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SUPPORTING IFORMATION

Nanocellulose enhances the dispersion and toxicity of ZnO NPs to green algae Eremosphaera viridis

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Methods

Text S1 Preparation and characterization of ZnO/CNCs hybrid

The ZnO/CNCs hybrid was prepared by mixing ZnO and CNCs stock suspensions at a ratio of 1:1 (V/V), and the mixed suspension was shaken at 120 r/min for 48 h at 25 °C to ensure adsorption equilibrium. The crystalline nature and phase of the samples were characterized by X-ray powder diffractometer (Bruker D8 Advance, Germany) with Cu Ka radiation in the 2θ range of 5-80° at scan rate of 5° min^{-1}. Fourier transform infrared (FTIR) spectra were obtained by using an ATR-FTIR spectrometer (Bruker Optics Tensor 27, USA). Data acquisitions were done in a range between 4000 and 400 cm^{-1} with a 4 cm^{-1} nominal resolution and 64 coadded scans. For X-ray photoelectron spectroscopy (XPS) analysis, it was performed on a Thermo Scientific ESCALAB 250 Xi photoelectron spectrometer using a monochromated Al Kα (1486.6 eV) X-ray source. All recorded peaks were analyzed using Casa XPS v2.3.13 software, and the O1s peaks were deconvoluted using Gaussian–Lorentzian component profile. The UV vis absorption spectra were recorded using a spectrophotometer (Agilent Technologies, USA) in the wavelength interval between 300 and 700 nm. A field emission scanning electron microscope (FESEM) (Carl Zeiss SIMGA-300, Germany) equipped with energy dispersive spectroscopy (EDS) was used to investigate the surface morphology of the samples.

Text S2 Dissolved Zn concentration

In order to obtain the concentration of dissolved Zn^{2+}, the ZnO NP suspensions (initial concentration of 1, 5, and 10 mg /L) with and without the addition of CNC (100 mg/L), were centrifuged at 15 000 rpm for 30 min at 4 °C. The supernatant was filtered through a syringe filter with 0.22 μm pore diameter (Antop 25, Whatman), acidified with 2% nitric acid and

analyzed by ICP-MS (PerkinElmer DRC II, USA). All centrifugation parameters and the filter pore size were selected based on the hydrodynamic diameter of the NPs in the test media (see hydrodynamic diameter in Figure S8), to ensure the large ZnO NPs can't pass through the filter, while the CNC associated with dissolved Zn^{2+} can successfully get through. The release of Zn ions from different concentration of ZnO NP suspensions were tested at 1h and 72h, respectively.

Text S3 Algal dry weight and chlorophyll a/b measurement

The dry weight of biomass per volume was obtained by filtering an aliquot (15ml) of algal samples on a pre weighed 0.45 µm filter membrane. DI water was used to wash sample on the filter for 3 times to wash out chemicals attached on the cell surface. The filter membrane along with algal cells were dried for 24 hours at 60 °C and the dry weight of algal cell per volume was calculated as follows:

$$\text{g/mL} = \frac{net\ weight\ of\ membrane\ before\ and\ after\ filtration}{filtration\ volume}$$

Chlorophyll a and chlorophyll b were measured by following the approach from Xin et al[1]. Briefly, the algae were exposed to ZnO at the concentration of 1,3, and 5 mg/L with and without the presence of CNCs for 72 h. For each treatment, 8 mL of algal medium was collected and centrifuged at 4500 rpm for 15 min, after which the supernatant was discarded. Then the precipitates were washed twice with phosphate buffered saline (PBS) and the cell residues were extracted with 8 ml 90% acetone at 75 °C for 5 min. 80 µL of $MgCO_3$ solution was added to prevent the conversion of chlorophylls to phaeophytin[2]. The absorbance of the supernatants were analyzed at 664 and 647 nm by a Cary-300 double beam UV-visible spectrophotometer (Agilent Technologies, CA, USA). The concentration of chlorophyll a and chlorophyll b was calculated in accordance with the Jeffrey and Humphrey equation[3].

[Chlorophyll a] (mg/L) = 11.93E664-1.93E647

[Chlorophyll b] (mg/L) = 20.36E647-5.50E664

Text S4 Reactive oxygen species (ROS) assay

The level of intracellular reactive oxygen species (ROS) was detected using 2,7-dichlorofluoresce in diacetate (DCFH-DA) fluorescence probe (Nanjing Jiancheng Institute of Biotechnology, China). Briefly, approximately 5×10^5 algal cells were collected after centrifugation at 1000 rmp/min for 5 min and then washed with PBS. The algal cells were incubated with 10 μM DCFH-DA in the dark at 37 °C for 30 min, collected and washed with 0.01M PBS for twice. 200 μL of the supernatant was transferred to a 96 well plate and the fluorescence intensity was measured using a microplate reader (BioTek, USA) with an excitation wavelength of 485 nm and an emission wavelength of 528 nm. The cellular green fluorescence was also confirmed by fluorescence microscopy (Zeiss, Denmark).

Text S5 Catalase (CAT) activity level

The decomposition rate of hydrogen peroxide by catalase (CAT) was used to assay the enzyme activity following the manufacturer's instruction (Nanjing Jiancheng Institute of Biotechnology, China). Briefly, cells were washed in cold PBS for three times and centrifuged for 10 min at 4500 rmp. Add 1 mL PBS to resuspended algal cells, and use Q55 sonicator for cell lysis at 4 ℃ (sonication 3s, interval 3s) for 10min. After cell lysis, the samples were centrifuged at 7000 r/min for 10min, and the supernatant was collected for the enzyme activity test and following MDA content determination. A reaction mixture of 0.1 mL algae enzyme solution and 2.5 mL

detection reagent were added in a quartz cuvette. The CAT activity level was measured using spectrophotometer based on the difference in absorbance at 240 nm with a recorder for 1 min.

CAT level ($U/10^4$ cell) = $[\Delta OD_{240} \times V_{overall}/(\varepsilon \times d) \times 10^9]/(V_{sample}/V_{cell\ lysis} \times 1)/T$

ε: molar extinction coefficient of H_2O_2 ($4.36 \times 10^4 L/mol/cm$)

d: path length of quartz cuvette (1cm)

Text S6 MDA content

MDA determination was based on the reaction of MDA with a chromogenic reagent (TBA) to yield a stable chromophore with maximal absorbance at 532 nm. Briefly, the algal cell lysis solutions or standards were placed in centrifuge tubes containing the detection reagents, then the samples were incubated for 40 min at 95 °C water bath. After cooling down under the ice bath, the samples were centrifuged at 4000 r/min for 10min, and the supernatant was collected for MDA content determination. The MDA concentrations of the algae samples were calculated by measuring maximal absorbance at 532 nm using a spectrophotometer.

MDA content (nmol/mL) = $(OD_{Sample} - OD_{Blank})/(OD_{Standard} - OD_{Blank})$ × concentration of the standard (10 nmol/mL) × sample dilution ratio

Text S7 Electron microscopy observations

The surface morphology of algal cells were investigated through SEM. Algal samples with 72 h of exposure to 5mg/L ZnO or ZnO-CNC were observed. The collected cells were prefixed in 2.5% glutaraldehyde, washed three times with 0.1M PBS, and then postfixed in 1% osmium tetroxide for 1 h. Subsequently, all the samples were washed with PBS for three times and dehydrated with increasing concentrations of ethanol (30%, 50%, 70%, 80%, 90%, 95%, and 100%) for 15 mins

in each step, and then washed with tertbutyl alcohol. Finally, all samples were critical point-dried, gold-coated, and observed using SEM. For TEM sample preparation, the dehydrated algal cells were transferred to acetone for 20 mins. Next, the samples were infiltrated in an acetone and Spurr resin mixture (1:1) for 1h, then transferred to another acetone and Spurr resin mixture (1:3) for 3h. Samples were subsequently blocked in capsules containing embedding medium and polymerized at 70 °C overnight. Ultrathin sections (90nm) were cut using an ultramicrotome, and then examined under TEM.

Text S8 Synchrotron-based FTIR spectromicroscopy

A Bruker Vertex 70v interferometer coupled with a liquid-nitrogen-cooled mercury cadmium telluride (MCT) detector was used to acquire the spectra, using synchrotron-based infrared light. An Agilent Cary 670 spectrometer equipped with a focal plane array (FPA) detector was applied to capture the FTIR mapping images. An FPA detector can collect several thousand spectra within minutes and provide the distribution of chemical components over a broad spectrum. Both the spectra and spectroscopic images were acquired over the range of 4000–800 cm^{-1} in transmission mode with a resolution of 4 cm^{-1} and the co-addition of 128 scans. For sample preparation, the algal cells were carefully washed in distilled water three times to remove the attached chemicals. The biomass was resuspended in 30 μL of heavy water (D_2O) and then loaded into a sandwich sample holder. This unit was based on a sandwich of two optical CaF_2 windows spaced by a thin layer of polymeric spacer of controlled thickness.

Text S9 Results and Discussion

The toxic effects of Zn ions and ZnO NPs on the green algae *Eremosphaera viridis* were compared using different biomarkers including cell density, chlorophyll-a, and reactive oxygen species (ROS). The stock solution of ZnO NPs was diluted to obtain different concentrations of ZnO NPs at 1, 5, and 10 mg/L, respectively. The concentrations of dissoluble Zn ions in the media were measured at 72 h and shown in Table 1. For comparison, $ZnSO_4 \cdot 7H_2O$ was used as the Zn ion salt. A stock solution of 200 mg/L $ZnSO_4 \cdot 7H_2O$ was prepared in sterile deionized water. According to Table T1, algae were exposed to ZnO NPs or Zn ions, with or without the presence of CNC:

(1) Control: blank algae medium;

(2) CNC: 100 mg/L nanocellulose were added;

(3) ZnO (A): 1 mg/L ZnO NPs were added;

(4) ZnO (B): 5 mg/L ZnO NPs were added;

(5) ZnO (C): 10 mg/L ZnO NPs were added;

(6) ZnO (A)-CNC: 1 mg/L ZnO NPs and 100 mg/L CNC were added;

(7) ZnO (B)-CNC: 1 mg/L ZnO NPs and 100 mg/L CNC were added;

(8) ZnO (C)-CNC: 1 mg/L ZnO NPs and 100 mg/L CNC were added;

(9) Zn ions (A): 0.82 mg/L Zn ions were added;

(10) Zn ions (B): 1.8 mg/L Zn ions were added;

(11) Zn ions (C): 2.2 mg/L Zn ions were added;

(12) Zn ions (A)-CNC: 0.82 mg/L Zn ions and 100 mg/L CNC were added;

(13) Zn ions (B)-CNC: 1.8 mg/L Zn ions and 100 mg/L CNC were added;

(14) Zn ions (C)-CNC: 2.2 mg/L Zn ions and 100 mg/L CNC were added.

Table T1. The dissolution of ZnO NPs

Treatment	ZnO NPs (mg/L)	Zn ions (mg/L)
A	1	0.82
B	5	1.8
C	10	2.2

1.1. Cell density and dry weight

Cell density was used to assess algal growth, which was determined by flow cytometer. The relative inhibition rate (IR) of algal growth was calculated as follows. T and C refer to the cell density in the treatments and the control, respectively.

IR (%)= (1-T/C)*100%

As shown in Table T2, the Zn ions or ZnO NPs at higher concentration led to a higher grow inhibition than at lower concentrations after 72h of exposure. The effects of Zn ions and ZnO NPs did not differ significantly (4%-6% difference), suggesting that dissoluble Zn ions mostly account for the impact of ZnO NPs on algal growth inhibition, which was consistent with many previous reports. The addition of CNC dramatically reduced the cell density of the algal cells, especially for the ZnO-CNC groups. Compared with Zn^{2+}-CNC groups, ZnO-CNC treatment had the strongest inhibition on algal growth, which might attributed to enhanced dissolution of Zn^{2+} from ZnO NPs. The adsorption between Zn ions and CNC exerted higher toxicity than pure Zn ions. This indicated that a higher Zn ions contact possibility with algae cells might occur with the addition of CNC. A similar trend was observed in the test of dry weight, as shown in Figure T1a. The contribution of Zn ions and ZnO NPs on the algal dry weight had negligible difference, suggesting that ZnO NPs is mostly attributed to released Zn ions.

Table T2. Cell density by flow cytometry

	Control	**ZnO**			**Zn^{2+}**		
		1 mg/L	5 mg/L	10 mg/L	0.82 mg/L	1.8 mg/L	2.2 mg/L
Cell/µL	44.0	32.0 (IR=27%)	21.3 (IR=52%)	7.7 (IR=83%)	34.7 (IR=21%)	23.3 (IR=47%)	9.3 (IR=79%)
	CNC	**ZnO-CNC**			**Zn^{2+}-CNC**		
		1 mg/L	5 mg/L	10 mg/L	0.82 mg/L	1.8 mg/L	2.2 mg/L
Cell/µL	42.2 (IR=4%)	27.8 (IR=37%)	6.8 (IR=85%)	1.3 (IR=97%)	31.8 (IR=28%)	18.2 (IR=59%)	7.0 (IR=84%)

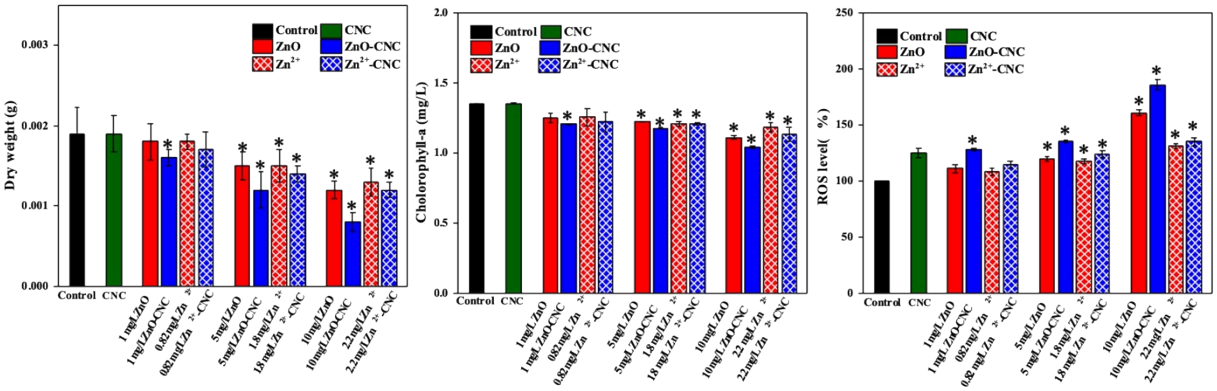

Figure T1. Algal toxicity of ZnO, ZnO-CNC, Zn^{2+}, and Zn^{2+}-CNC at 72 h. (a) Dry weight; (b) chlorophyll a content; (c) relative ROS level. The "*" symbols denote p-values <0.05 compared to the control.

1.2. Chlorophyll a content and ROS generation

In Figure T1b, chlorophyll a content in algae was influenced by ZnO NPs and Zn ions at higher concentration, presumable because Zn ions inhibited the reductive steps in the biosynthetic pathway of chlorophyll a. It was noted that the reduction of chlorophyll a was not as significant as the cell density. This may due to the fact that chlorophyll a plays important role in the inhibition of lipid peroxidation. This defense mechanism made them more active under the toxic stress. However, the overall inhibition trend was almost same as the cell density, where the toxicity of ZnO NPs was dependent on the released Zn ions. In addition to the dissolved metal ions, ROS generation was proposed to be a general paradigm for the toxic action of metal oxide

NPs. As shown in Figure T1c, 5 mg/L and 10 mg/L ZnO NPs generated extremely significantly higher ROS than the control group. Comparably, the free Zn ions induced less oxidative stress, suggesting that the toxic effect of ZnO NPs was not only dependent on the dissolved Zn ions but also particle-related. The ZnO NPs may adhere on the surface of the algal cell wall and generate the extracellular ROS. The presence of CNC could promote dissolution of ZnO NPs on the cell wall, resulting shift of particle-related toxic effect to ion-related toxic effect.

In conclusion, the findings showed that dissolved Zn ions were the important contributors to ZnO NPs toxicity. The toxic effect of ZnO NPs to algal cells was slightly higher than Zn ions, probably due to the mechanical damage of the cell walls by the nano particles. CNC could adsorb the Zn ions and induce higher toxicity as compared to pure Zn ions. This indicated that CNC might enhance the bioavailability of Zn ions to algal cells, leading to growth inhibition, affected photosynthesis and oxidative damage. The ZnO-CNC treatment exhibited the most severe toxicity in all groups. This could be explained by the presence of CNC promoting dissolution of ZnO NPs on the cell wall, resulting shift of particle-related toxic effect to ion-related toxic effect.

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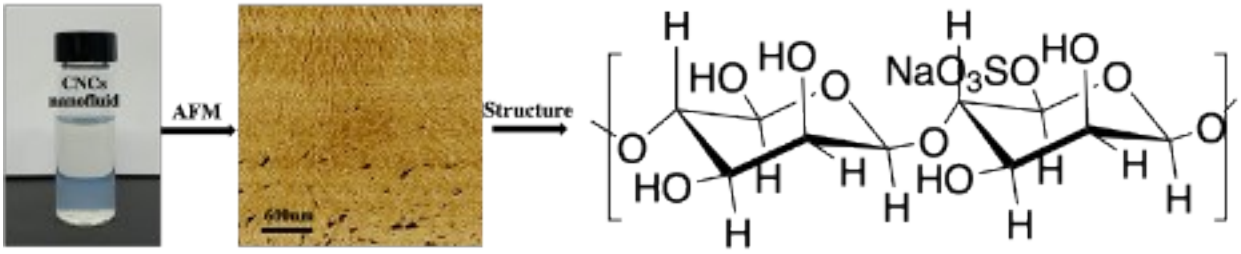


Figure S1. Atomic force microscopy images and chemical structure of CNCs nanofluid.

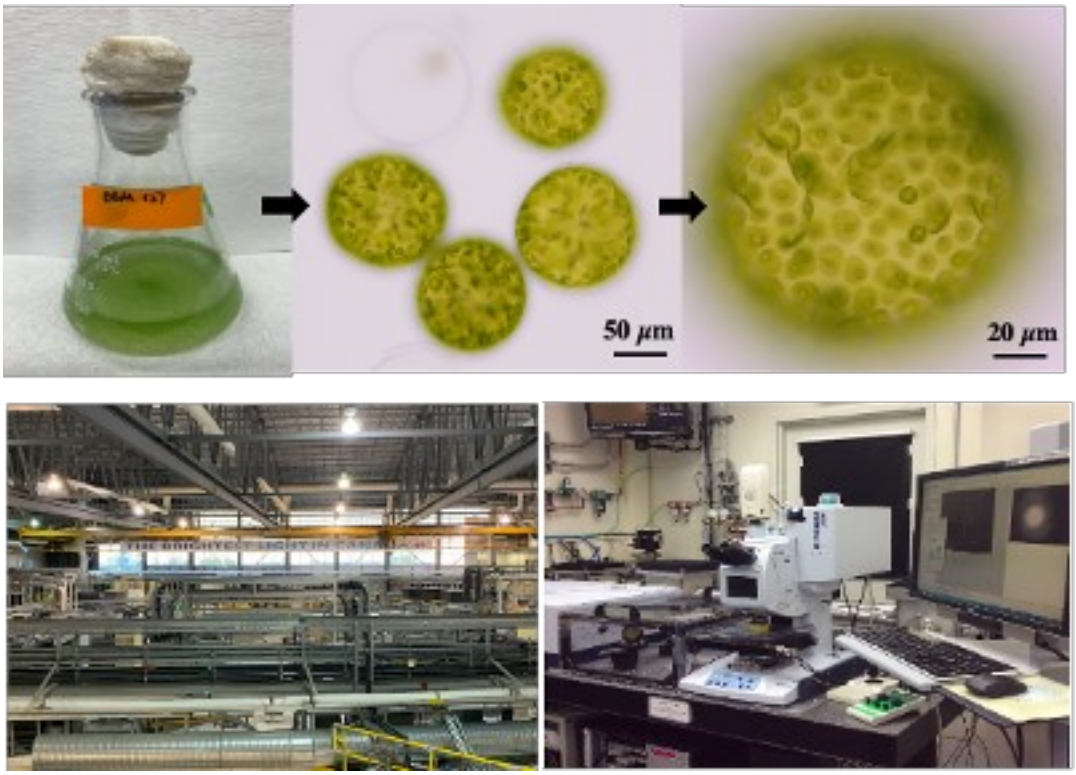


Figure S2. (a) The microscopic image of algae *Eremosphaera viridis* at different magnifications; (b) The Canadian light source and Bruker Vertex 70v Interferometer/ Hyperion 3000 IR Microscope.

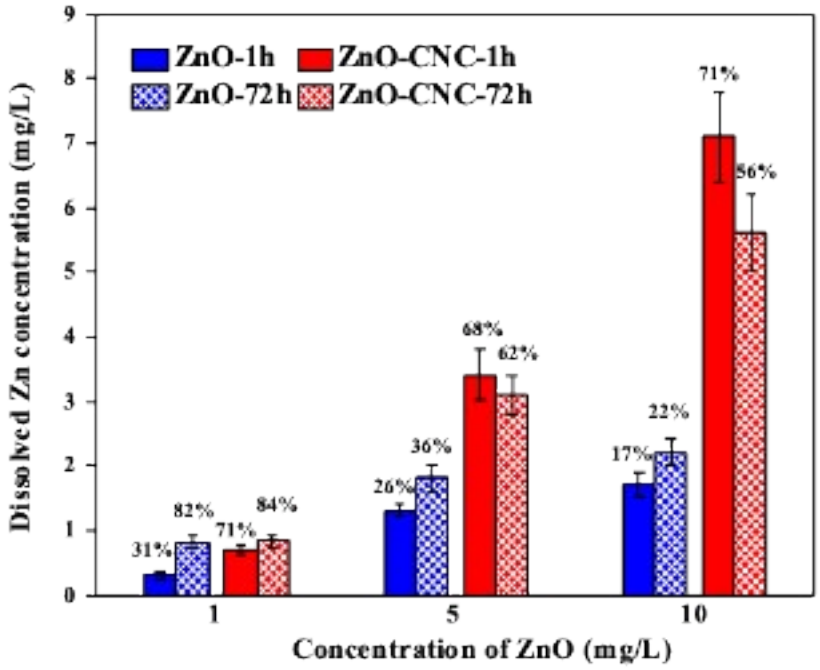


Figure S3. Dissolved zinc ions released from ZnO NPs at three initial concentrations over time.

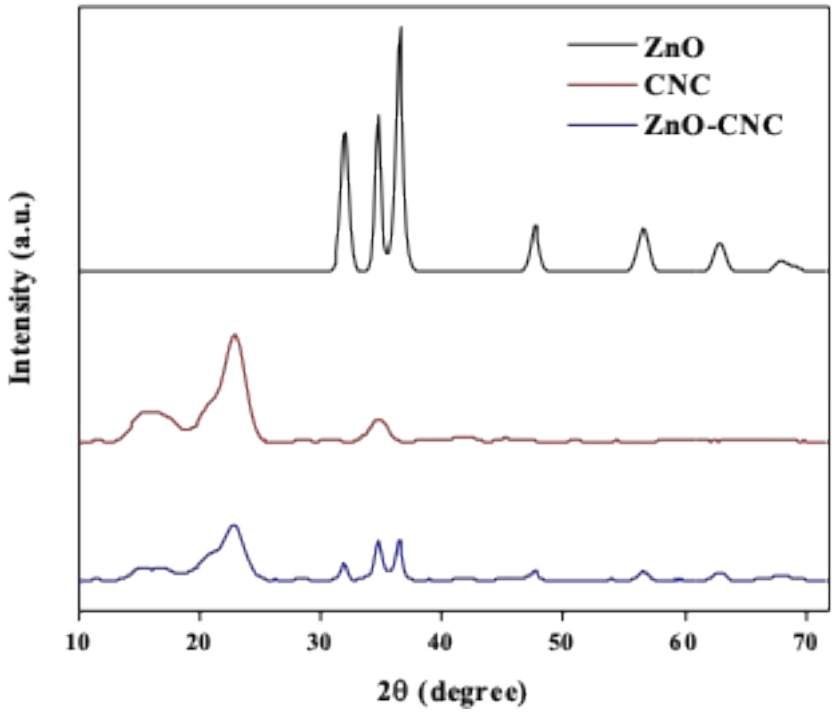
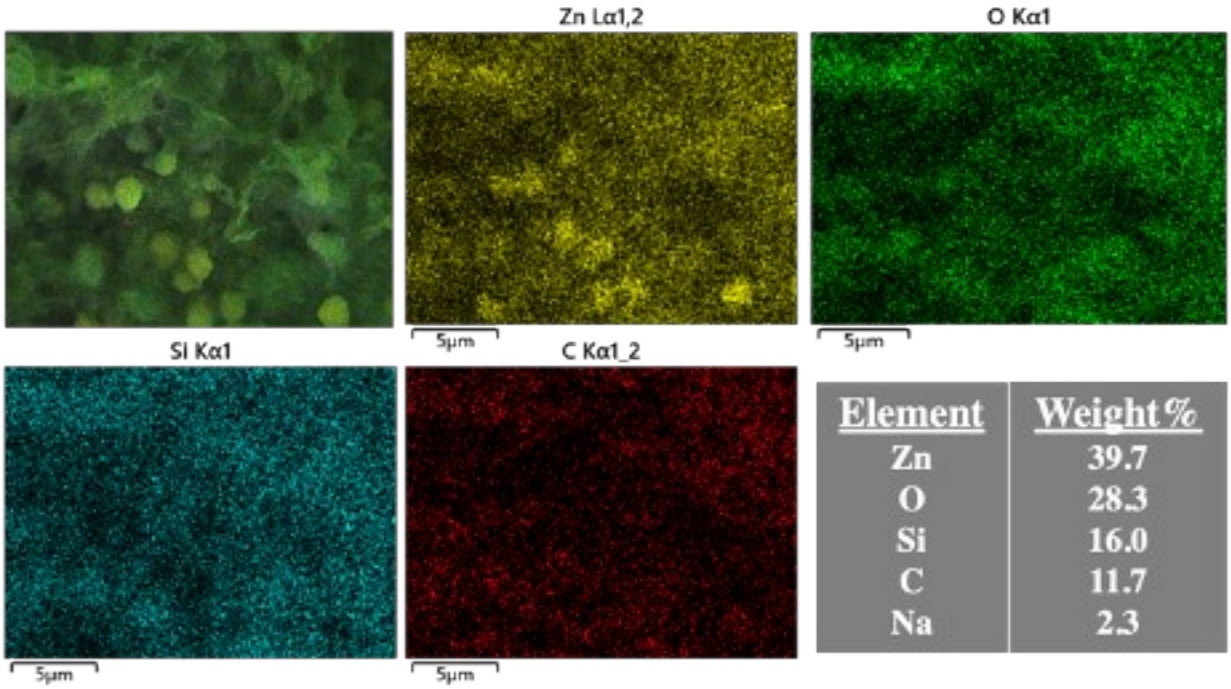


Figure S4. XRD pattern of ZnO, CNC, and ZnO-CNC.



Element	Weight%
Zn	39.7
O	28.3
Si	16.0
C	11.7
Na	2.3

Figure S5. SEM-EDS analysis of ZnO-CNC nanocomposite.

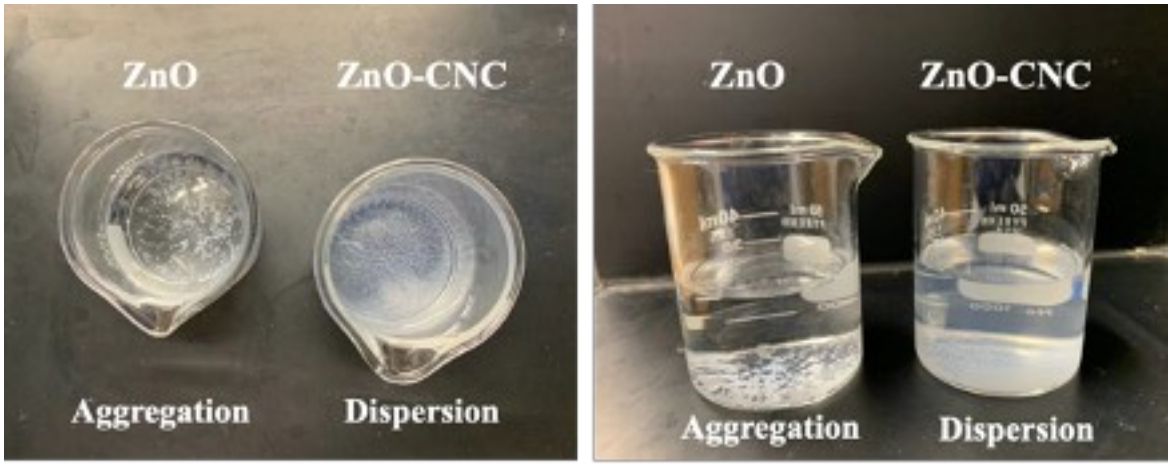


Figure S6. Top and side view of ZnO and ZnO-CNC suspension.

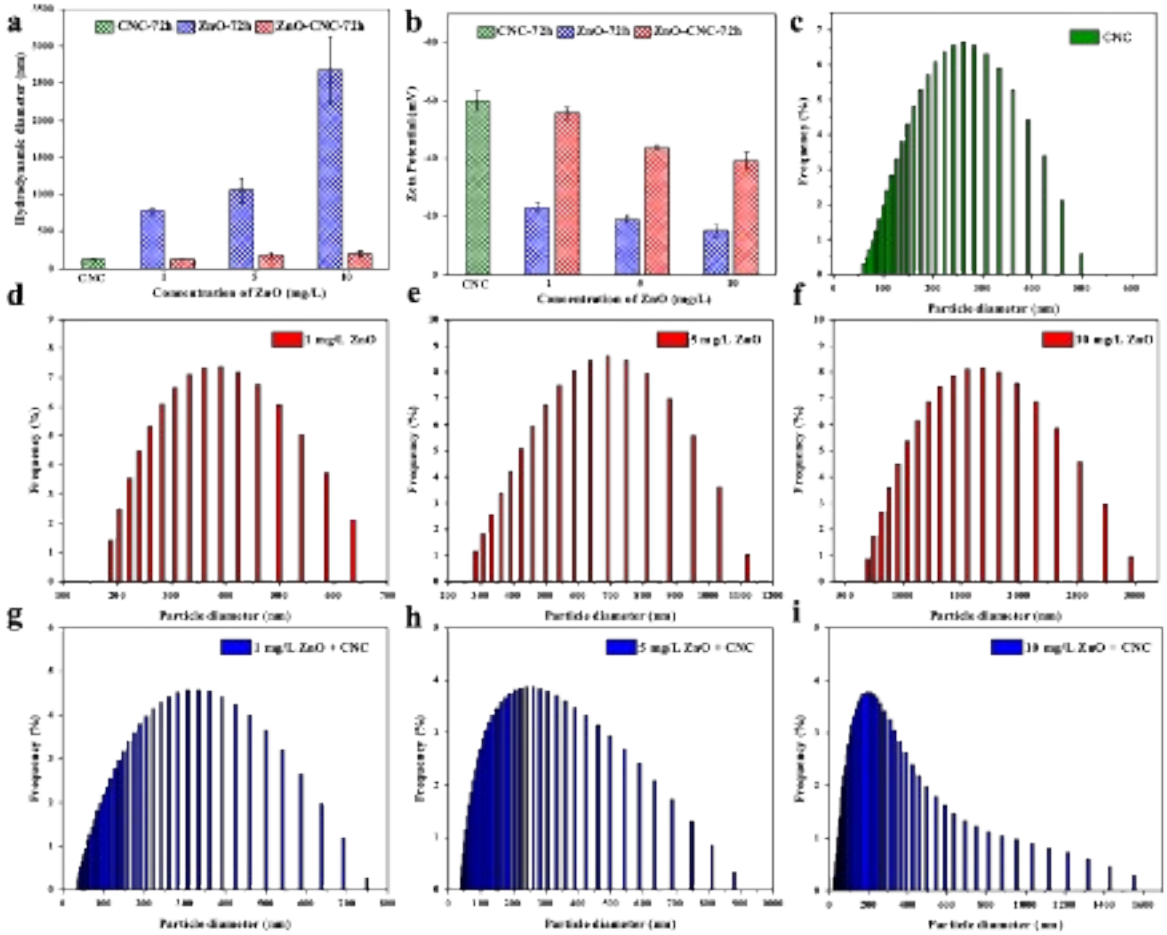


Figure S7. (a) Hydrodynamic diameter; (b) zeta potential; (c-i) size distribution of CNC, ZnO and ZnO-CNC in the pure water at 72h.

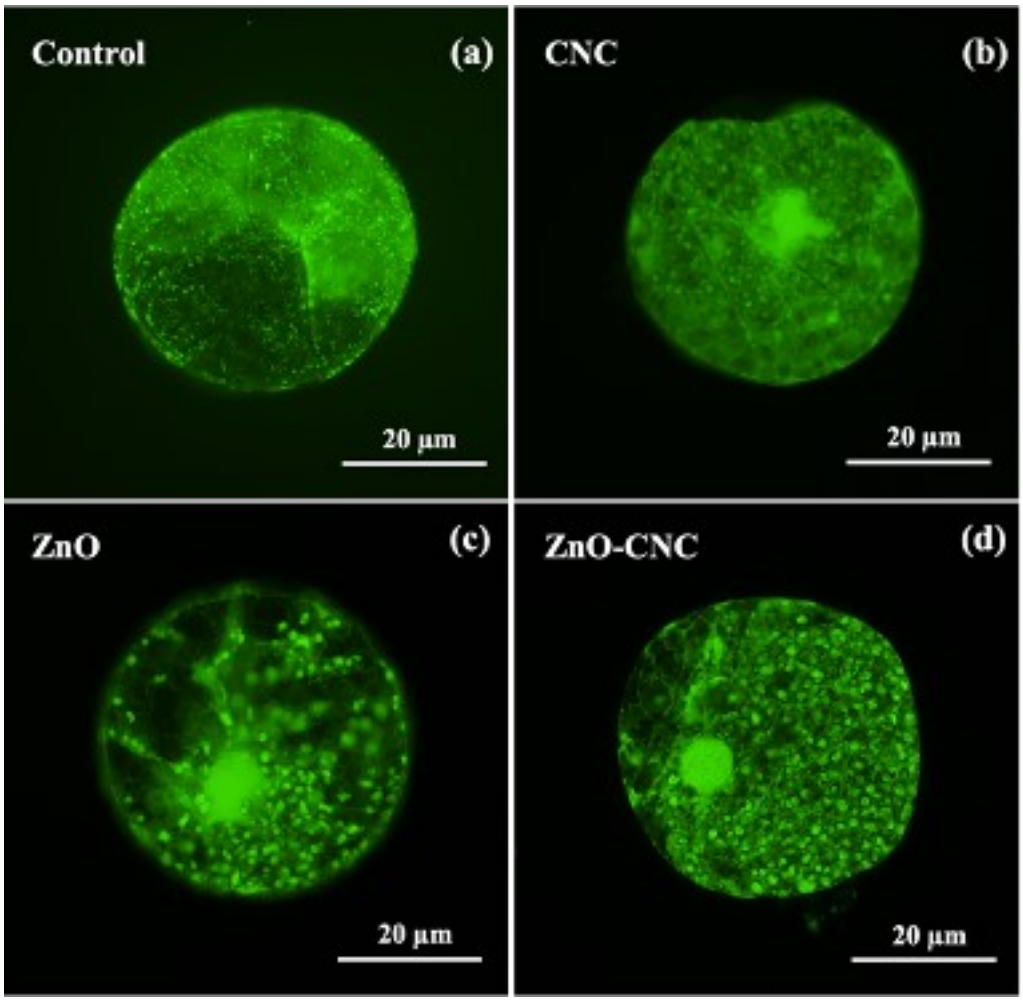


Figure S8. Algal cell stained with DCFH-DA dye after 72 h exposure to (a) control; (b) 100 mg/L CNC; (c) 5mg/L ZnO; (d) 5mg/L ZnO-CNC.

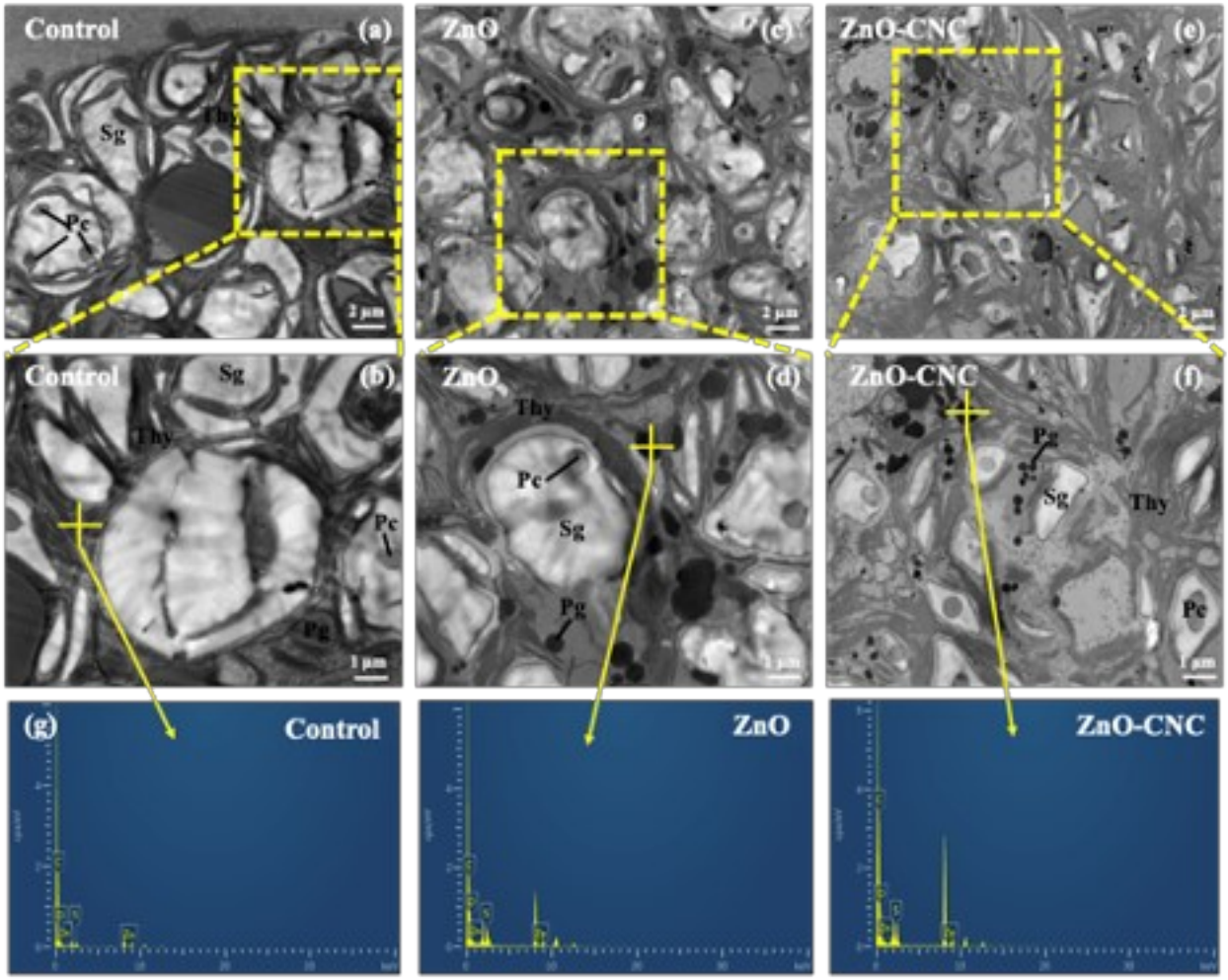


Figure S9. The algal ultrastructure and Zn uptake. TEM images of the algae in the absence of nanoparticles (a-b) and presence of 10 mg/L ZnO (c-d), 10 mg/L ZnO-CNC (e-f), and EDS spectra (g). Abbreviation: plastoglobule (Pg), starch granule (Sg), thylakoid (Thy), and pyrenoid center (Pc).

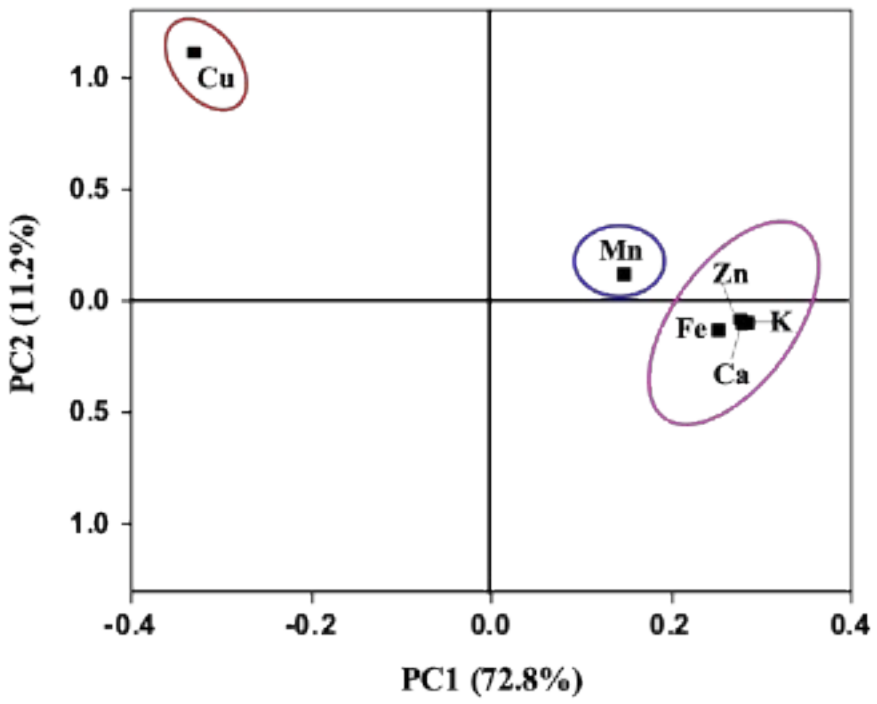


Figure S10. Principal component analysis (PCA) score plot of six elements in XRF mapping.

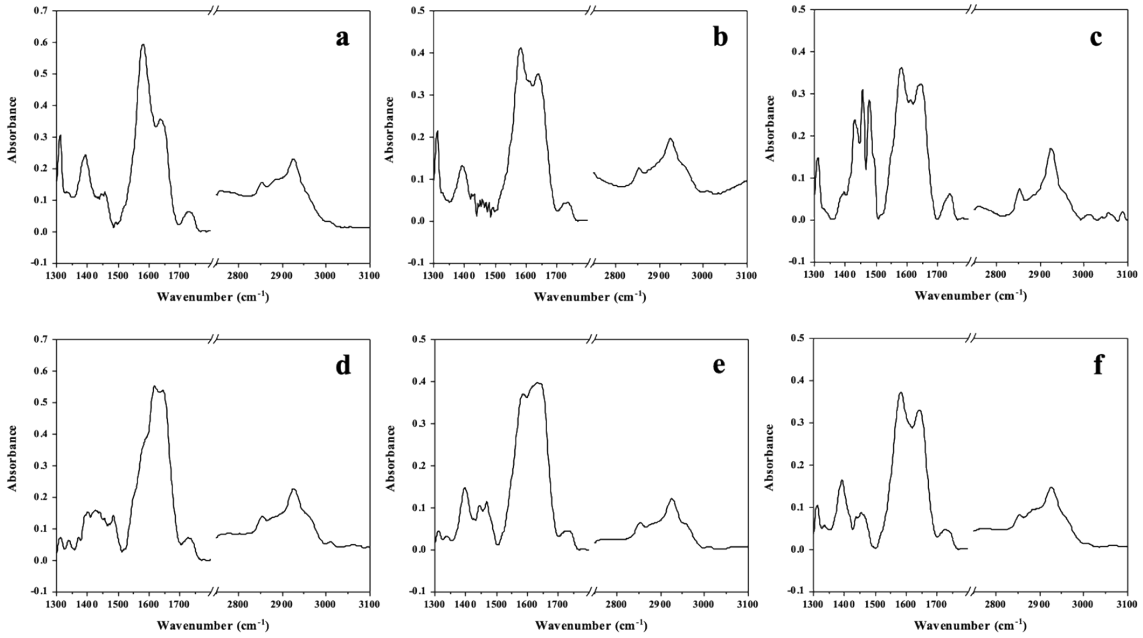


Figure S11. SR-FTIR spectra of microalgae *E. viridis* under the exposure of (a) 1 mg/L ZnO; (b) 5 mg/L ZnO; (c) 10 mg/L ZnO; (d) 1 mg/L ZnO-CNC; (e) 5 mg/L ZnO-CNC; (f) 10 mg/L ZnO-CNC.

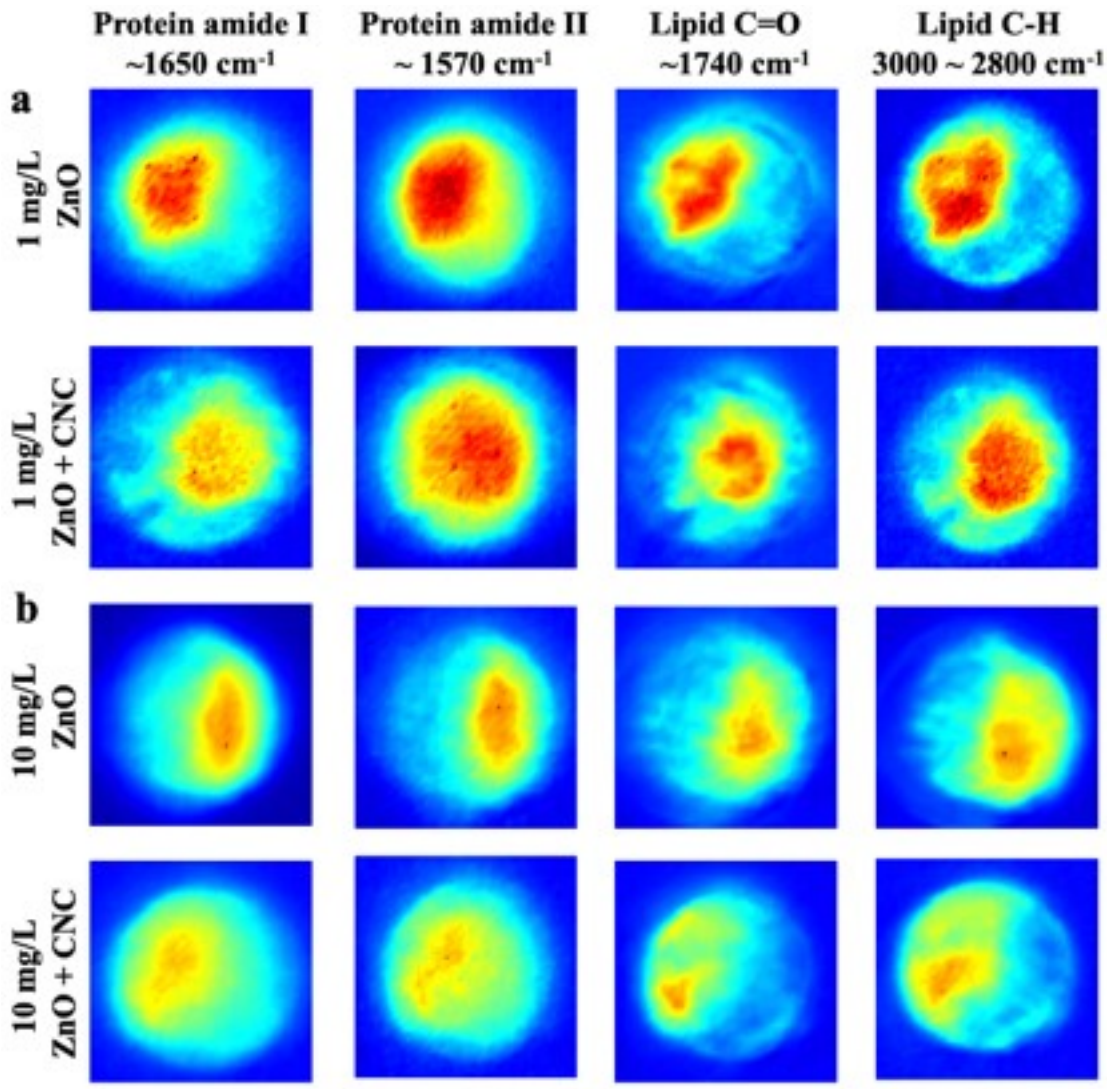


Figure S12. FTIR mapping of protein and lipids in algal cell under exposure to (a) 1 mg/L ZnO and co-exposure to 1 mg/L ZnO and CNC; (b) 10 mg/L ZnO and co-exposure to 10 mg/L ZnO and CNC.

List of Tables

Table S1. Elemental assay of ZnO, CNC and ZnO-CNC.

Name	Binding energy (eV)			Atomic (%)		
	ZnO	CNC	ZnO-CNC	ZnO	CNC	ZnO-CNC
C 1s	-	285.81	286.33	-	62.71	47.74
O 1s	530.65	532.50	532.04	44.52	36.09	45.7
Zn 2p	1021.13	-	1021.78	55.48	-	5.01
Na 1s	-	1071.1	1072.25	-	1.2	1.55