Supporting Information

Cellular response of *Chlorella pyrenoidosa* to oxidized multi-walled carbon nanotubes

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Number of tables: 4

Composition of the OECD medium

Algal cell culture medium recommended by OECD is composed of ultrapure water and the following ingredients (mg/L): NH_4Cl 15, $MgCl_2•6H_2O$ 12, $CaCl_2•2H_2O$ 18, $MgSO_4•7H_2O$ 15, KH_2PO_4 1.6, $FeCl_3•6H_2O$ 0.08, $Na_2EDTA•2H_2O$ 0.1, H_3BO_3 0.185, $MnCl_2•4H_2O$ 0.415, $ZnCl_2$ 3×10^{-3}, $CoCl_2•6H_2O$ 1.5×10^{-3}, $CuCl_2•2H_2O$ 10^{-5}, $Na_2MoO_4•2H_2O$ 7×10^{-3}, $NaHCO_3$ 50.

Preparation of oxidized MWCNTs

The pristine MWCNTs were ultrasonically treated with a mixture of concentrated H_2SO_4 and HNO_3 (3:1, V/V) at room temperature for 1, 2, 4 and 8 h, respectively. The oxidized MWCNTs (o-MWCNTs) were then separated from the concentrated acid solution by dilution and centrifugation, and were thoroughly washed with ultrapure water to neutral. Finally, the o-MWCNTs were obtained through lyophilization, and were stored in tightly capped vials.

Settling experiment

Individual MWCNTs and algal cell suspensions, as well as the mixtures of MWCNTs and algal cells, were transferred to cuvettes to monitor changes in absorbance at 660 nm over time for 12 h. The settling curves, which involve the normalized absorbances versus settling time curves, were subsequently plotted.

Density gradient centrifugation

Sucrose solutions over a concentration range of 10% to 60% (with an increment of 10%) were prepared. Our preliminary experiment had shown that the serial sucrose concentrations with an increment of 10% were able to separate the MWCNTs from the algal cells. Beginning with the highest concentration, density gradients of sucrose were created by carefully and slowly pipetting layers of sucrose into a 22 mL glass vial inclined at an angle of 30° with decreasing concentration. The glass vials containing the density gradients were kept static for about 6 h before use. Two mL

aliquots of the mixtures were layered on the top of the sucrose gradients, and immediately centrifuged at 1000*g* for 25 min, resulting in an obvious separation between algal cells and MWCNTs (as shown in Fig. S2). MWCNTs distributed in the upper layer of sucrose gradient due to their low density. Algal cells could be distinguished by their green color, and compact agglomerates containing both algal cells and MWCNTs settled to the bottom of the vial.

Analysis of total lipids

The obtained cell pellets were lyophilized for 48 h. Then 9 mL of a chloroform/methanol solution (1:2, V/V) were added into 22 mL glass vials containing approximately 10 mg of the cell powders. After bath sonication (100 W, 40 kHz, 25 °C) for 30 min, 3 mL chloroform was added and the sample was sonicated for 5 min. Three mL water was subsequently added and the sample was sonicated for another 5 min. After centrifugation (1500 *g*, 15 min, 25 °C), the chloroform layer was then pipetted and transferred to a pre-weighed test tube. The above procedures were repeated three times until the chloroform layer became colorless. Finally, the extract and test tube was dried under nitrogen in water bath at 45 °C, and the mass was determined. The total lipid content was calculated by the difference between the two masses and was expressed as % of dry cell weight

Analysis of fatty acid composition

The cell pellets were saponifed in a 1 mL 15% NaOH-methanol solution at 100 °C for 30 min, followed by methylation in 2 mL 6 mol/L HCl-methanol solution at 85 °C for 10 min, and extraction with 1 mL methyl tertiary butyl ether/hexane solution (1:1, V/V). After being washed with 3 mL 0.03 mol/L NaOH solution and 0.5 mL saturated NaCl, the extracted organic phase was then analyzed by gas chromatography (GC, Agilent 7890A, USA) with flame ionization detection (FID). The system utilized a DB-5MS capillary column coated with 5% diphenyl cross-linked with 95% dimethylpolysiloxane (30 m×250 μm inner diameter, 0.25 μm film thickness). The samples (1

μL) were injected into the GC column in splitless mode with Helium as the carrier gas at a constant flow rate of 1.2 mL/min. The initial temperature was kept at 80 °C for 1 min, raised to 320 °C at a rate of 12°C/min, and then kept for another 8 min. The injection and ion source temperatures were 275 and 230°C, respectively. The energy was -70eV in electron impact mode. The mass spectrometry data were acquired in full-scan mode with the range of 50-800 m/z.

Analysis parameters for metabolite profiling analysis and data analysis

The system utilized a DB-5MS capillary column coated with 5% diphenyl cross-linked with 95% dimethylpolysiloxane (30 m×250 μm inner diameter, 0.25 μm film thickness). The samples (1 μL) were injected into the GC column in splitless mode with Helium as the carrier gas at a constant flow rate of 1 mL/min. The initial temperature was kept at 50 °C for 1 min, raised to 310 °C at a rate of 10°C/min, and then kept for another 8 min. The injection, transfer line, and ion source temperatures were 280, 270, and 220 °C, respectively. The energy was -70 eV in electron impact mode. The mass spectrometry data were acquired in full-scan mode with the m/z range of 50-500 at a rate of 20 spectra/sec after a solvent delay of 6.1 min.

The raw data was pre-processed using interquartile range de-noising method. The missing values were then adjusted to half of the minimum value. An internal standard normalization method was employed during data analysis. Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed by SIMCA14.1 software package (Umetrics, Umea, Sweden) using the normalized data. Variable importance values (VIP) were obtained from OPLS-DA, and differential metabolites with VIP>1 and $p<0.05$ were subsequently selected. The differential metabolites were then subjected to hierarchical cluster analysis (HCA). And HCA was performed using a similarity metrics of Euclidian distances and complete linkage grouping. Commercial databases including KEGG (http://www.genome.jp/kegg/) and NIST

(http://www.nist.gov/index.html) were used to search for the metabolite pathways using the MetaboAnalyst website (www.metaboanalyst.ca/).

Measurement of SOD activity

The obtained cells were washed with 0.01 M PBS (pH 7.0) twice, and were resuspended in 2 mL PBS. After sonication (100 W, 40 kHz, 25℃) for 20 min, 1.5 mL ethanol and 1.5 mL chloroform were added to the samples, followed by 10 minutes' vibration (150 rpm, 25℃). The aqueous layer containing crude enzymes was separated from organic layer by centrifugation (3500*g*, 10 min, 25℃), and was pipetted to an 8 mL vial. The samples were mixed with four reagents provided by the assay kit, and were incubated at 37℃ for 40 min. Two mL chromogenic reagent was added and the absorbance at 550 nm was recorded. The result was normalized by the cell number.

Table S1. Sequences of primer pairs used for real-time PCR.

Target gene	Description	Primer sequence (5'-3')	Length (bp)
*acc*A	acetyl-CoA carboxylase	F: CGCAATACCAAGGAGAACAT R: ACATCTCACGCAGGTTGAC	202
*acc*D	acetyl-CoA carboxylase	F: TAGTTTGTGCTTCGGGTGG R: CAATAAGGGCTTTCGGTTCA	227
*dgat*7494	diacylglycerol acyltransferase	F: GCTCGCTGGGCCTGATGCTGTT R: GCGGATGAGCGGGAAGTAGA	186
*dgat*2354	diacylglycerol acyltransferase	F: TTTGGCGAGAATGAGCAGTA R: ACCTTTGAAGGCGGGCAG	236
ME	malic enzyme	F: CCCTCTCGTTCCCCTTTTATT R: AAATGCTGACGCAAGTGTGA	158
PEPC	phosphoenolpyruvate carboxylase	F: AAGGAGTGGGACGAGGATAAG R: GGTGTGGGACATTGAGATGAT	238
*CAH*2	putative carbonic anhydrase	F: GACTCCAACATTGCGAAGAT R: GGAAGAGGTCGGTCAGGT	109
*rbc*L	Rubisco large subunit	F: CTTTCCAAGGTCCTCCTCAC R: TCTCTCCAACGCATAAATGG	208
*HLA*3	putative inorganic carbon transporter	F: TGATGTGCTTCCTCACCCT R: TCCAAAGTGTCCTGGTCCT	189
*psb*B	photosystem II P680 chlorophyll A apoprotein	F: CACCGTCTGATTGAAGAGTTGC R: GATGTTCCTTTCCGTCGTTCTG	187
*cox*2	*cytochrome c* oxidase subunit Ⅱ	F: GAAGTGGATAATCGTATGGTTG R: CTGCATCACATTTTGCTCCTAA	115
*atp*B	ATP synthase CF1 beta subunit	F: GTTTCGTTCAAGCTGGTTCT R: GTTCTTGTAAGCCACCCATT	105
*fts*H	cell division protein FtsH	F: ACAAAGTGACCGAAATCCAGAA R: TTACGAATTGGGAGACTAGAAG	129
actin	reference gene	F: GCTCAACTCCTCCACGCT R: GTCCTTGCGGATGTCCAC	187

Table S2. Selected properties of the MWCNTs.

Materials	Outer diameter (nm)	Length (μm)	Elemental contents (%)				Specific surface area (m^2 g^{-1})	Pore volume (cm^3 g^{-1})	Electrophoretic mobility (μm cm/Vs)	Hydrodynamic size (nm)
			C	H	N	O				
p-MWCNTs	43±18	4.2 ± 0.9	97.55	0.08	ND[a]	2.82	26.4	0.050	-2.86 ± 0.13	748 ± 120
o-MWCNT1	45±18	3.5 ± 0.7	93.99	0.17	ND	5.22	53.2	0.080	-4.27 ± 0.01	284 ± 5
o-MWCNT2	45±16	2.2 ± 0.6	93.75	0.15	0.11	5.36	57.6	0.086	-4.34 ± 0.07	249 ± 6
o-MWCNT4	47±17	2.1 ± 0.5	92.44	0.20	0.12	6.25	58.9	0.100	-4.35 ± 0.01	250 ± 6
o-MWCNT8	44±18	1.7 ± 0.6	91.06	0.21	ND	7.25	76.0	0.111	-4.43 ± 0.12	228 ± 3

[a] ND stands for data not detected. The data were from Ref. 1.

Table S3. Concentration of selected metal ions in the OECD medium with and without o-MWCNT8.

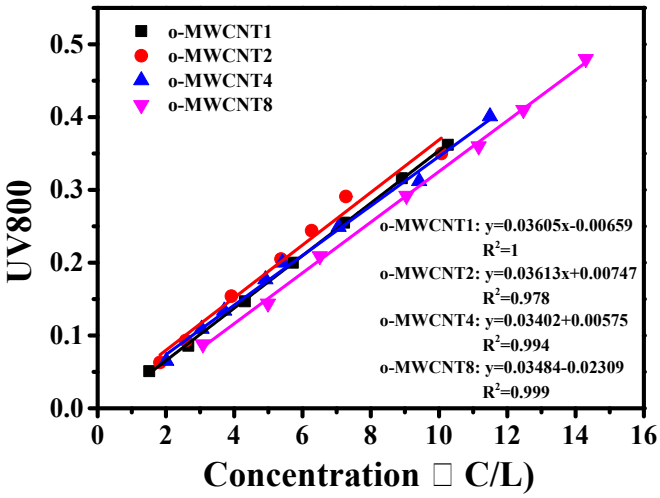
Cations	Zn (μg/L)	Mn (μg/L)	Co (μg/L)	Ni (μg/L)	Cu (μg/L)	Mo (μg/L)	Mg (mg/L)	Ca (mg/L)
Without o-MWCNTs	1.45	132	0.26	<0.1	<0.1	6.67	3.64	5.73
With o-MWCNT8	<0.1	8.57	0.15	16.0	<0.1	5.51	2.51	2.74

Note: The presence of o-MWCNT8 decreased the concentrations of Zn, Mn, Co, Mo, Mg, and Ca cations in the OECD medium, suggesting cation adsorption. The o-MWCNT8 released Ni into the OECD medium, and thus increased the concentration of Ni in the presence of o-MWCNT8.

Table S4. Effect of o-MWCNT8 on the fatty acid content (%) of algal cells.

Fatty acids	Control cells	5 mg/L o-MWCNTs treated cells	10 mg/L o-MWCNTs treated cells
SFA			
C12:0	0.13 ± 0.02	0.14 ± 0.01	0.14 ± 0.03
C14:0	0.66 ± 0.05	0.67 ± 0.01	0.66 ± 0.04
C15:0 iso	0.10 ± 0.01	0.12 ± 0.02	0.12 ± 0.01
C15:0 2OH	1.49 ± 0.04	1.69 ± 0.24	1.8 ± 0.01
C15:0 3OH	0.17 ± 0.15	0.31 ± 0.01	ND
C16:0	38.40 ± 1.01	33.30 ± 0.45	32.22 ± 1.12
C16:0 anteiso	9.94 ± 0.56	12.25 ± 0.89	13.16 ± 0.76
C16:0 iso 3OH	0.74 ± 0.64	ND	ND
C17:0	0.51 ± 0.02	0.33 ± 0.04	0.32 ± 0.02
C17:0 iso	0.33 ± 0.01	0.32 ± 0.01	0.27 ± 0.01
C17:0 anteiso	0.34 ± 0.13	0.60 ± 0.14	0.62 ± 0.15
C17:0 10-methyl	2.76 ± 0.31	3.46 ± 0.18	3.46 ± 0.1
C17:0 iso 3OH	0.55 ± 0.11	0.62 ± 0.07	0.82 ± 0.11
C18:0	1.32 ± 0.17	0.72 ± 0.18	0.58 ± 0.18
C18:0 iso	ND	0.76 ± 0.04	0.81 ± 0.04
Sum	57.44 ± 1.61	55.25 ± 0.63	54.95 ± 0.52
UFA			
C16:1 w7c alcohol	0.80 ± 0.10	0.82 ± 0.13	0.75 ± 0.06
C17:1 w8c	0.86 ± 0.06	0.91 ± 0.01	0.95 ± 0.01
C18:1 w5c	ND	3.53 ± 0.88	3.83 ± 0.59
C18:2 w6,9c	40.85 ± 0.65	38.78 ± 1.06	39.00 ± 1.00
Sum	42.51 ± 0.76	44.03 ± 0.04	44.53 ± 0.33
SFA/UFA ratio	1.35 ± 0.02	1.25 ± 0.01	1.23 ± 0.02

Note: ND stands for not detected.



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108 **Fig. S1 Correlation between the UV800 and the TOC-based concentration of stabilized o-**

109 **MWCNT suspensions.**

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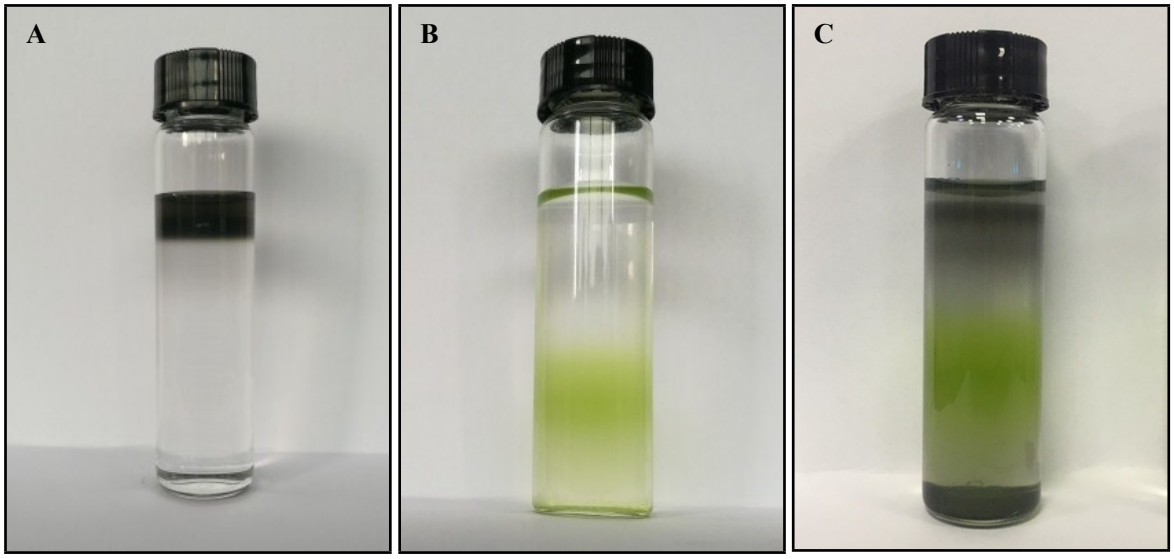

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120 **Fig. S2 Images of vials after the density gradient centrifugation for o-MWCNT8 (A), the algal**

121 **cells (B), and the mixture of algal cells and o-MWCNT8 (C).**

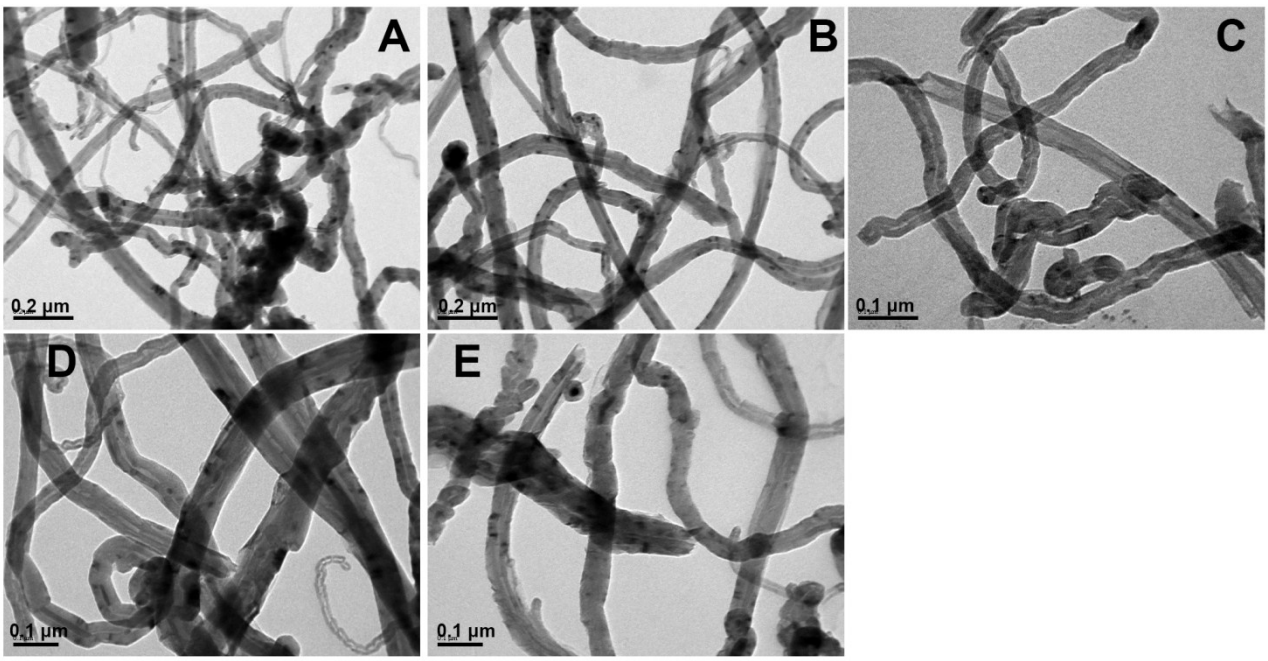

Fig. S3 TEM images of p-MWCNTs (A), o-MWCNT1 (B), o-MWCNT2 (C), o-MWCNT4 (D) and o-MWCNT8 (E). The images were from Ref. 1.

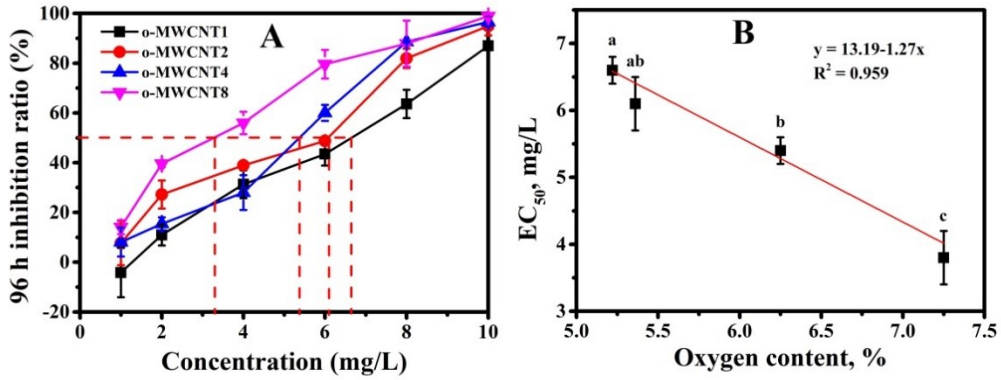

Fig. S4 (A) Dose-response curves and (B) the correlation between calculated EC_{50} values and oxygen contents of the o-MWCNTs. Values with different letters (a–c) differ significantly ($p < 0.05$). Error bars represent standard deviations (n=3).

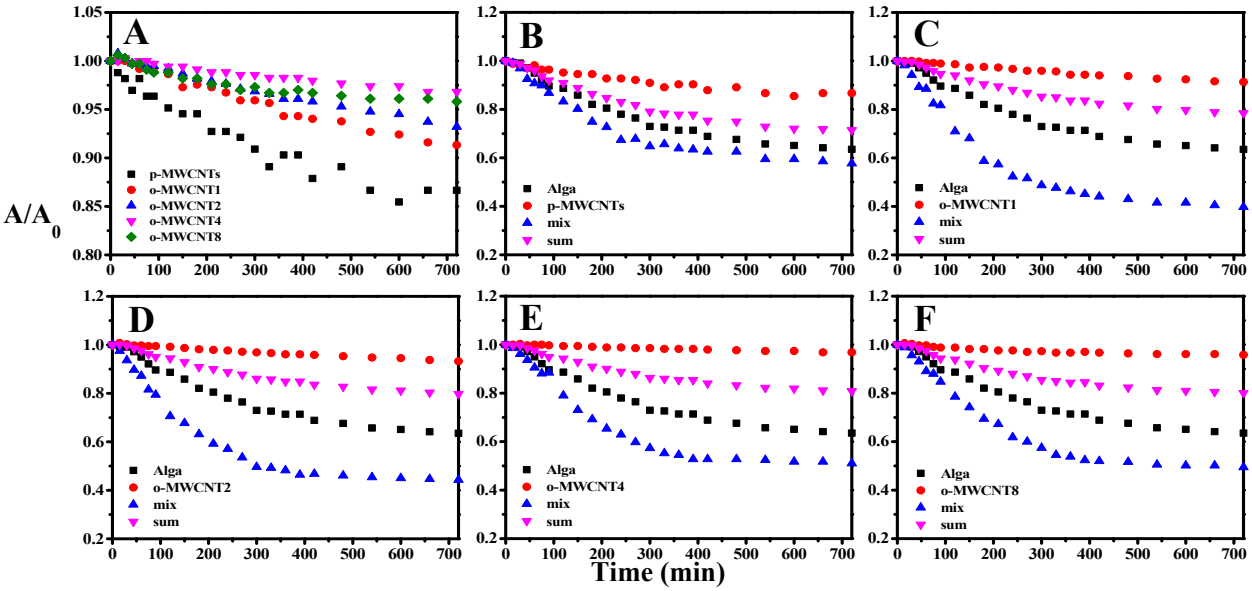

Fig. S5 The individual settling curves of MWCNTs (A), and co- (mix) and additive- (sum) settling curves of MWCNTs and algal cells (B-F) in the OECD medium.

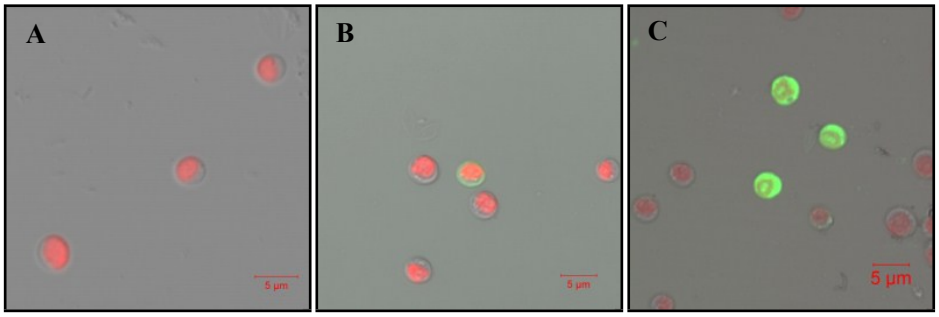

Fig. S6 CLSM images of untreated algal cells (A) and algal cells treated with FITC (B) and FITC labeled o-MWCNT8 (C) under high magnification.

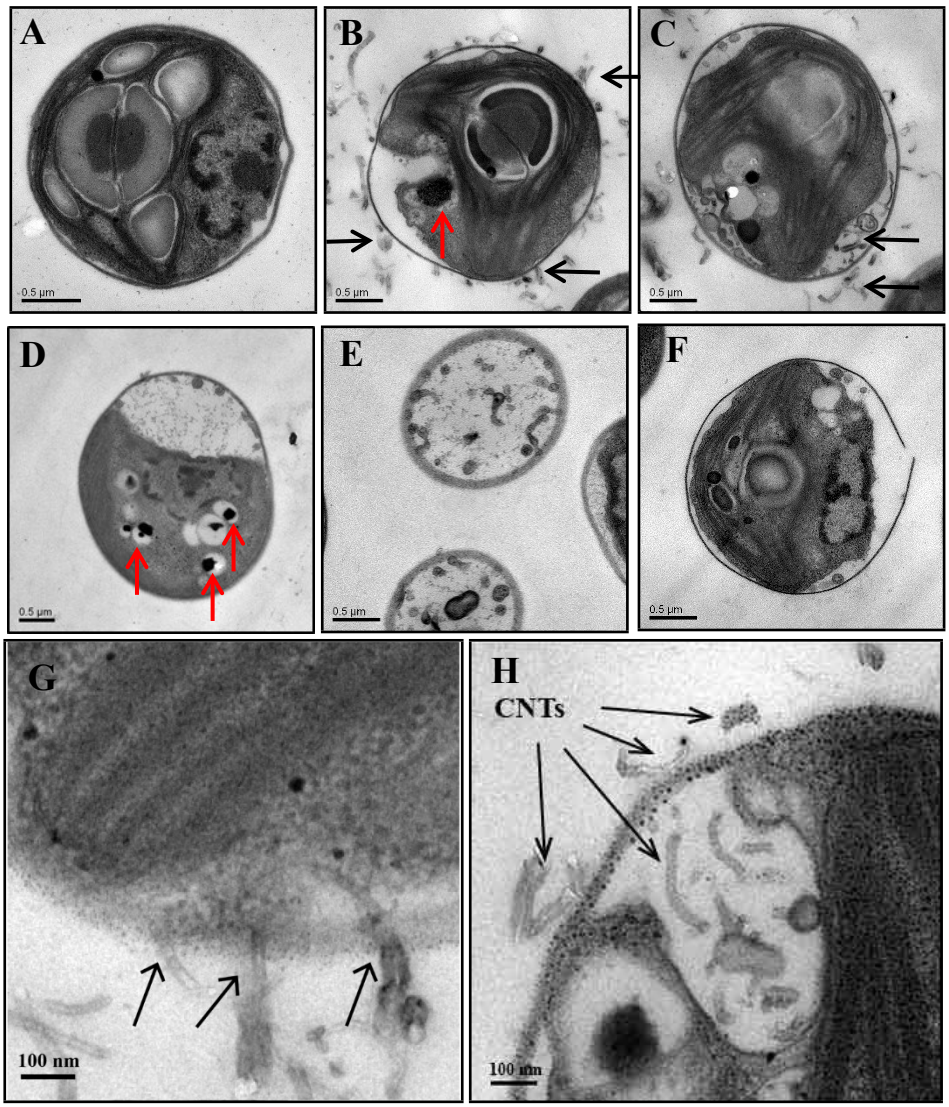

Fig. S7 TEM image of untreated algal cells untreated (A) and cells treated with 10 mg/L o-MWCNT8 (B-H). Panels G and H are the magnified images of the rectangle located areas in Figure 2 B2 and B3, respectively. Black and red arrows in the images point to o-MWCNT8 and electron-density granules in vacuoles, respectively.

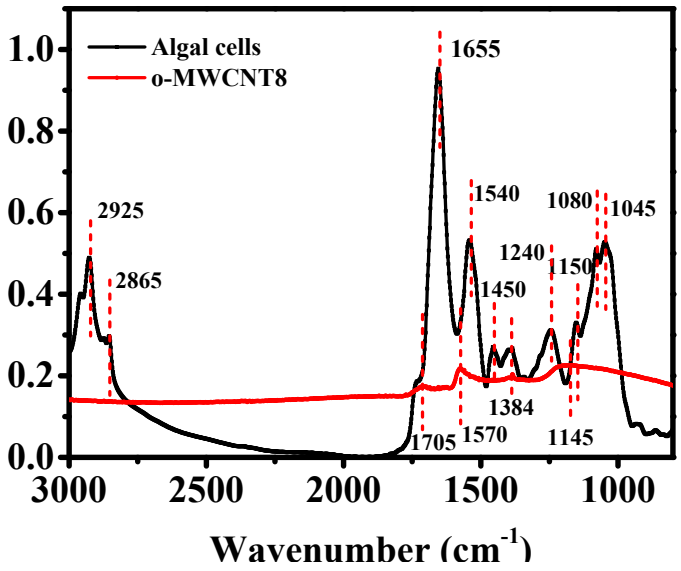

Fig. S8 FTIR spectra of algal cells and o-MWCNT8.

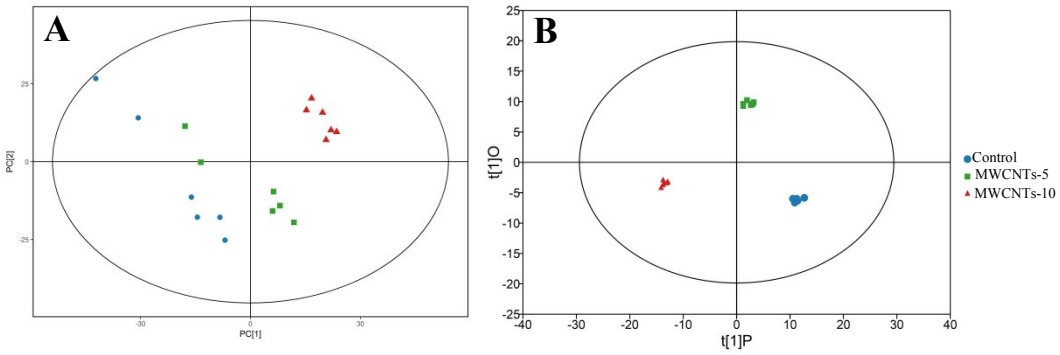

Fig. S9 Score scatter plots of PCA (A) and the OPLS-DA (B) model for the control group, and groups treated with 5 mg/L o-MWCNT8 (MWCNTs-5) and 10 mg/L o-MWCNT8 (MWCNTs-10).

Reference cited:

1. L.Q. Zhang, M.H. Wang, J. Fang, K. Yang, D.H. Lin, The effect of oxidation on physicochemical properties and aqueous stabilization of multiwalled carbon nanotubes: Comparison of multiple analysis methods, *Sci. China Chem.*, 2016, **59**, 1498-1507.