

Supporting Information

Cellular Response of Freshwater Algae to Halloysite Nanotubes: Alteration of Oxidative Stress and Membrane Function

Xiaochen Huang,^a Yichao Huang,^{b,c} Dali Wang,^d Mingxian Liu^{e,*}, Jing Li,^{d,*} and Da Chen^d

^a *School of Agriculture, Sun Yat-Sen University, Shenzhen 518107, P. R. China*

^b *Department of Toxicology, School of Public Health, Anhui Medical University, Hefei 230032, China*

^c *Key Laboratory of Environmental Toxicology of Anhui Higher Education Institutes, Hefei 230032, China*

^d *Guangdong Key Laboratory of Environmental Pollution and Health, School of Environment, Jinan University, Guangzhou 514443, P. R. China*

^e *Department of Materials Science and Engineering, Jinan University, Guangzhou 510632, P. R. China*

*Corresponding authors: *Mingxian Liu*. E-mail: liumx@jnu.edu.cn, *Jing Li*. E-mail: jli909@163.com

■ METHODS AND MATERIALS

Text S1. FTIR determination

Briefly, the halloysite nanotubes (HNTs), nanosized SiO₂ (nSiO₂), nanosized Al₂O₃ (nAl₂O₃), and nSiO₂+nAl₂O₃ mixture were ground with infrared quality KBr (w/w, 1:100), vacuum-pressed and measured with the range of 400-4000 cm⁻¹. 16 cycles were co-added with a resolution of 2 cm⁻¹ to enhance signal to noise ratio.

Text S2. Zeta potential and particle size

The suspension of tested nanomaterials were individually prepared in the BG-11 medium and treated with sonication (100 W, 40 kHz, 25 °C) for 30 min. The final concentrations of HNTs, nSiO₂, and nAl₂O₃ were 1 mg/mL, 0.55 mg/mL, and 0.32 mg/mL, respectively. The 0.55 mg/mL nSiO₂ and 0.32 mg/mL nAl₂O₃ were mixed and suspended under sonication treatment and quickly moved in the cuvette for measurements of hydrodynamic diameters using the Zetasizer. The hydrodynamic diameters of nanomaterials were recorded at 0-30 min.

Text S3. SEM observation

The surface morphology of tested nanomaterials, algal cells, and their interactions were determined using SEM (ULTRA 55, ZEISS, Germany). Briefly, the nanomaterials were prepared with absolute ethanol and treated with sonication for 2 h. The interactions between algal cells and nanomaterials were observed after 96-h exposure. Prior to the SEM observations, the samples were rinsed thrice with PBS (0.1 M, pH 7.0), filtered with membrane, freeze-dried, and gold-coated.

Text S4. TEM observation

The algal cells exposed to nanomaterials were centrifuged (1500 g, 10 min) and fixed with 2.5% glutaraldehyde (v/v) at 4 °C for overnight. Prior to fix with 1% osmium tetroxide (v/v) for 1-2 h, samples were thoroughly rinsed thrice (15 min/time) with PBS (0.1 M, pH 7.0). After repeatedly washing step by PBS, the samples were dehydrated with the increasing concentrations of ethanol (30%, 50%, 70%, 80%, 90%, 95%, and 100%) in sequence for 15-20 min and 100% acetone for 20 min. Epoxy resin and 100% acetone mixed at 1:1 and 3:1 (v/v) were subsequently used for permeation and embedding. Ultramicrotome (EM UC7, Leica, Germany) was employed to obtain ultra-thin algal samples (70-90 nm), which were then strained with lead citrate and uranyl acetate for 5-10 min. The ultrastructure of algal cells were mounted on copper grids and observed using a TEM (Tecnai Spirit, FEI, USA).

Text S5. Determination of reactive oxygen species (ROS), membrane integrity, and mitochondrial membrane potential (MMP)

In brief, treated algal cells were centrifuged and then rinsed thrice with PBS (0.1 M, pH 7.0) to resuspend in the PBS solution. For ROS detection, the centrifuged cell pellets were stained with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) in the dark for 15 min and measured fluorescence intensity at 488 nm excitation and 525 nm emission with a fluorescence spectrophotometer (HITACHI F-4600, Japan). Propidium iodide (PI, 1 mg/mL) was used to stain with algal cells in the dark for 15 min to determine membrane integrity using flow cytometer (Gallios, Beckman coulter, USA) at 488 nm excitation and 620 nm emission with a FL3 detector. The JC-1 strained algal cells incubated in the

dark for 15 min were employed to determine MMP. The excitation wavelength for MMP detection was 488 nm, measuring with FL1 detector (green fluorescence) at 520 nm and FL2 detector (red fluorescence) at 580 nm. The loss of MMP was quantified according to ratio value of red to green fluorescence intensity. At least 10000 algal cells were collected and analyzed for each replicate.

Text S6. Adsorption of Nutrients and Release of Al and Si

The adsorption of nutrients by nanomaterials were determined according to the method by Zhao et al.¹ Briefly, HNTs, nSiO₂, nAl₂O₃, and nSiO₂+nAl₂O₃ were individually prepared with sterile ultrapure water, added in algal medium, stirred with shaker (150 rpm) at 25 °C for 96 h. The solution was filtered through a 0.22-μm membrane filter to obtain the supernatants. The microelements (B, Co, Cu, Zn, Mn, and Fe) and macroelements (Ca, Mg, K, and P) in the supernatants were determined using inductively coupled plasma optical emission spectrometry (ICP-OES, iCAP 7000 Series, Thermo Scientific, USA). The sterile supernatants were applied to cultivate algae for 96 h. Cell numbers were counted to investigate the effects of nutrient removal on algal growth.

To determine the release of Al and Si during treatments, the suspension of HNTs, nSiO₂, nAl₂O₃, or nSiO₂+nAl₂O₃ was sonicated for 30 min and then stirred (150 rpm) at 25 °C for 96 h. The suspension was collected every 24 h and filtered through 0.22-μm membrane. Concentrations of Al and Si were determined using ICP-OES. To investigate the toxicities of Si or Al ions to algae, Na₂SiO₃·9H₂O (1.2, 23.5, and 48.2 mg/L) and Al(NO₃)₃·9H₂O (0.06, 0.34, and 0.86 mg/L) were separately added in sterile medium to cultivate algae for 96 h.

Text S7. Pretreatment for fatty acid (FA) analysis

An aliquot of 500 μL methylbenzene and 20 μL C17:0 (1 mg/mL) were added in sequence and

blended by Vortex oscillator (9454FIALUS, Fisher Scientific, USA). The samples were added with 2 mL NaOH-methanol solution (3:50, m/V), heated in water bath (80 °C) for 15 min for saponification. Subsequently, the extract of FAs was treated with acetylchloride-methanol solution (1:10, V/V) at 80 °C for another 15 min for transmethylation. 1 mL K₂CO₃ and 1 mL *n*-hexane were added sequentially to extract the methyl esters prior to analysis by Gas Chromatography-Mass Spectrometry (GC-MS).

GC separation was achieved using a J&W DB-23 column (60 m × 250 μm × 0.25 μm, Agilent Technologies, USA). Detection used 5977B MSD detector (Agilent Technologies, USA) under electron impact (EI) ionization source followed our previous method.² Samples (1 μL) were injected with a split ratio of 20:1 at 250 °C. Helium was used as the carrier gas at a flow rate of 1.2 mL/min. GC oven programs are set as follows: 50 °C for 1 min, a linear ramp to 175 °C at 25 °C/min and held for 2 min, linear ramp to 230 °C at 3 °C/min and held for 5 min. MS ion source was set at 300 °C. The MS detector was operated in the selected ion monitoring (SIM) mode and detailed parameters have been previously summarized.² The FAME peaks were identified and quantified according to FAME standard mixture GLC-463 (Nu-Chek, USA) using MassHunter software (Agilent Technologies). Each FA was quantified as their relative weight percentage to the total FAME.

■ RESULTS AND DISCUSSION

Text S8. Ions release and adsorption depended on media and nanomaterials

Nanomaterials have ability to release their dissolved ions and absorb ions existed in aqueous phase, depending on nanomaterials' species and cultivation media. These properties could further have impacts on algal biological responses. The 96-h cell number of algae after nutrients removal by four

tested nanomaterials (Fig. S7) suggested that there were no significant differences in cell number between the control and any four treatments, although several nutrients were significantly reduced (Fig. S8). Hoecke et al. also ruled out the hypothesis that CeO₂ nanoparticles induced nutrient depletion was responsible for the toxicity to *Pseudokirchneriella subcapitata* in the presence of OECD medium.³ In addition, although previous work reported that dissolved ions released from both metallic nanoparticles and HNTs was the predominant contribution to toxicity,^{4,5} our study revealed that only 0.23% Al released from nAl₂O₃ (Figs. S5 and S6). It is worth noting that 0.74 mg/L of Al ions (corresponding to 0.07% Al ions release in Al₂O₃ NPs) could induce 45.9% growth inhibition, accounting for 76.5% of total growth inhibition. Thus, the mass release of Al from Al₂O₃ NPs could be the main toxic mechanism to algae. Different biological responses between HNTs and nSiO₂+nAl₂O₃ were not attributed to the released ions.

REFERENCES:

1. J. Zhao, X. Cao, Z. Wang, Y. Dai and B. Xing, Mechanistic understanding toward the toxicity of graphene-family materials to freshwater algae, *Water Res.*, 2017, **111**: 18-27.
2. Y. Huang, F. Sun, H. Tan, Y. Deng, Z. Sun, H. Chen, J. Li and D. Chen, DEHP and DINP induce tissue-and gender-specific disturbances in fatty acid and lipidomic profiles in neonatal mice: a comparative study, *Environ. Sci. Technol.*, 2019, **53**, 12812-12822.
3. K. V. Hoecke, J. T. Quik, J. Mankiewicz-Boczek, K. A. D. Schamphelaere, A. Elsaesser, P. V. D. Meeren, C. Barnes, G. McKerr, C. V. Howard, D. V. D Meent, K. Rydzyński, K. A. Dawson, A. Salvati, A. Lesniak, I. Lynch, G. Silversmit, B. D. Samber, L. Vincze and K. Rydzyński, Fate and effects of CeO₂ nanoparticles in aquatic ecotoxicity tests, *Environ. Sci. Technol.* 2009, **43**, 4537-

4546.

4. F. Chen, Z. Xiao, L. Yue, J. Wang, Y. Feng, X. Zhu, Z. Wang and Xing, B, Algae response to engineered nanoparticles: current understanding, mechanisms and implications, *Environ. Sci.: Nano*. 2019, **6**, 1026-1042.
5. X. Wang, J. Gong, Z. Gui, T. Hu and X. Xu, Halloysite nanotubes-induced Al accumulation and oxidative damage in liver of mice after 30-day repeated oral administration, *Environ. Toxicol.* 2018, **33**, 623-630.

■ Tables

Table S1 Composition of BG-11 medium used for the culture of *Chlorella vulgaris*.

	Components	Concentration (g/L)
Regular solution	NaNO ₃	1.5
	K ₂ HPO ₄	0.04
	MgSO ₄ ·7H ₂ O	0.075
	Citric acid	0.006
	CaCl ₂ ·2H ₂ O	0.036
	Na ₂ CO ₃	0.02
	Ferric ammonium citrate	0.006
	Na ₂ ·EDTA·2H ₂ O	0.001

A5	H ₃ BO ₃	2.86
(trace metal mix solution, *10 ⁻³)	MnCl ₂ ·4H ₂ O	1.81
	ZnSO ₄ ·7H ₂ O	0.222
	Na ₂ MoO ₄ ·2H ₂ O	0.39
	CuSO ₄ ·5H ₂ O	0.079
	Co(NO ₃) ₂ ·6H ₂ O	0.049

■ Figures:

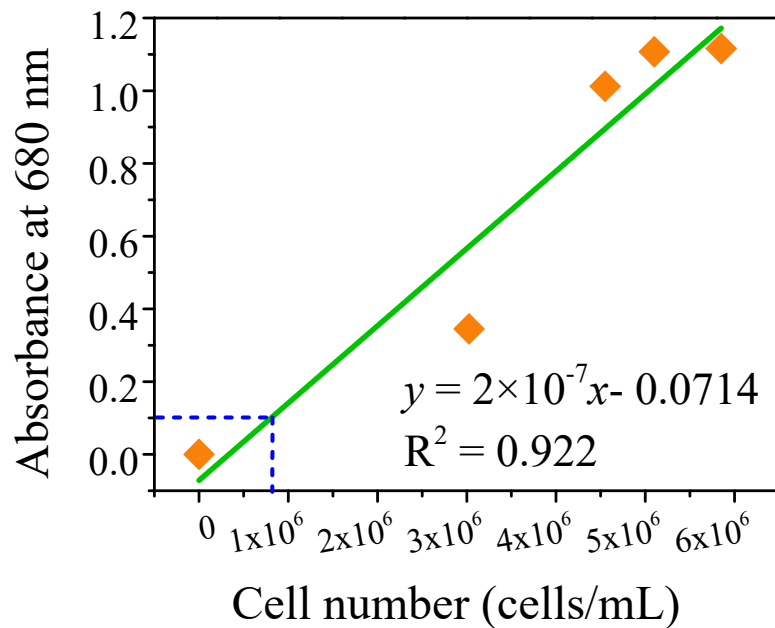


Fig. S1. The correlation between algal cell number and absorbance at 680 nm.

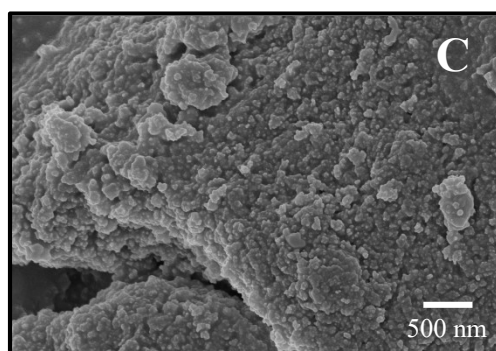
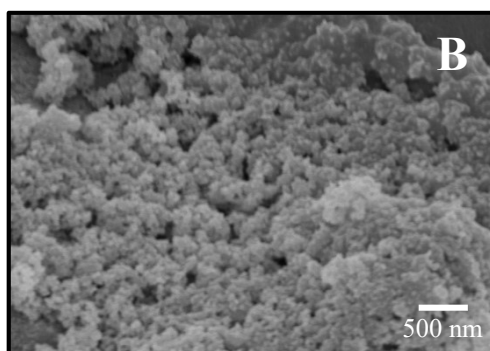
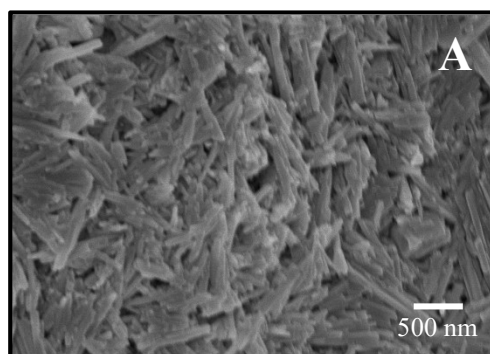


Fig. S2 The scan electron microscope (SEM) images of HNTs (A), nSiO₂ (B), and nAl₂O₃ (C).

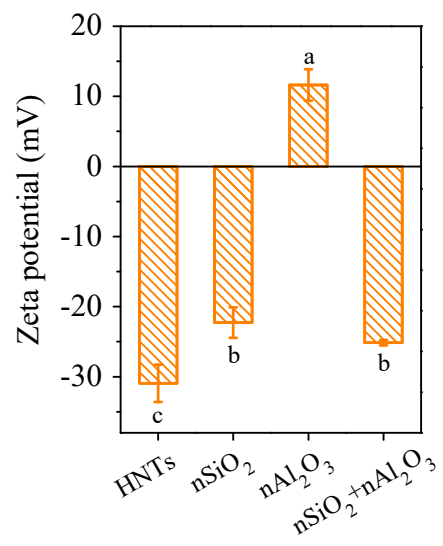


Fig. S3. The zeta potentials of tested nanomaterials in BG-11 medium.

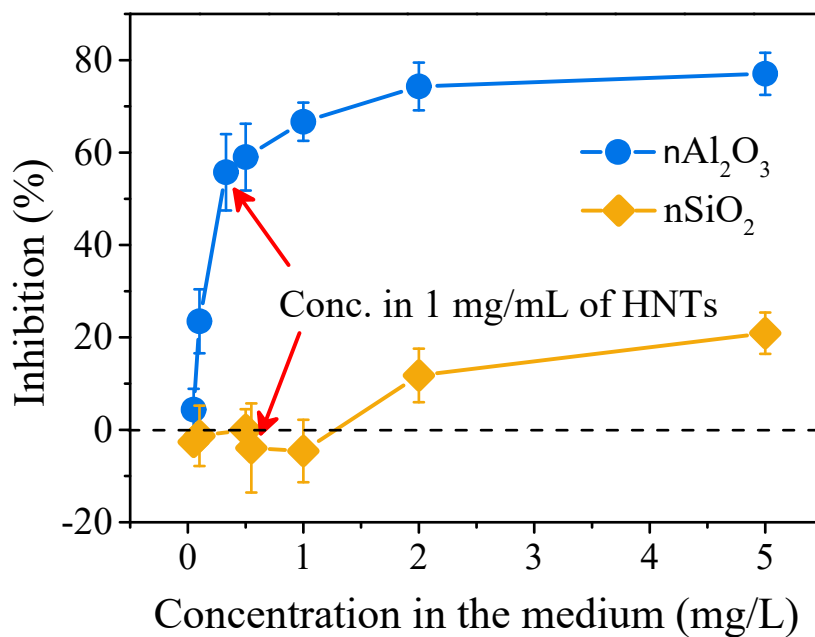
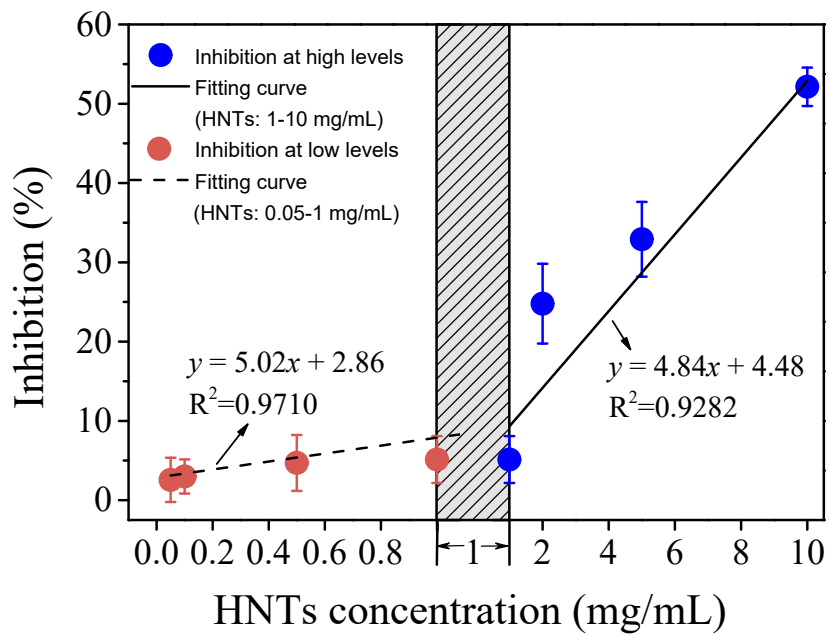


Fig. S4. The algal growth inhibition when exposed to different concentrations of HNTs, $n\text{SiO}_2$, and $n\text{Al}_2\text{O}_3$.

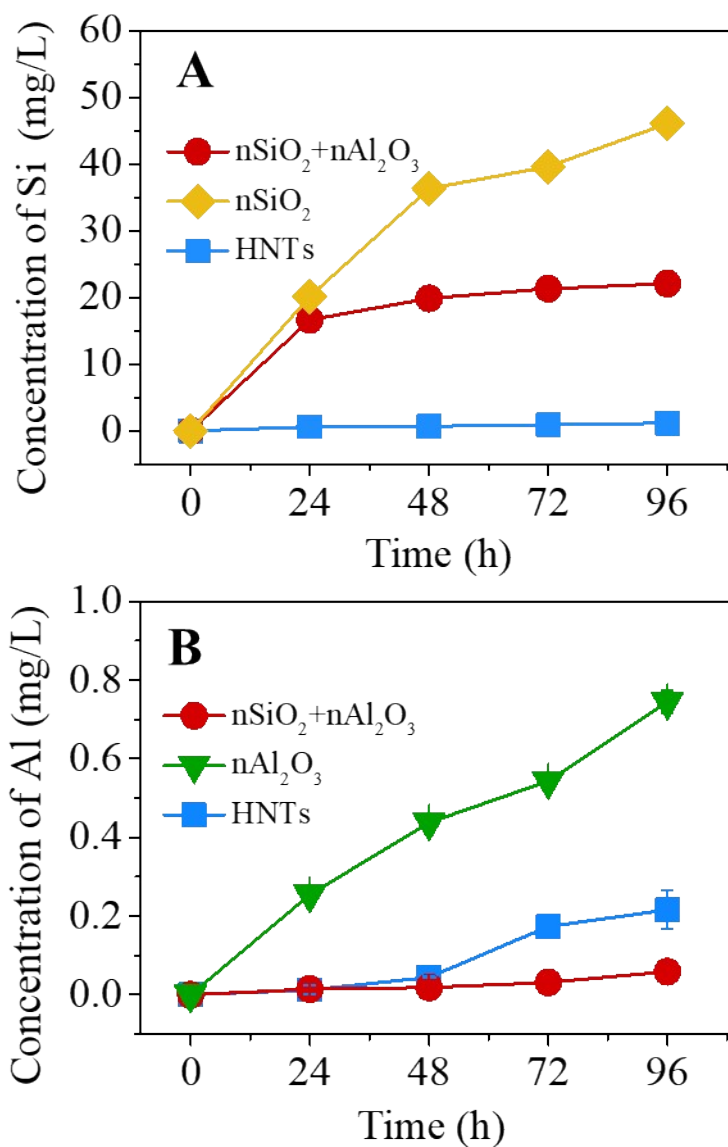


Fig. S5 The time-course of released Si (A) from HNTs, nSiO₂, and nSiO₂+nAl₂O₃ and Al (B) from HNTs, nAl₂O₃, and nSiO₂+nAl₂O₃ in the algal medium.

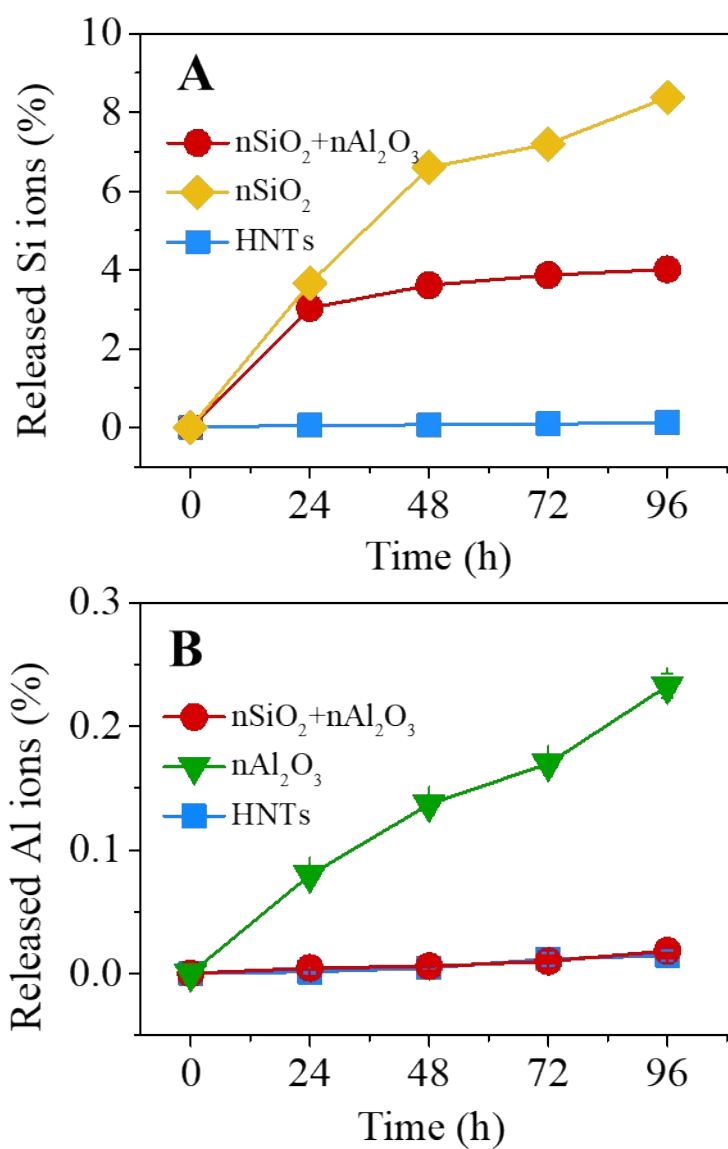


Fig. S6 The time-course of percentage of released Si (A) from HNTs, nSiO₂, and nSiO₂+nAl₂O₃ and Al (B) from HNTs, nAl₂O₃, and nSiO₂+nAl₂O₃ in the algal medium.

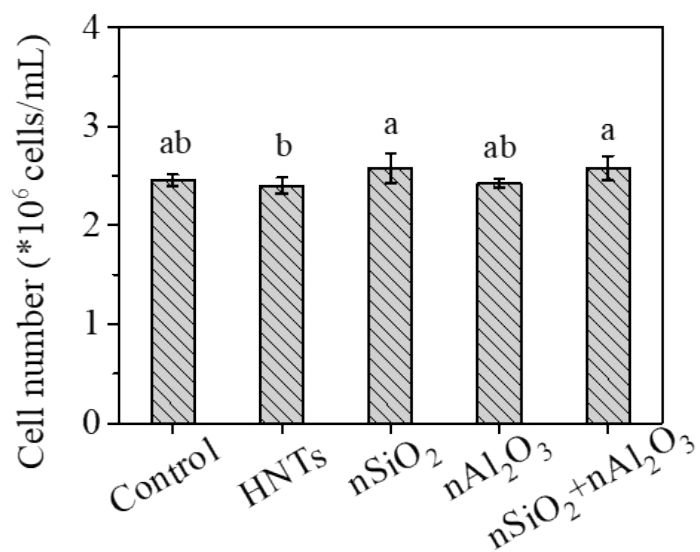


Fig. S7 The 96-h algal growth inhibition cultured under control and NMs-adsorbed medium. Lowercase letters represent significance in cell number among five treatments.

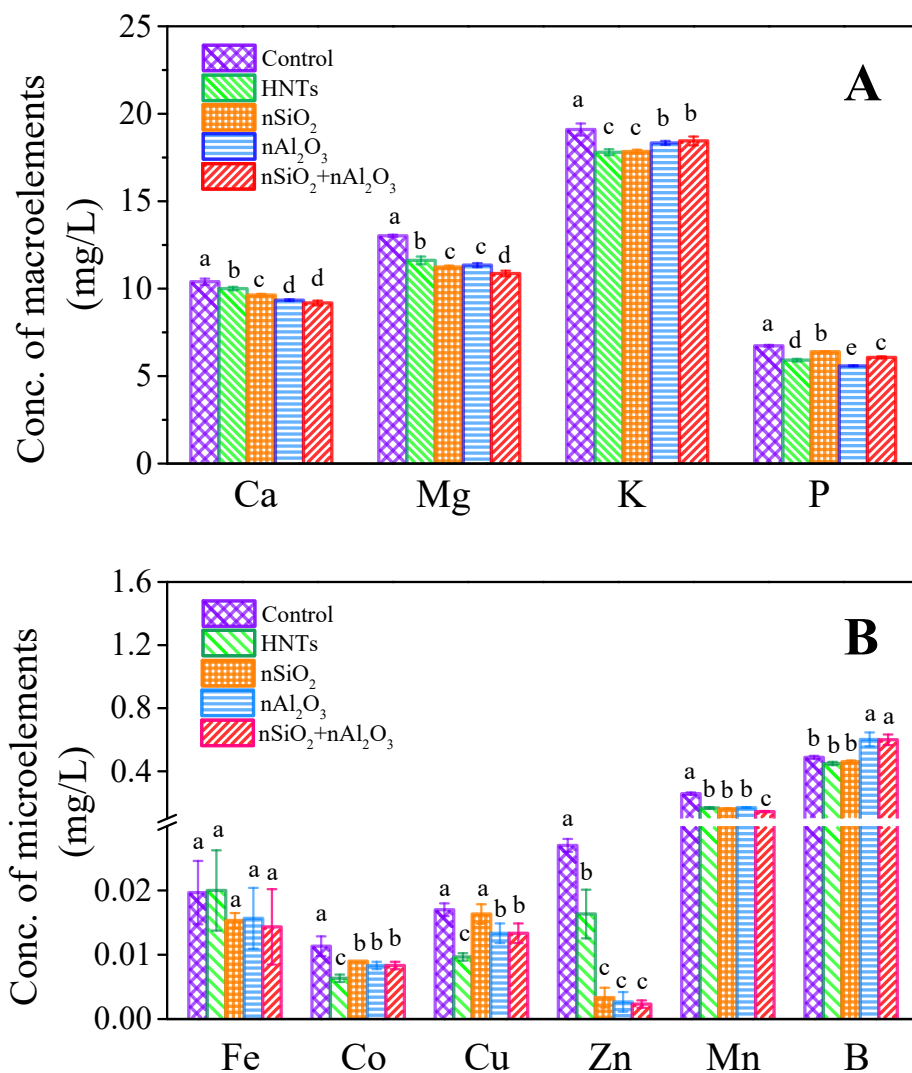


Fig. S8 Concentrations of macroelements (A) and microelements (B) in supernatants after adsorption for 96 h. Lowercase letters represent significance in macroelements and microelements among five treatments.

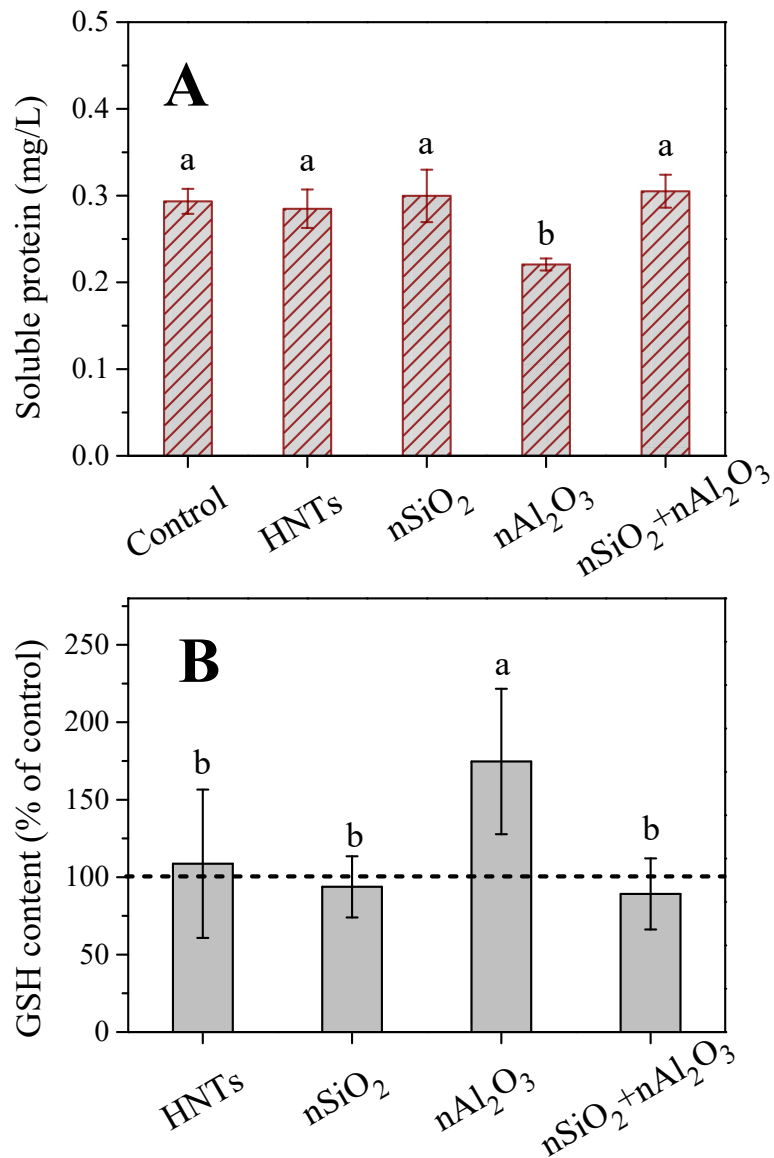


Fig. S9 The change of soluble protein (A) and GSH content (B) in algae under five treatments for 96 h. Lowercase letters represent significance in soluble protein and GSH content among five treatments.

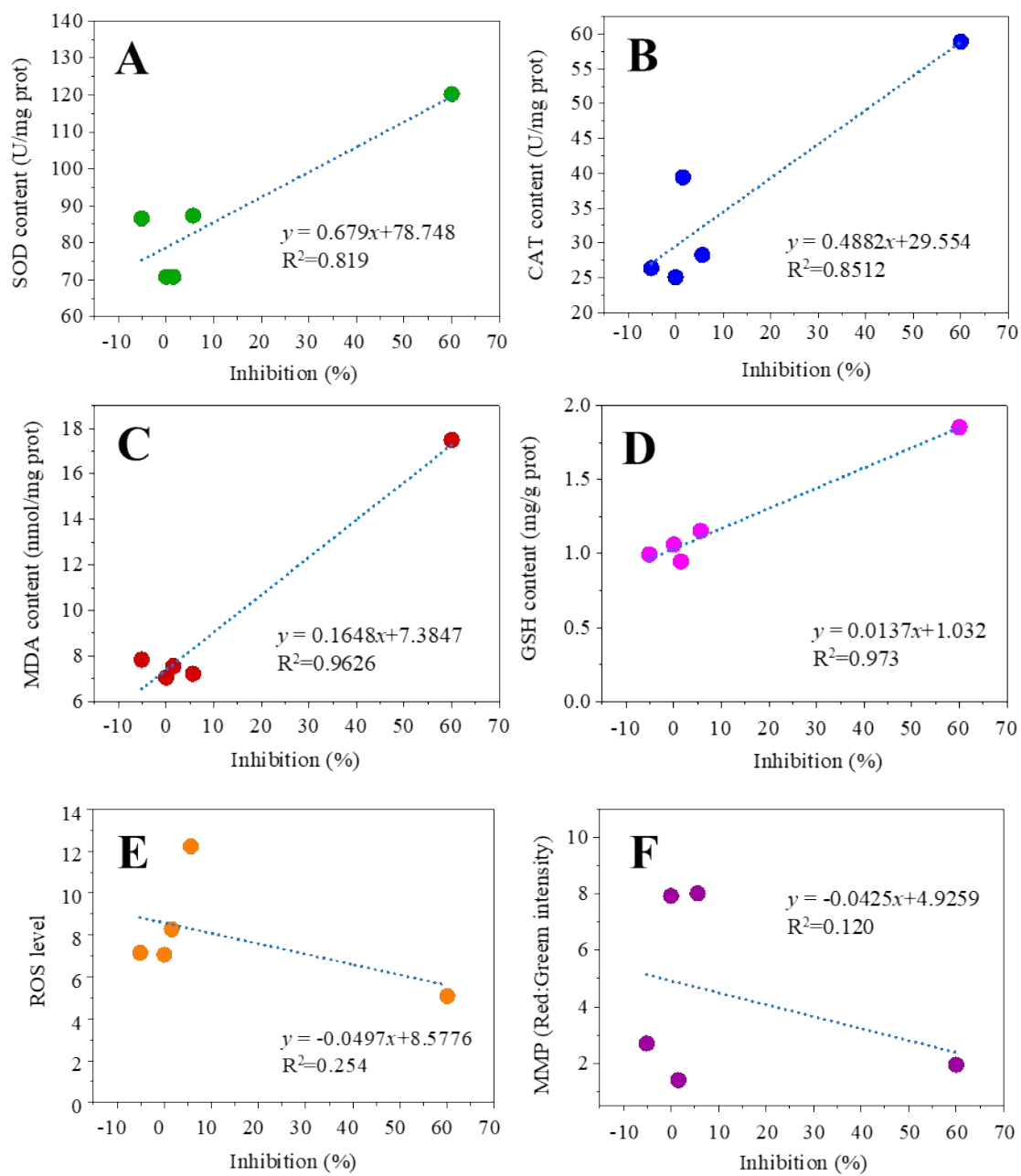


Fig. S10 The correlation between growth inhibition and SOD activity (A), CAT activity (B), MDA content (C), GSH content (D), ROS (E), and MMP (F).

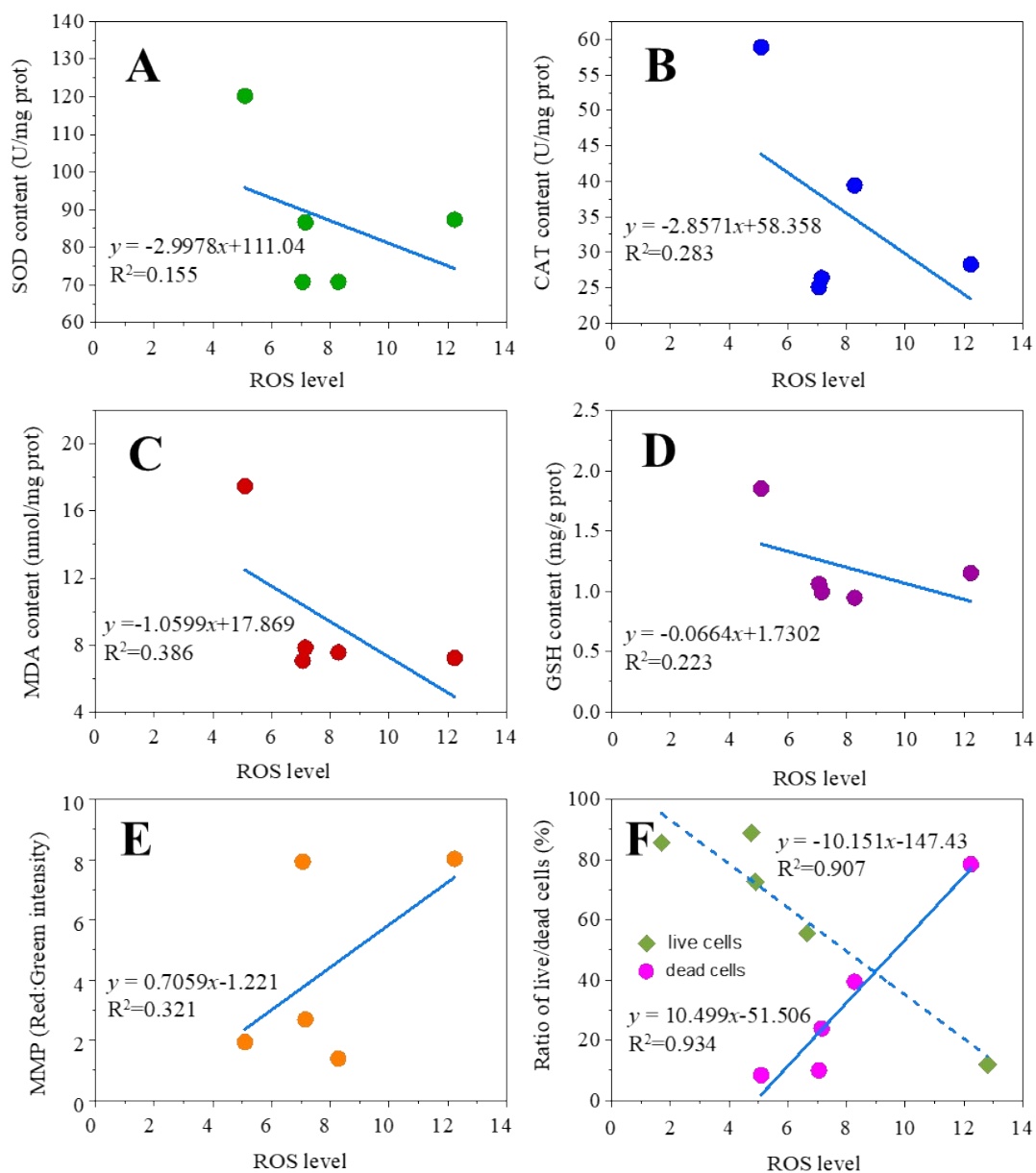


Fig. S11 The correlation between ROS level and SOD activity (A), CAT activity (B), MDA content (C), GSH content (D), MMP (E), and ratio of intact/injured cells (F).

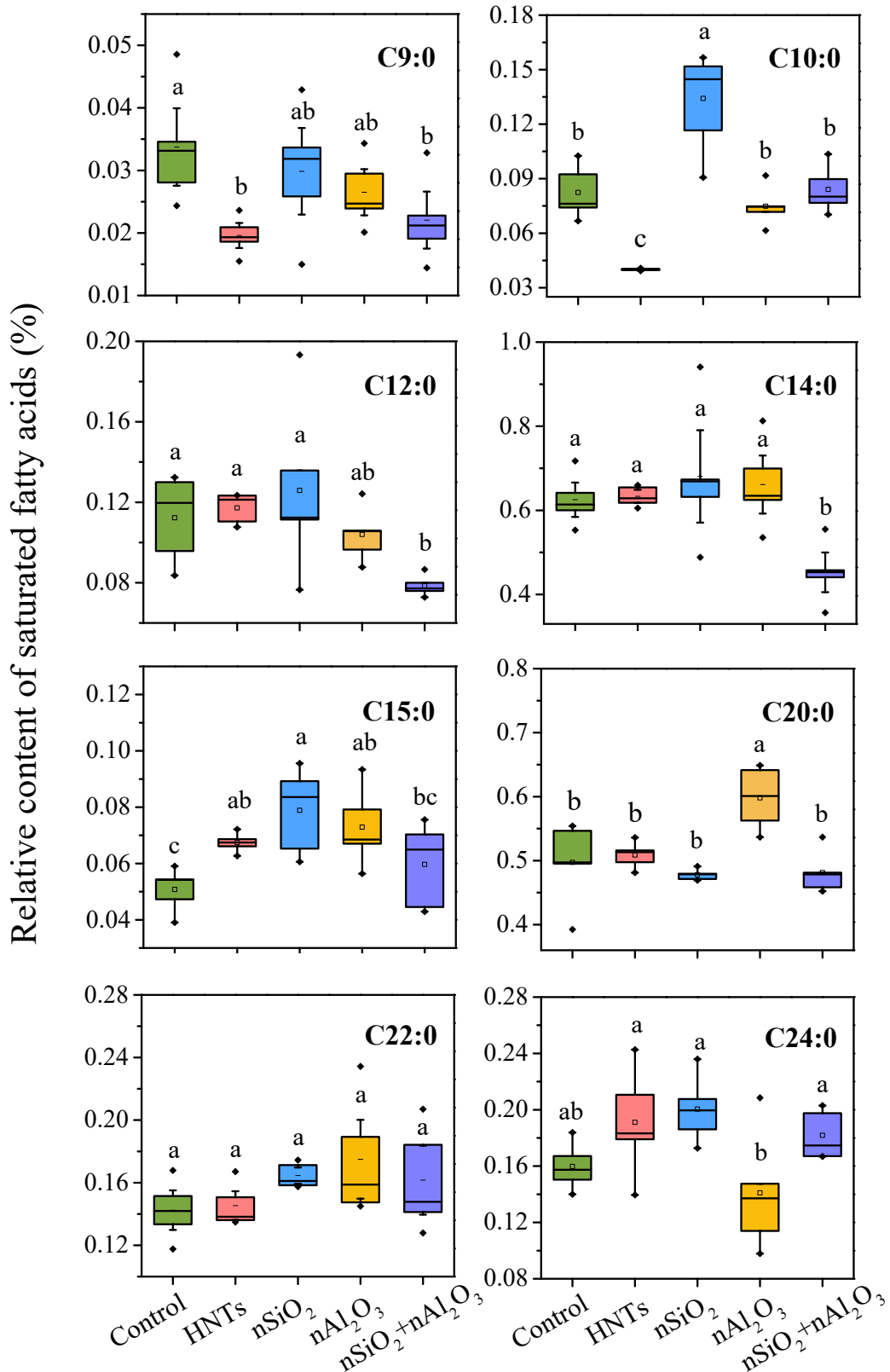


Fig. S12 The change of relative content (%) of several saturated fatty acids (SFAs) under HNTs, nSiO₂, nAl₂O₃, and nSiO₂+nAl₂O₃ exposure. Lowercase letters represent significance in relative content of SFAs among five treatments.

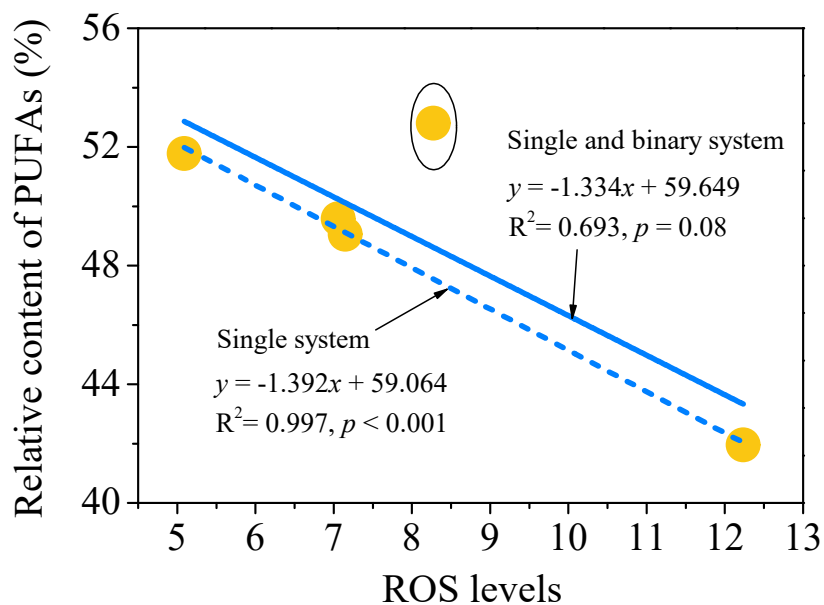


Fig. S13 The correlation between ROS and relative content of polyunsaturated fatty acids (PUFAs). The blue solid line and dash line represent linear fitting in the “single and binary” and “single” system, respectively.

Note: The difference in these two system is to fit with or without $n\text{SiO}_2+n\text{Al}_2\text{O}_3$ mixture-related data.