were more agglomerated. The low availability of diuron sorbed to industrial CNT could also reflect the slower desorption kinetics of industrial CNT.32 Bulky structures in transmission electron microscopy images, and the high condensed carbon content of the industrial CNT, were the main differences of the industrial CNT to the purified CNT. This suggests that, rather than the oxygen content, the amorphous carbon impurities helped the dispersion of industrial CNT and made them suspendable more like soot.32 At the same time, amorphous carbon may have hampered the diffusion to/from the sorption sites, leading to less bioavailable diuron.

The increase of the \(c_w^0\) with time32 was most likely triggered by a change of size and shape of CNT agglomerates when they interacted with algal cells (for detailed discussion see Schwab et al.32) and contributed to the absolute increase of PSII inhibition with time in dose–response curves using \(c_w^0,\text{init}\). However, the contribution of redissolved diuron canceled out if \(c_w^0\) and not this \(c_w^0,\text{init}\) was used for the graphs. The changes of toxicity can therefore only partially be explained by redissolved diuron. Furthermore, increases of \(c_w^0\) by ~20%32 can only contribute a few % to the toxicity increase due to the logarithmic relationship of dose and response.

The findings of the experimental setup with constant diuron concentration and variable CNT concentration suggest that, at low CNT concentrations ≤10 mg CNT/L, LEEC in the algae-CNT-pollutant mixtures led to increased bioavailability of diuron especially for long pre-equilibration times of CNT and diuron and long incubation times of pre-equilibrated CNT and diuron with algae. Whether or not LEEC prevails at present environmentally relevant CNT concentrations remains to be elucidated, but organic pollutants sorbed to CNT are clearly shown in this work the first time to still be bioavailable and toxic for an aquatic organism. Toxicity increase due to LEEC becomes overridden only at high (environmentally irrelevant) concentrations ≥10 mg CNT/L, where toxicity decrease due to sorption of diuron to the CNT32 becomes the dominant process. The experimental setups with constant CNT or soot concentrations confirm these results, since also there, increased
toxicity with time or duration of the pre-equilibration time was observed, especially for the purified CNT, but only little for the industrial CNT and not for soot.

The fact that the ratio of measured per predicted PSII inhibition (I/I') was highly concentration dependent shows that c<sub>e</sub>, in presence of CNT clearly did not correspond to the bioavailable diuron fraction. Moreover, at low environmentally more relevant diuron concentrations between 2 and 10 μg/L diuron, bioavailability of diuron in presence of CNT will be greatly underestimated using this assumption, which was already used to predict risks for carbonaceous nanomaterials. For purified CNT, the diuron toxicity increase due to LEEC compensated or even overrode the (predicted) toxicity reduction due to sorption to CNT.

With the current predicted environmental exposure concentrations of CNT (0.021–0.0034 ng/L), underestimation of bioavailability of organic pollutants sorbed to CNT will result in no additional (or specific) environmental hazard, because the relative importance of CNT compared to black carbon is negligible. However, in view of the rapidly growing CNT production volumes and first reports of carbonaceous nanoparticle bioaccumulation in food chains, alternative concepts such as chemical activity could be used to predict bioavailability of organic pollutants sorbed to CNT, and the potential of CNT to increase the toxicity of organic pollutants should be taken into account. Future studies on the effects of sorbed pollutants could aim at analytical determination of intracellular concentrations of exposed organisms, or the LEEC itself. The enhanced bioavailability of pollutants in presence of CNT reported in this study and elsewhere should be considered when assessing the impact of carbonaceous nanomaterials in the environment.

**ASSOCIATED CONTENT**

Supporting Information
Details on the CNT characterization and raw data. This information is available free of charge via the Internet at http://pubs.acs.org/.

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Notes
The authors declare no competing financial interest.

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