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

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SUPPORTING INFORMATION CONTENT:

This supporting information contains 15 pages: 10 figures, 2 tables, and sections containing more details on the diuron quantification, photosynthetic inhibition by diuron, bioavailable diuron, the Hill slope, and the factor of toxicity increase of sorbed diuron as compared to diuron alone.

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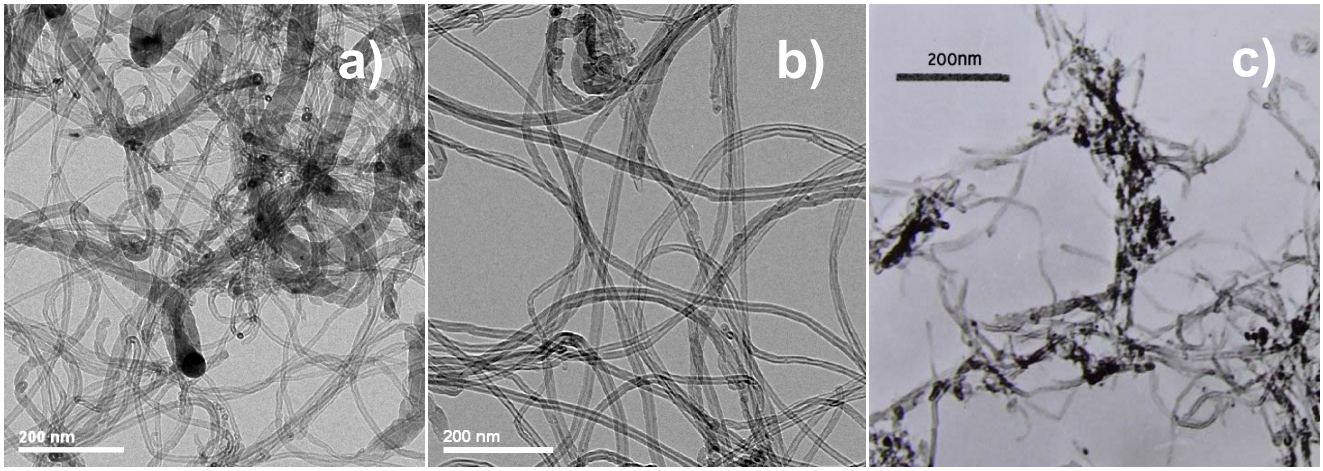
Figure S1: Transmission electron microscopy (TEM) and high resolution TEM (c) of the purified pristine (a), the purified functionalized (b), and the industrial pristine (c) multi-walled carbon nanotubes (CNT) used in this study. The image of the industrial CNT was taken from the product characterization of Cheap Tubes Inc., Brattleboro, VT 05301, USA.



Diuron Quantification. The dissolved diuron concentrations c_w in the particle-diuron-algae 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) using the same analytical method as described and tested in detail in Schwab *et al.*[4] After filtration with a disposable 0.44 µm hydrophobic PTFE syringe filter (Millipore, Switzerland), the internal standard D6-diuron was added, and the $\boldsymbol{c_w}$ was measured on a high-performance liquid chromatography system coupled with a tandem mass spectrometer (HPLC-MSMS, Varian 1200L, VarianInc, Walnut Creek, CA). The total uncertainty of the quantification method was <3% of the initial diuron concentrations (including filtration).

PSII Inhibition by Diuron. The photosynthetic activity of the algal cells was measured by *in vivo* fluorescence using a pulse amplitude modulated fluorometer (ToxY-PAM Dual Channel Yield Analyzer, Heinz Walz GmbH, Effeltrich, Germany). The ToxY-PAM measures *Y*, the uniteless quantum yield of photosystem II (PSII) in the thylakoids, wherefrom the PSII inhibition *I* (%) relative to the control without diuron (3 independent samples) was calculated. Each

replicate was measured five times. The background fluorescence signal of CNT or diuron alone was negligible. Further description of the fluorescence technique is available in ref.[1] or ref.[2]

The effect concentration at which 50% inhibition occurred (EC_{50}) was calculated by nonlinear regression using the software Statistica (StatSoft, Inc., STATISTICA version 9.0.; www.statsoft.com). To account for inter-assay reproducibility of the algal photosynthesis and to increase the robustness of the EC_{50} of diuron, eight independent diuron dose-response curves were measured for different algal batches and for different incubation times between 0.5-3.5 h, and an overall dose-response curve was generated using the pooled data. The effect range covered -3.1-78.2% PSII inhibition and thus complies with the requirements to calculate EC_{50} values.[3] Within the same experiments and varying incubation times between 30 min and 3.5 h, the EC_{50} values of the eight individual repetitions of the diuron dose-response curve differed maximally by approximately 60% (Incubation for 45 min, minimal EC_{50}: 62.2 $[49.6, 74.7]_{95\%}$, incubation for 1.5 h, maximal EC_{50}: 106.3 $[72.7, 139.7]_{95\%}$).

Bioavailable Diuron. The terms "bioavailability" or "availability" were used thorough the whole MS according to the official OECD guidance document for the testing of chemicals on toxicokinetics, which defines "bioavailability" as "Fraction of an administered dose that reaches the systemic circulation or is made available *at the site of physiological activity*".[6] In case of the herbicide diuron, the site of physiological activity is the PSII reaction center complex,[1] and the most established procedure to determine the activity of this reaction center is fluorescence technique.[1,7] Therefore, and because it is a widely accepted practice to determine the bioavailability of toxic compounds in microorganisms via the toxicological response,[5,8,9] the photosynthetic activity was used in this work to study the "bioavailable" diuron. For further discussion on how to relate bioavailability to effects, see ref.[9]

Figure S2: Diuron dose-response curves for the green algae *C. vulgaris* used to generate the global diuron dose-response curve shown in Figure 1A. Incubation time of diuron + algae was 0 to 3 h.

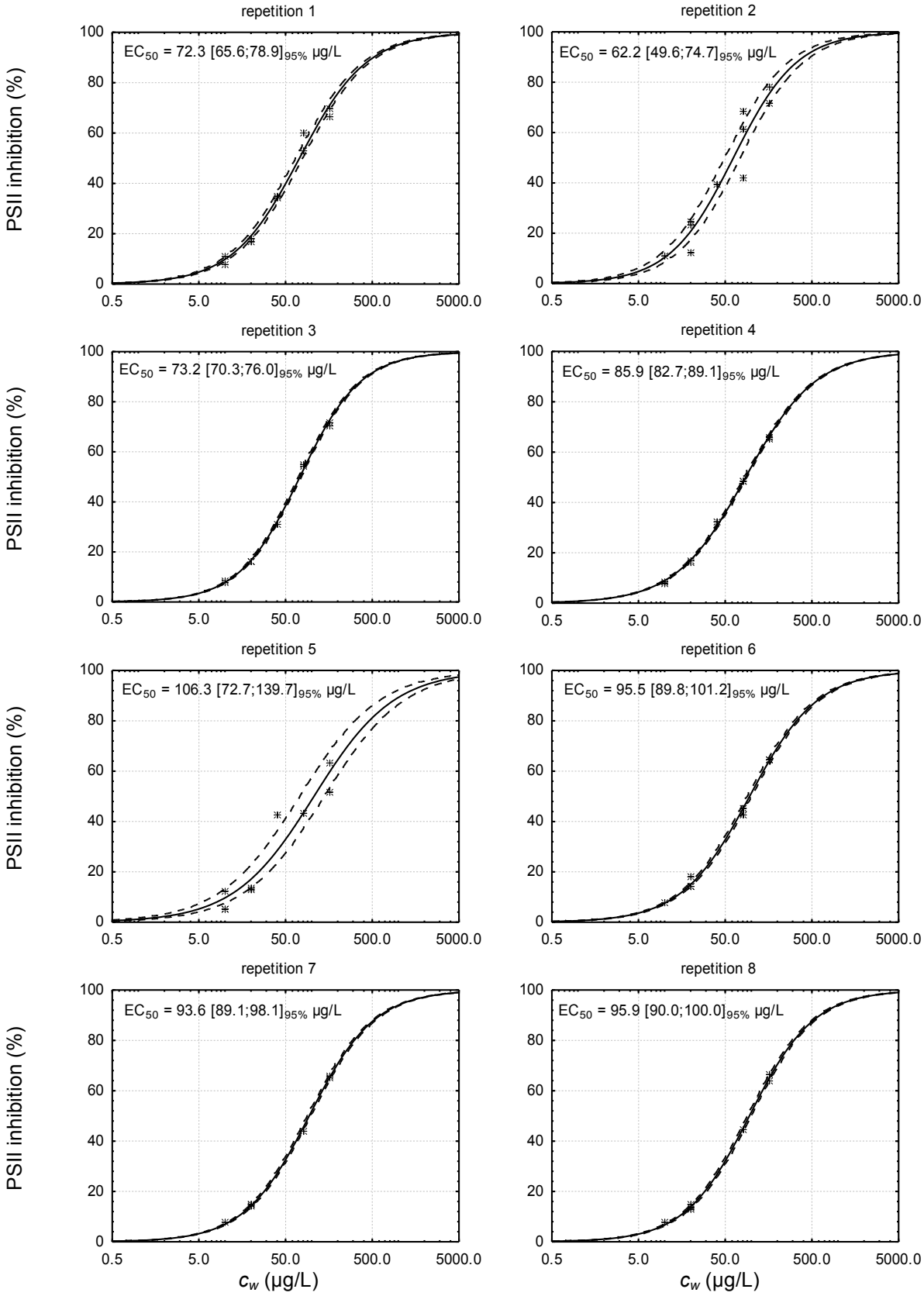


Figure S3: Photosynthetic yield of *Chlorella vulgaris* in control suspensions, run in parallel to the ternary trials, exposed to carbon nanotubes (CNT) and soot without diuron. No values were significantly different from the control treatments (Kruskal-Wallis analysis of variances, $H(4, N=110)=13.79652$ $p=0.0080$). Data from industrial pristine CNT (ipCNT), purified pristine CNT (ppCNT), and purified oxidized CNT (poxCNT) are shown.

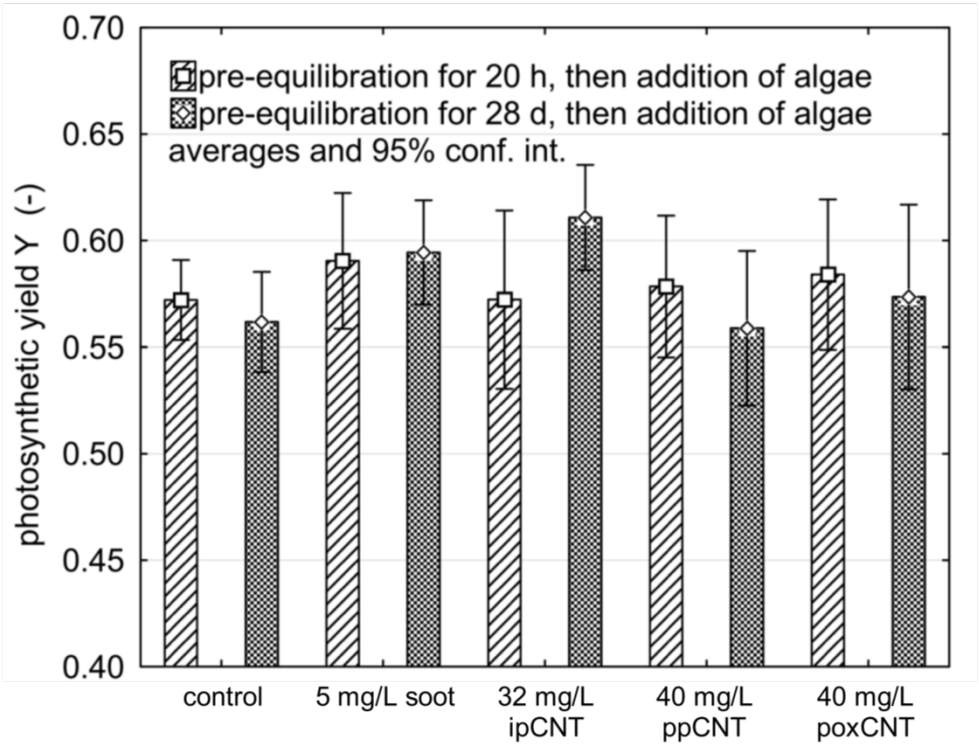


Figure S4: PSII inhibition as a function of dissolved diuron concentration c_w in all ternary batch experiments. Nanoparticle (NP) concentrations were held constant (10.0 mg CNT/L or 5.0 mg_{Soot}/L). CNT or soot were pre-equilibrated for 20 h (left panels) or 28 d (right panels) with a series of diuron concentrations and then incubated for 3, 6, 15, and 24 h with *C. vulgaris*.

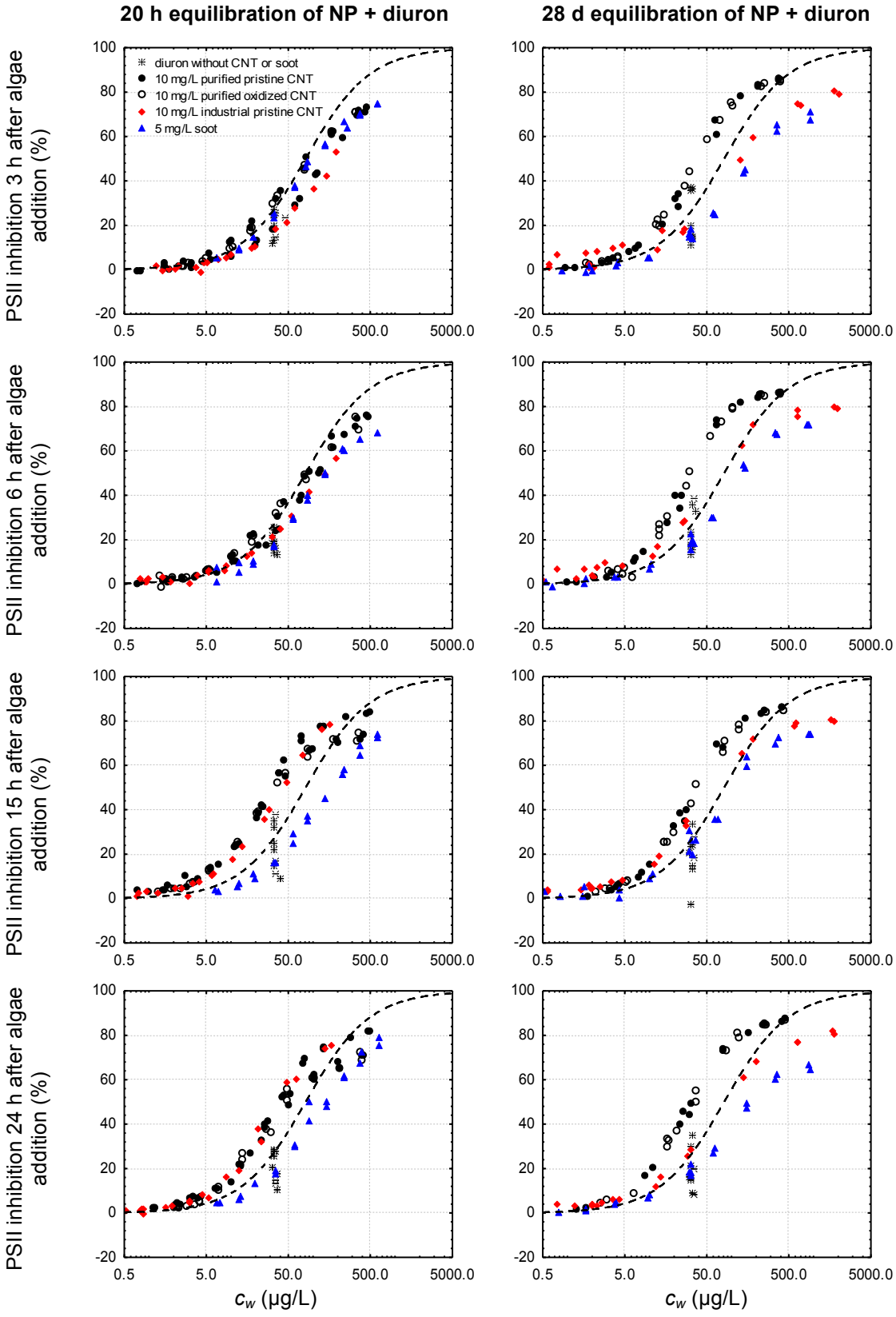


Figure S5: PSII inhibition of green algae as a function of initially established diuron concentration $c_{w,init}$ of all trials performed with a constant CNT (10 mg CNT/L) or soot (5.0 mg_{Soot}/L) concentration. The trials were pre-equilibrated for 20 h (left panels) or 28 d (right panels).

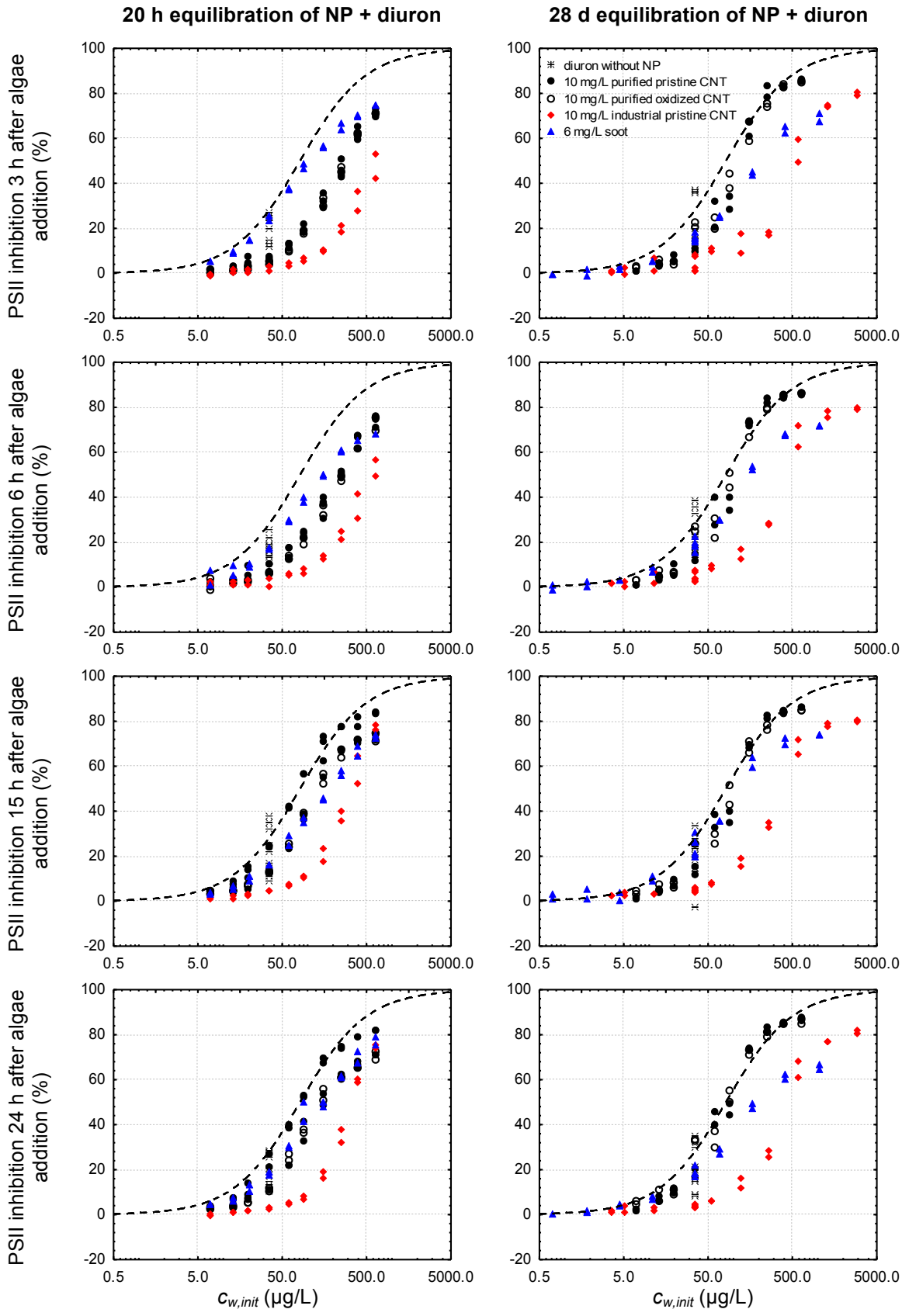


Figure S6: Results of experiments in which different CNT concentrations c_{NP} (mg_{NP}/L) and a constant diuron concentration (40.0 and 35.0 µg/L) were pre-equilibrated for 20 h (left panels) or 28 d (right panels) respectively. After pre-equilibration, algae were added and PSII inhibition was measured at different time points. ---: Algal response to diuron if no CNT were present.

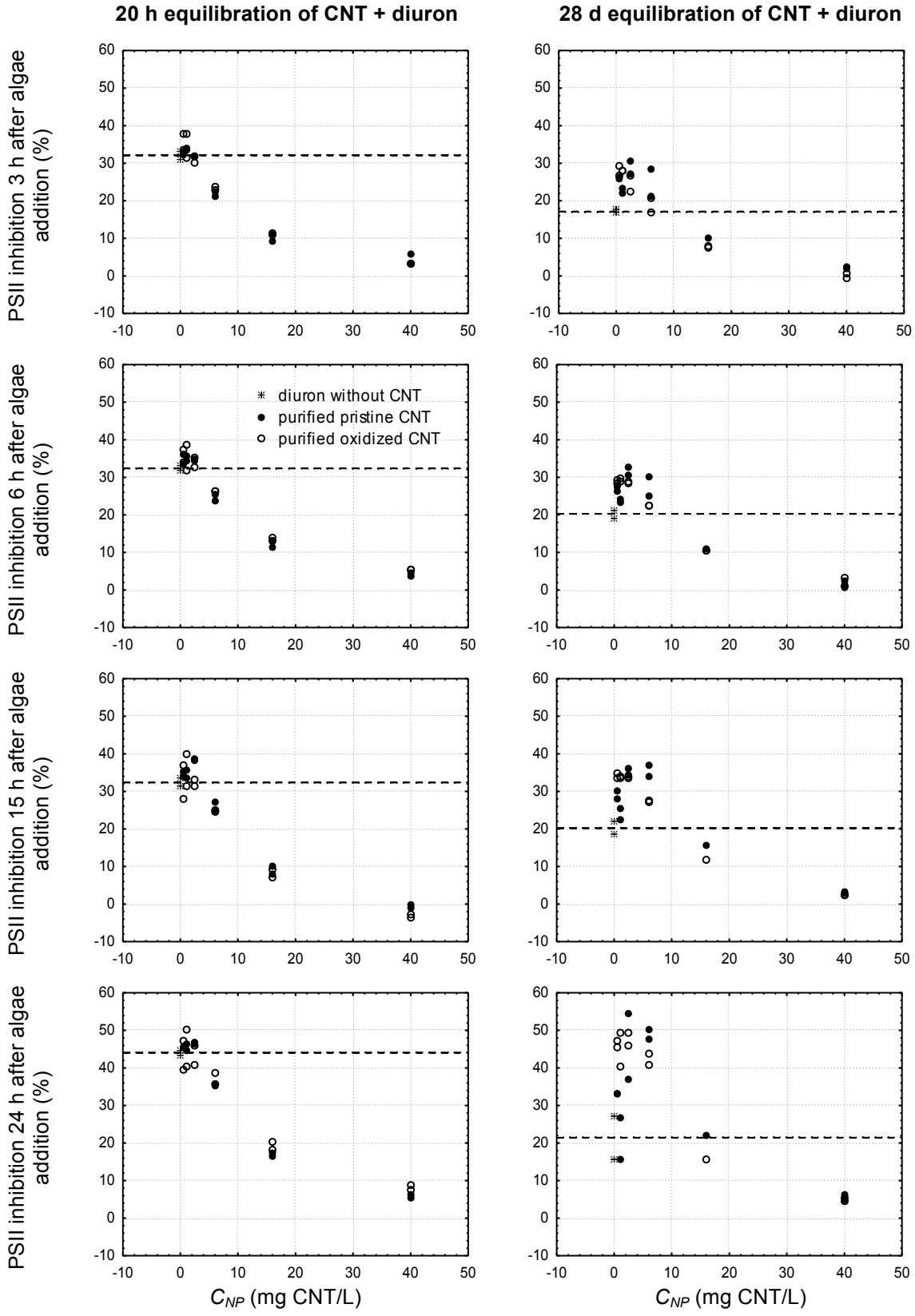


Table S1: Effect concentrations of 50% (EC_{50}) and slope factors[1] of the initially added diuron in the presence of 10.0 mg_{CNT}/L and 5.0 mg_{soot}/L respectively. The EC_{50} of diuron alone was 80.8 [74.8, 87.3]$_{95\%}$ µg/L (*N*=94) (see also Figure 1A).

			EC_{50} (µg/L)				slope factor (-)			
material	**equili-bration time (d)**	**time after addition of algae (h)**	**esti-mate**	**lower 95% conf. limit.**	**upper 95% conf. limit.**	**p-value**	**esti-mate**	**lower 95% conf. limit.**	**upper 95% conf. limit.**	**p-value**
purified pristine CNT	0.83	3	284	274	294	<10E-14	0.82	0.78	0.86	<10E-14
		6	244	235	254	<10E-14	0.84	0.80	0.89	<10E-14
		15	112	97	126	<10E-14	0.93	0.82	1.08	<10E-14
		24	133	114	151	<10E-14	0.99	0.87	1.16	<10E-14
	28.00	3	121	108	134	1.6E-13	0.63	0.55	0.75	8.1E-11
		6	103	90	115	9.2E-13	0.65	0.56	0.78	3.0E-10
		15	104	93	115	1.5E-13	0.69	0.60	0.81	2.2E-11
		24	87	78	95	2.2E-14	0.74	0.66	0.84	1.3E-12
purified oxidized CNT	0.83	3	289	277	301	<10E-14	0.78	0.74	0.82	<10E-14
		6	273	258	287	<10E-14	0.83	0.78	0.89	<10E-14
		15	155	136	175	4.3E-12	0.94	0.83	1.09	1.6E-11
		24	176	150	201	5.4E-11	0.99	0.86	1.17	1.1E-10
	28.00	3	117	105	129	3.6E-14	0.74	0.65	0.84	2.3E-12
		6	98	87	108	2.4E-13	0.73	0.64	0.85	1.4E-11
		15	100	90	111	1.4E-13	0.78	0.69	0.90	3.9E-12
		24	84	74	94	5.4E-13	0.83	0.73	0.96	5.4E-12
industrial pristine CNT	0.83	3	698	629	767	3.5E-14	0.73	0.64	0.84	4.1E-12
		6	607	554	661	<10E-14	0.75	0.67	0.85	7.3E-13
		15	326	307	345	<10E-14	0.60	0.55	0.67	3.5E-14
		24	341	329	353	<10E-14	0.55	0.51	0.59	<10E-14
	28.00	3	625	524	725	2.0E-11	0.86	0.73	1.03	4.5E-11
		6	473	395	552	3.1E-11	0.88	0.76	1.05	2.2E-11
		15	424	364	484	1.4E-12	0.89	0.78	1.02	7.2E-13
		24	491	426	555	3.9E-13	0.83	0.73	0.96	9.8E-13
soot	0.83	3	125	112	137	4.2E-14	1.18	1.08	1.31	1.2E-14
		6	168	150	187	2.3E-12	1.12	1.01	1.26	1.8E-12
		15	186	176	197	<10E-14	1.09	1.03	1.15	<10E-14
		24	150	135	164	3.5E-14	1.09	0.99	1.20	2.7E-14
	28.00	3	254	223	284	4.4E-13	1.23	1.13	1.35	<10E-14
		6	191	164	218	5.9E-12	1.25	1.13	1.40	4.7E-14
		15	138	113	163	4.2E-10	1.28	1.13	1.49	5.1E-12
		24	257	214	300	5.1E-10	1.44	1.29	1.63	1.9E-12

Table S2: Effect concentrations of 50% (EC_{50}) and slope factors[1] of the dissolved diuron c_w in the presence of 10.0 mg CNT/L and 5.0 mg_{soot}/L respectively. The EC_{50} of diuron alone was 80.8 [74.8, 87.3]$_{95\%}$ µg/L (N=94) (see also Figure 1A).

			EC_{50} (µg/L)				slope factor (-)			
material	**equili-bration time (d)**	**time after addition of algae (h)**	**esti-mate**	**lower 95% conf. limit.**	**upper 95% conf. limit.**	**p-value**	**esti-mate**	**lower 95% conf. limit.**	**upper 95% conf. limit.**	**p-value**
purified pristine CNT	0.83	3	124	111	138	<10E-14	1.15	1.05	1.28	<10E-14
		6	104	97	111	<10E-14	1.13	1.06	1.21	<10E-14
		15	38	33	43	<10E-14	1.19	1.07	1.33	<10E-14
		24	53	46	59	<10E-14	1.22	1.09	1.37	<10E-14
	28.00	3	44	17	71	2.8E-03	0.91	0.58	2.09	1.5E-03
		6	36	31	40	1.8E-12	0.88	0.79	1.00	1.5E-12
		15	41	37	46	6.2E-13	0.92	0.83	1.03	4.0E-13
		24	35	31	39	4.3E-11	0.99	0.89	1.12	9.6E-11
purified oxidized CNT	0.83	3	99	91	106	<10E-14	1.14	1.07	1.23	<10E-14
		6	93	84	102	5.6E-14	1.19	1.11	1.29	<10E-14
		15	45	36	54	1.2E-08	1.29	1.12	1.52	3.0E-10
		24	64	50	79	9.2E-08	1.31	1.11	1.59	2.3E-09
	28.00	3	41	37	45	<10E-14	0.94	0.87	1.03	<10E-14
		6	34	30	38	4.2E-13	0.87	0.78	0.98	5.0E-13
		15	42	38	46	<10E-14	0.96	0.88	1.05	<10E-14
		24	35	32	39	1.3E-11	0.96	0.87	1.08	3.0E-11
industrial pristine CNT	0.83	3	183	168	197	<10E-14	1.04	0.97	1.12	<10E-14
		6	141	133	149	<10E-14	1.12	1.08	1.18	<10E-14
		15	44	42	46	<10E-14	0.94	0.90	0.99	<10E-14
		24	43	39	47	2.1E-14	0.96	0.88	1.05	<10E-14
	28.00	3	156	126	186	5.0E-10	1.43	1.29	1.60	<10E-14
		6	97	75	120	1.3E-08	1.45	1.29	1.65	1.5E-13
		15	82	65	99	1.8E-09	1.42	1.27	1.60	2.2E-14
		24	111	92	130	5.1E-11	1.35	1.23	1.50	<10E-14
soot	0.83	3	119	108	131	3.6E-14	1.18	1.08	1.30	<10E-14
		6	159	141	177	2.5E-12	1.11	1.00	1.25	2.1E-12
		15	178	167	188	<10E-14	1.08	1.02	1.14	<10E-14
		24	146	132	160	7.4E-14	1.09	0.99	1.21	1.1E-13
	28.00	3	223	195	251	7.2E-13	1.21	1.10	1.34	<10E-14
		6	165	142	188	7.0E-12	1.22	1.09	1.37	1.0E-13
		15	127	105	149	2.4E-10	1.26	1.10	1.46	4.8E-12
		24	228	191	264	2.6E-10	1.42	1.27	1.60	1.4E-12

Hill Slope. The slope factor (Hill slope) of the diuron dose-response curve (0.901 [0.847, 0.962]$_{95\%}$) was very close to 1. A slope factor of 1 reflects that the dose-response curve of the antagonist diuron, which competes with the receptor binding site of plastoquinone, followed almost precisely a receptor binding curve and the binding of the agonist plastoquinone was almost completely suppressed.[10] The observed toxicity of the CNT-diuron mixture therefore still seems to be based on the very selective receptor binding mechanism of diuron,[7] since the shape of the dose-response curves in presence of particles remains largely unchanged and did not flatten, as this is the case if *e.g.* unspecific modes of toxic action come into play.

Factor of Toxicity Increase. To quantify the factor by which diuron toxicity (and thus PSII inhibition) is increased, the factor $(c_w/I)/(c_w'/I')$ was calculated for every replicate. The predicted dissolved concentration c_w' (µg/L) was calculated analogously to I' using a nonlinear regression of the diuron dose-response curve (Equation 1 solved for c_w) and I. The ratio c_w/I quantifies the diuron concentration resulting in 1% PSII inhibition in the sample replicate, and the ratio c_w'/I' quantifies the diuron concentration per 1% PSII inhibition in the control dose-response curve of diuron alone. The resulting data (Figure S7) show that at low c_w, the diuron toxicity is 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.

Figure S7: The factor diuron toxicity increase normalized by the toxicity of diuron alone (c_w/I)/(c_w'/I') as a function of dissolved diuron concentration c_w 24 h after addition of green algae to a 28 d pre-equilibrated mixture of varying diuron concentrations and a constant concentration of CNT or soot. The data demonstrate that diuron toxicity increased up to a factor of 5 due to the presence of CNT. In presence of soot, no increase was observed.

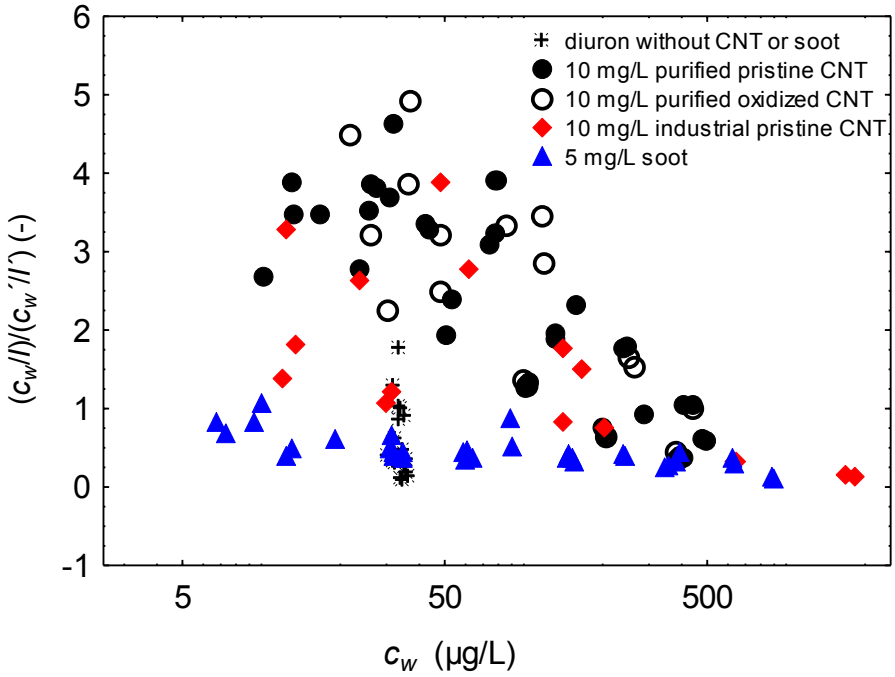


Figure S8: Scatterplot predicted / measured PSII inhibition as a function of dissolved diuron concentration for all performed trials. Predicted / measured PSII inhibition ratios >4 are not shown for reasons of readability of the ratios <1. Data for purified pristine and oxidized CNT (pCNT and oxCNT), industrial pristine CNT (pCT), and soot are shown.

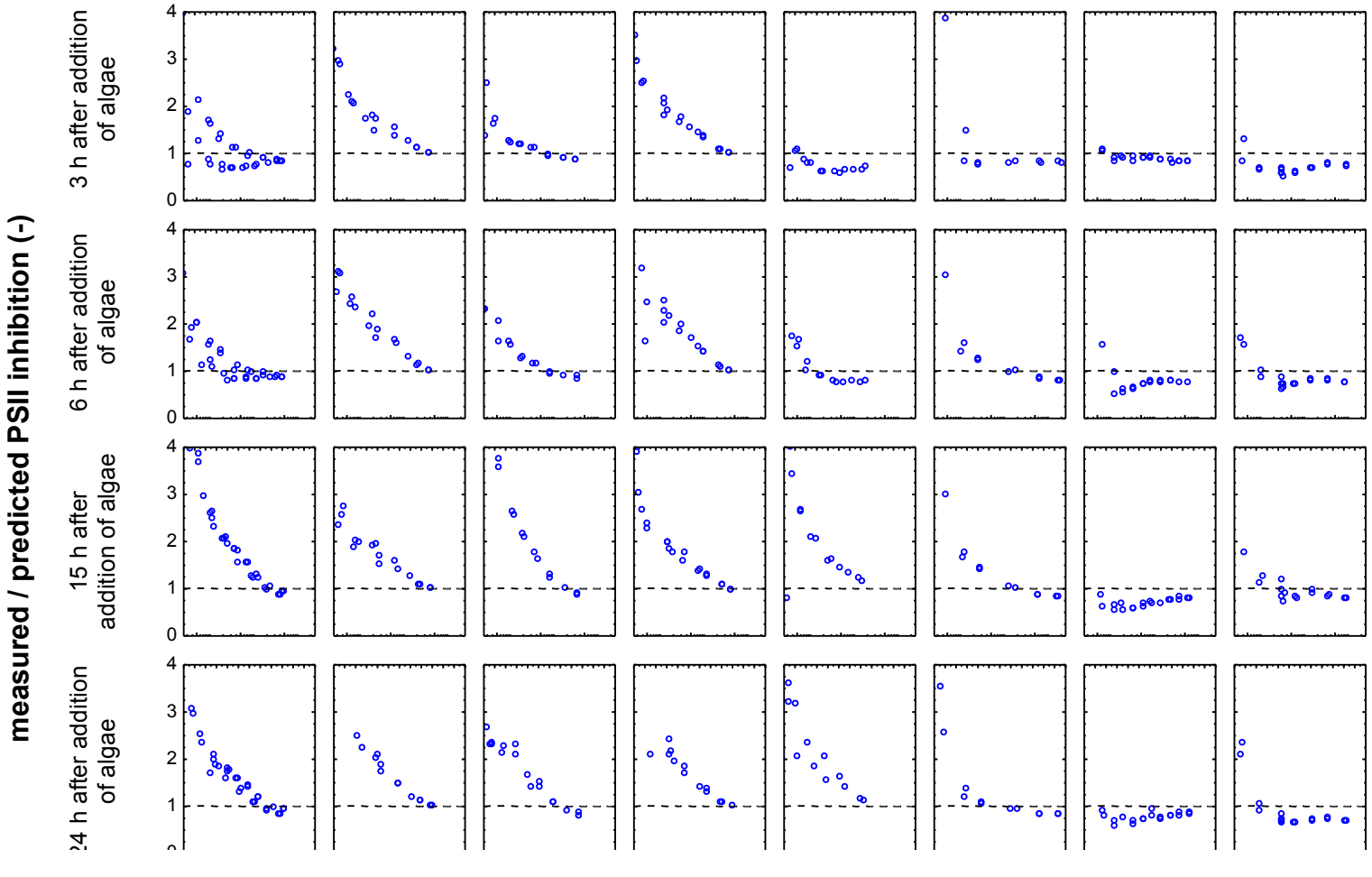


Figure S9: Microscopic images of carbon nanotubes (CNT) in presence of the green algae *P. subcapitata* (bottom pictures) established using hyperspectral imaging. All pixels possessing the same spectral signature as CNT are colored by red *pseudo* color. The spectral library of CNT is available in Figure S10.

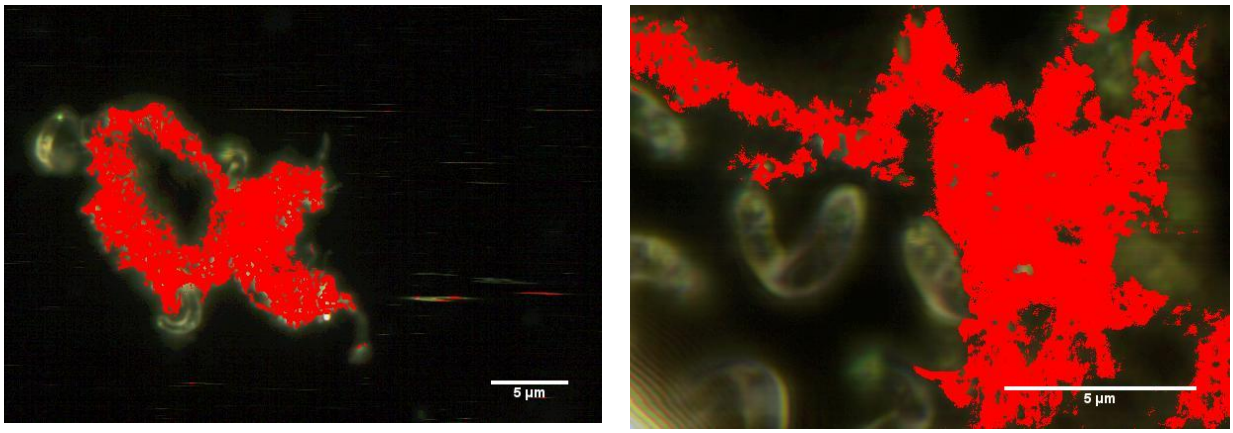
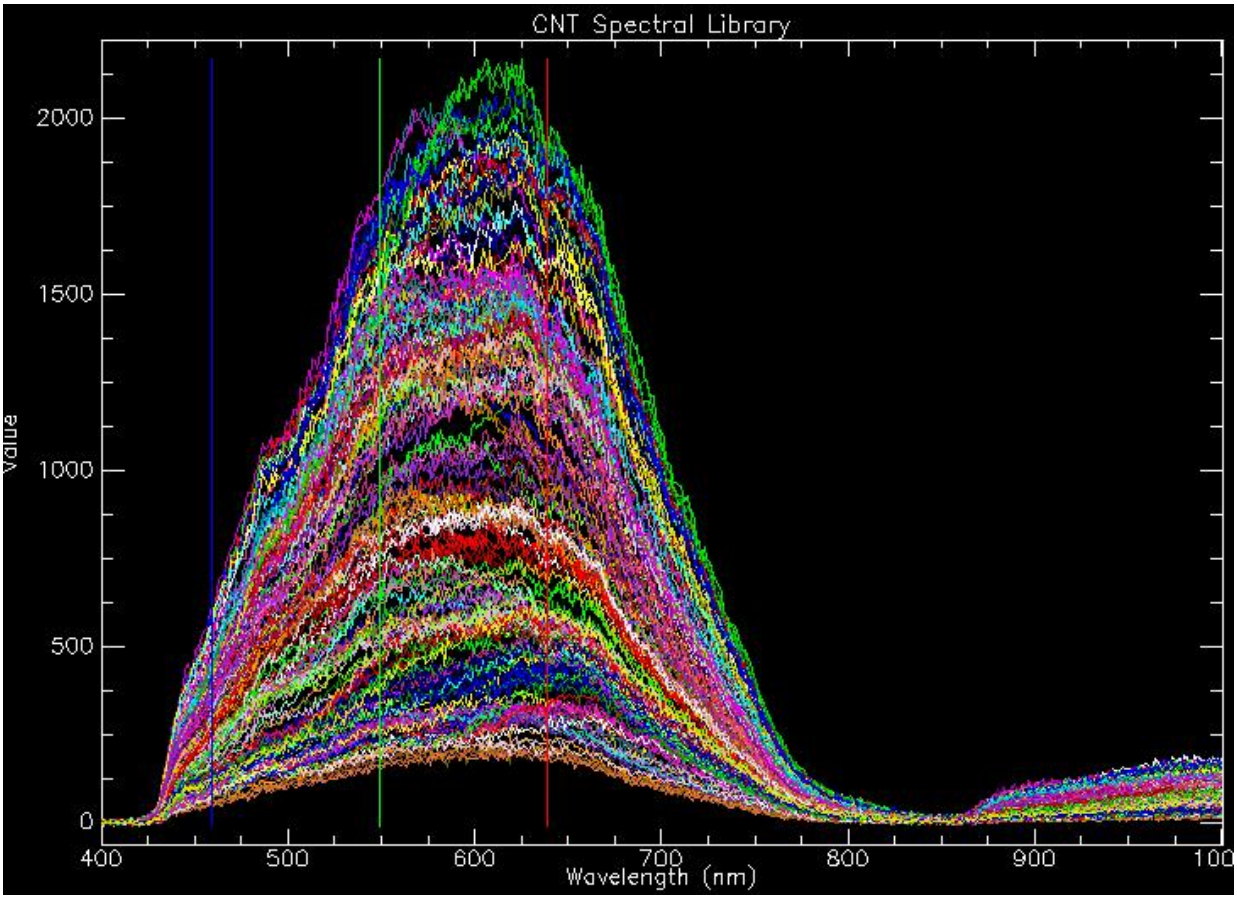


Figure S10: Spectral library of carbon nanotubes (CNT) recorded using hyperspectral imaging of a sample containing nanotubes only. The spectral library was employed to color pixels in microscopic images belonging to CNT in Figure 1 and Figure S9.



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