Supplementary Materials

Humic acid can mitigate or magnify nanoplastic toxicity to freshwater microalgae: What are the factors driving these contrasting effects?

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Text S1. Characterization and preparation of stock nanoplastic suspensions

The morphology of nanoplastics (NPs) was examined using a field emission scanning electron microscope (FESEM, SUPRA 55VP-4132, Carl Zeiss, Germany) and a transmission electron microscope (TEM), equipped with an ultra-high-resolution field emission gun (JEOL, TEM 2100 F) and operated at 200 kV. Fourier transform infrared (FTIR) spectrometer (Nicolet iS5 FTIR, Thermo-Fisher Scientific, USA) was used to identify the chemical functional groups of NPs. Additionally, the hydrodynamic diameters and zeta potentials of NPs were evaluated in deionized water and BG11 medium using dynamic light scattering (DLS, Zetasizer Nano ZS90, Malvern Analytical Ltd., UK).

Text S2. Adsorption of humic acid onto nanoplastics

The physicochemical interaction between HA and NPs was determined by adsorption experiments, where 10 mg L^{-1} and 25 mg L^{-1} of HA solution and NP suspension were added to 25 mL of BG11 medium in a glass vial. These concentrations were chosen based on the results of a preliminary toxicity experiment (data not shown). The pH value of the solution was adjusted to 7.0 using 0.1 M HCl or 0.1 M NaOH. The vials were then shaken at 25 °C and 150 rpm for 24 h. After solution transfer to centrifuge tube and centrifugation at 10000 rpm for 30 min, the HA concentration in the supernatants was determined at 254 nm using a UV-vis spectrometer (Evolution 201, Thermo Fisher Scientific, USA). The remaining samples were freeze-dried using a freeze dryer (BUCHI Lyovapor L-200, Singapore), and analysed using FTIR and SEM to determine chemical bonds, morphology and cellular surface alterations.

Text S3. Analysis of metabolic activity

Esterase activity is associated with cell metabolism and is commonly used to evaluate the growth status of microalgae. To determine esterase activity, a fluorescein diacetate (FDA) probe was used. When taken up by viable cells, FDA is converted into fluorescein that can be measured using a fluorescence reader. To perform the procedure, 1 mL of algal suspensions were centrifuged at 3000 rpm for 10 min, and washed three times with BG-11 medium. Then, the microalgae were incubated with 1 μM FDA in the dark at room temperature for 30 min, centrifuged again and washed three times with BG-11 medium. Finally, the fluorescence intensity was measured using a

fluorescence spectrometer (Edinburgh FS5 Spectrofluorometer, UK), with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.[1]

Text S4. Analysis of oxidative stress and antioxidant response

The cellular reactive oxygen species (ROS) level of microalgae was measured using the oxidant-sensing fluorescent probe, with 2′,7′-dichlorofluorescein diacetate (DCFH-DA) as the dye. Briefly, after exposure to the experimental conditions for 96 h, the microalgae were centrifuged and washed three times with PBS. The cell culture was then stained with 10 μM DCFH-DA in the dark at 37 °C for 30 min. The stained algal cells were washed three times with PBS, centrifuged and resuspended in PBS. The level of ROS was then analysed using a fluorescence spectrometer (Edinburgh FS5 Spectrofluorometer, UK), with an excitation wavelength of 488 nm and an emission wavelength of 525 nm based on DCF detection. The relative ROS level was calculated based on the fluorescence intensity (FI) using the following equation:

$$\text{Relative ROS level (\%)} = \text{DCF FI ((treatment))/ FI (control)} \times 100$$

The MDA content was measured by the thiobarbituric acid-reactive substances (TBARS) assay.[2] Briefly, 10 mL of an algae sample was centrifuged (5000 rpm, 25 °C, 15 min) and the supernatant was discarded. Then, 5 mL of 10% trichloroacetic acid solution was added, followed by ultrasonication for 15 min (100 W, 40 kHz, 25 °C) and centrifugation for 15 min (4000 rpm, 25 °C). 2 mL of the supernatant was added into the colorimetric tube, followed by adding 2 mL of 0.6% thiobarbituric acid (dissolved in 10% trichloroacetic acid solution). The mixture was incubated in a water bath at 95 °C for 30 min and then immediately cooled in an ice bath. The absorbance of the supernatant was measured at 450 nm, 532 nm and 600 nm after centrifugation for 15 min (4000 rpm, 25 °C). The MDA content was calculated using the following equation.

$$CMDA = 6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$$

To assess the toxicity of NPs, HA and their combination on microalgae, hydrogen peroxide (H_2O_2) content was analysed as per Patterson et al. (1984).[3] 2 mL of an algae sample was collected in Eppendorf tubes and centrifuged at 5000 rpm for 15 min. The supernatant was discarded, and 2 mL of 0.1% w/v trichloroacetic acid (TCA) solution was then added and mixed thoroughly. The mixture was centrifuged again at 10000 rpm for 10 min. Subsequently, 0.5 mL of the supernatant was taken and mixed with 0.5 mL of 10 mM PBS (pH 7.0) and 1 mL of 1 M potassium iodide (KI).

The absorbance was measured at 470 nm, and a calibration curve was formulated using H_2O_2 as a standard with the following equation:

$$Concentration\ of\ H_2O_2\ =\ 0.128 + (3.880 \times Absorbance)$$

SOD activity was measured by the use of xanthine/xanthine oxidase as the superoxide ($O_2 \cdot -$) generator and nitro blue tetrazolium (NBT) as the detector.[4] Sodium carbonate working solution (50 mM, 0.1 mM xanthine, 0.025 mM NBT, 0.1 mM EDTA), xanthine oxidase (0.1 μM mL^{-1} in 2 M ammonium sulfate) and treatment sample/blank (potassium phosphate buffer) were mixed in a 96-well microplate. The change in absorbance was measured every 30 s over 5 min at 560 nm using the BioTek Epoch 2 Microplate Reader (Agilent, USA). One unit of SOD activity is defined as the amount of enzyme needed to inhibit the reaction of $O_2 \cdot -$ with NBT by 50%. Enzymatic activity was expressed in U mg^{-1} protein.

Catalase activity was assayed according to the method of Cohen et al. (1970).[5] The reaction mixture in a final volume of 3 mL contained 100 mL of an algae sample with 1.90 mL of potassium phosphate buffer (50 mM, pH 7.0). The enzyme reaction was initiated by the addition of 1.0 mL of hydrogen peroxide solution. The absorbance at 240 nm was determined for 3 min at an interval of 30 s. One enzyme unit is defined as the amount of enzyme decomposing 1 mM H_2O_2 per min at 25 °C.

Reduced glutathione activity was determined by the method of Jollow et al. (1974),[6] which is based on the reaction with 5,5'-dithiobis- (2-nitrobenzoic acid) (DTNB or Ellman's reagent) to give a yellow compound that absorbs light at 412 nm. The samples (0.1 mL algal substances) were precipitated with 5% trichloroacetic acid (TCA) and the precipitate was removed by centrifugation at 12000 rpm at 4 °C for 15 min. The supernatant (0.4 mL) was taken and mixed with 2.2 mL of potassium phosphate buffer (0.1 M, pH 7.4). The reaction was initiated by the addition of 0.4 mL DTNB (5,5′-dithiobis-2-nitrobenzoic acid) and the absorbance at 412 nm measured within 30 s of reaction initiation against a blank containing TCA instead of sample. The amount of glutathione was expressed as U mg^{-1} protein.

Text S5. Analysis of membrane damage

The mitochondrial membrane potential (MMP) of microalgae after experimental exposure was assessed using the TMRE MMP assay kit (Sigma Aldrich, USA).[7] To do so, the microalgae were collected through centrifugation at 12000 rpm for 10 min, and then washed with assay buffer three times. The precipitated cells were resuspended in 500 μL of BG11 medium. MMP was assessed using a fluorescent dye, tetramethylrhodamine ethyl ester (TMRE), which was incorporated into aliquots at the concentration approximately between 1×10^5 and 5×10^5 cells L^{-1}. The algal cells were stained with TMRE dye to the final concentration of 200 nM at 37 °C for 30 min. After staining, the cells were washed three times with assay buffer to remove excess dye. Finally, 100 μL of assay buffer was added and the fluorescence was measured using a fluorescence spectrometer (Edinburgh FS5 Spectrofluorometer, UK), with excitation and emission wavelengths of 549 nm and 575 nm, respectively (Xin et al., 2020).

The membrane integrity was measured according to the cellular fluorescence stained with propidium iodide using a flow cytometer (BD FACS Calibur, USA). After 96 h exposure, the microalgae were collected by centrifugation (5000 rpm, 10 min), washed with PBS (10 mM, pH = 7.0) for three times and then stained with propidium iodide (10 mg L^{-1}) in the dark for 20 min. Propidium iodide, a fluorescent dye, can pass through the damaged cell membrane and integrate with nucleic acids to produce red fluorescence (535 nm excitation, 615 nm emission).[8] The fluorescence intensity of the damaged algal cells was determined with PE channel (585/42 nm). At least 10000 algal cells were measured per sample and the data were analysed with software FlowJo V10 (FlowJo LLC, Ashland, OR, USA). Also, the visualised membrane integrity of algal cells was stained with a fluorescent probe of propidium iodide (PI, 100 μg mL^{-1}, Sigma). The stained cells were also collected and placed on a microscope slide, and then observed under a fluorescent microscope after 10 min of dark incubation (Nikon ECLIPSE 50i, Tokyo, Japan) (Hu et al., 2020).

Text S6. Morphological observation and uptake of nanoplastics by microalgae

A scanning electron microscope (FESEM, SUPRA 55VP-4132, Carl Zeiss, Germany) was used to investigate the interaction of microalgae with NPs and HA on the cell surface after exposure for 96 h. The algal suspension was collected and centrifuged at 14000 rpm for 15 min. The

supernatant was washed three times with PBS at pH 7.2, fixed with glutaraldehyde for 24 h and rewashed three times with PBS. The microalgae were then fixed in 1% osmium tetroxide for 1 h and washed three times again with PBS. Ethanol solution of varying concentrations (30%, 50%, 70%, 80% and 90%) was used for dehydration for 15 min, and the samples were completely dried in an automatic critical point dryer (Leica EM CPD300, Germany). The algal cells were evenly spread on a single sheet and attached to a conductive adhesive coated in gold for observation under SEM at 5 kV.[9]

Fourier Transform Infrared (FTIR) analysis was conducted to assess the changes in the surface biomacromolecules of microalgae after exposure for 96 h. To obtain a more distinct effect, a higher concentration of the test materials was used when exposing the microalgae for FTIR characterization. The microalgae for control, 25 mg L^{-1} NPs, 10 mg L^{-1} HA in isolation and their combination were tested, following the previous method with minor modifications.[10] Briefly, the algal cells were collected via centrifugation, washed twice with PBS and freeze-dried. The freeze-dried cells were then blended with potassium bromide at a ratio of 1:100 and compressed into tablets for functional group analysis. FTIR spectra (Nicolet iS5 FTIR, Thermo-Fisher Scientific, USA) ranging from 400 to 4000 cm^{-1} with a resolution of 2 cm^{-1} were obtained.

To analyse the interaction between test materials and algal cells, the zeta potentials of NPs with and without HA in BG-11 medium and algal suspensions (pH = 7.0) were measured at 0 h and 96 h by dynamic light scattering using a Zetasizer Nano (Zetasizer Nano ZS90, Malvern Panalytical Ltd., UK).

To quantify the accumulation of NPs in microalgae after 96 h exposure, the alkaline lysis method was used.[11] Briefly, 2 mL of algal suspension was centrifuged at 14000 rpm at 4 °C for 10 min and the supernatant was then removed. The cells were lysed by vortexing them at 300 rpm for 24 h with an addition of 1 mL of 10 M NaOH. The mixture was then filtered using 0.2 μm hydrophilic polycarbonate membranes (GTTP04700, Merck Millipore Ltd., Switzerland) and the resulting solution was incubated in a water bath at 80 °C for 30 min with 10 mL of 100% ethanol. After centrifugation at 2500 rpm for 10 min, the bottom liquid obtained after centrifugation at 2500 rpm for 10 min was subject to UV-vis spectrophotometer (Evolution 201, Thermo-fisher scientific) at a wavelength of 260 nm, which showed a high degree of absorbance by NPs. Standard curves were used to calculate the concentrations of NPs using the following formula:

$$\mathrm{Abs}_{\mathrm{absorbed}} = \left(\mathrm{Abs}_{\mathrm{NPs+Algae}} - \mathrm{Abs}_{\mathrm{Algae}}\right) - \mathrm{Abs}_{\mathrm{Filtered}}$$

where $\mathrm{Abs}_{\mathrm{Filtered}}$ and $\mathrm{Abs}_{\mathrm{NPs+Algae}}$ are the absorbance of the microalgae-NP bead solution before and after filtration, respectively; $\mathrm{Abs}_{\mathrm{Algae}}$ is the absorbance of the microalgae alone.

Text S7. Data analysis

(1) IA model calculation for combined toxicity assessment:

The independent action (IA) was used to assess the combined effects of NPs and HA by examining several indicators, including SOD, CAT, GST activities, GSH, MDA, ROS, H_2O_2 levels, cell membrane integrity and mitochondrial membrane damage. IA model equation[12,13] was used to calculate the expected IR:

$$E(C_{mix}) = 1 - \prod_{i=1}^{n} (1 - E(C_i))$$

where C_i represents the concentration of the ith component in the mixture, while $E(C_{mix})$ denotes the anticipated IR for the entire mixture. $E(C_i)$ corresponds to the IR observed for each individual component in separate toxicity assessments.

The interaction type in a mixture is determined by comparing the observed and predicted effects, and is classified into three types:

- Synergistic: The observed mixture effect is significantly greater than the predicted effect.
- Antagonistic: The observed mixture effect is significantly lower than the predicted effect.
- Additive: The observed mixture effect is not significantly different from the predicted effect.

(2) Calculation of Integrated Biomarker Response (IBR):

The calculation of IBR involved the following steps[14,15]:

1. The mean value of each biomarker in a given treatment (denoted as X_i) was standardised using the overall mean (m) and standard deviation (s) calculated across all treatments:

$$Y_i = \frac{X_i - m}{s}$$

2. Each standardised value (Y_i) was converted to a Z_i value by assigning a positive or negative sign, based on the nature of the biological response (positive for activation and negative for inhibition).

$$Z_i = Y_i \; or \; Z_i = -Y_i$$

3. The score value (S_i) for each biomarker in each treatment was obtained by summing the absolute minimum value and the Z_i value:

$$S_i = Z_i + |min|$$

4. The S_i values for all biomarkers in each treatment were plotted on a radar chart. The IBR value was then calculated as the total area of the radar chart, using the following equations:

$$A_i = \frac{S_i \times S_{i+1} \times \sin\left(\frac{2\pi}{k}\right)}{2}$$

The total IBR value for each treatment was computed as the sum of the areas of all sectors in the radar chart:

$$IBR = \sum_{i=1}^{k} A_i$$

where k is the number of observed biomarkers.

5. Given that there are 8 biomarkers, a total of 2520 unique radar chart permutations, $((k-1)!/2)$ were generated ($k = 8$). The mean IBR value from all permutations was considered as the final IBR score for each treatment.

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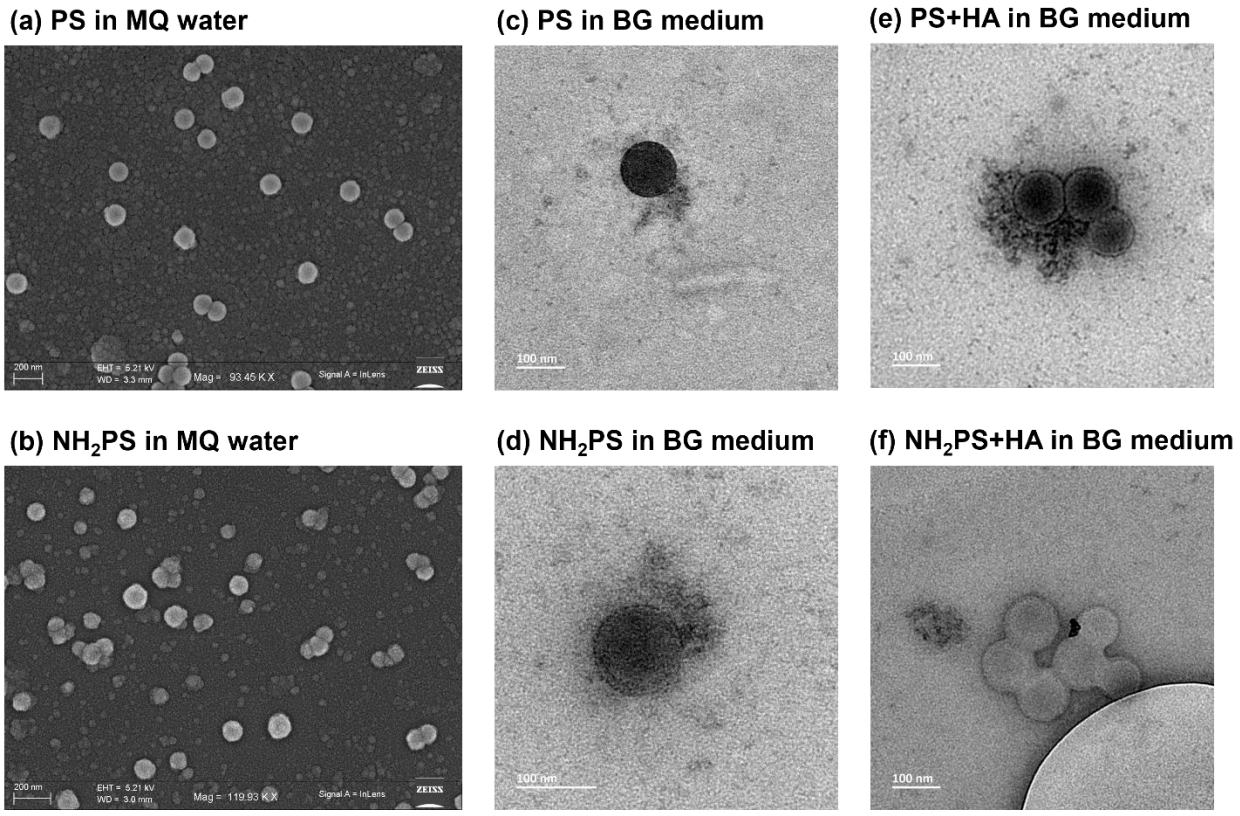

Fig. S1 SEM images showing (a) PS and (b) NH_2PS with uniform spheres (average diameter: 100 nm) in MQ water; TEM images showing (c) PS (25 mg L^{-1}), (d) NH_2PS (25 mg L^{-1}), (e) PS-HA complexes (10 mg L^{-1}, 25 mg L^{-1}) and (f) NH_2PS-HA complexes (10 mg L^{-1}, 25 mg L^{-1}) in BG11 medium.

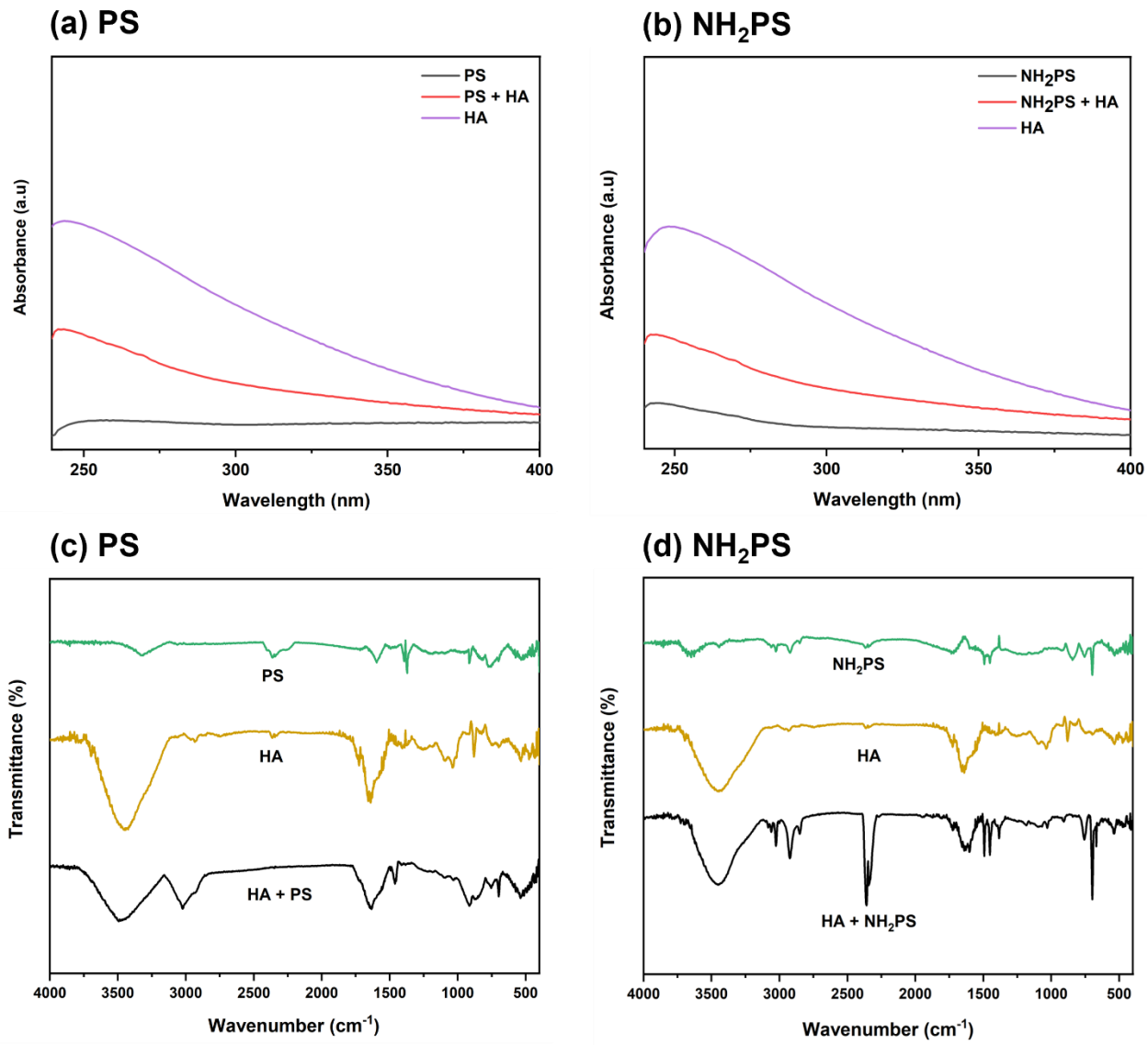

Fig. S2 The UV absorption spectra of (a) PS and (b) NH_2PS, as well as FTIR transmission spectra of (c) PS and (d) NH_2PS with or without HA.

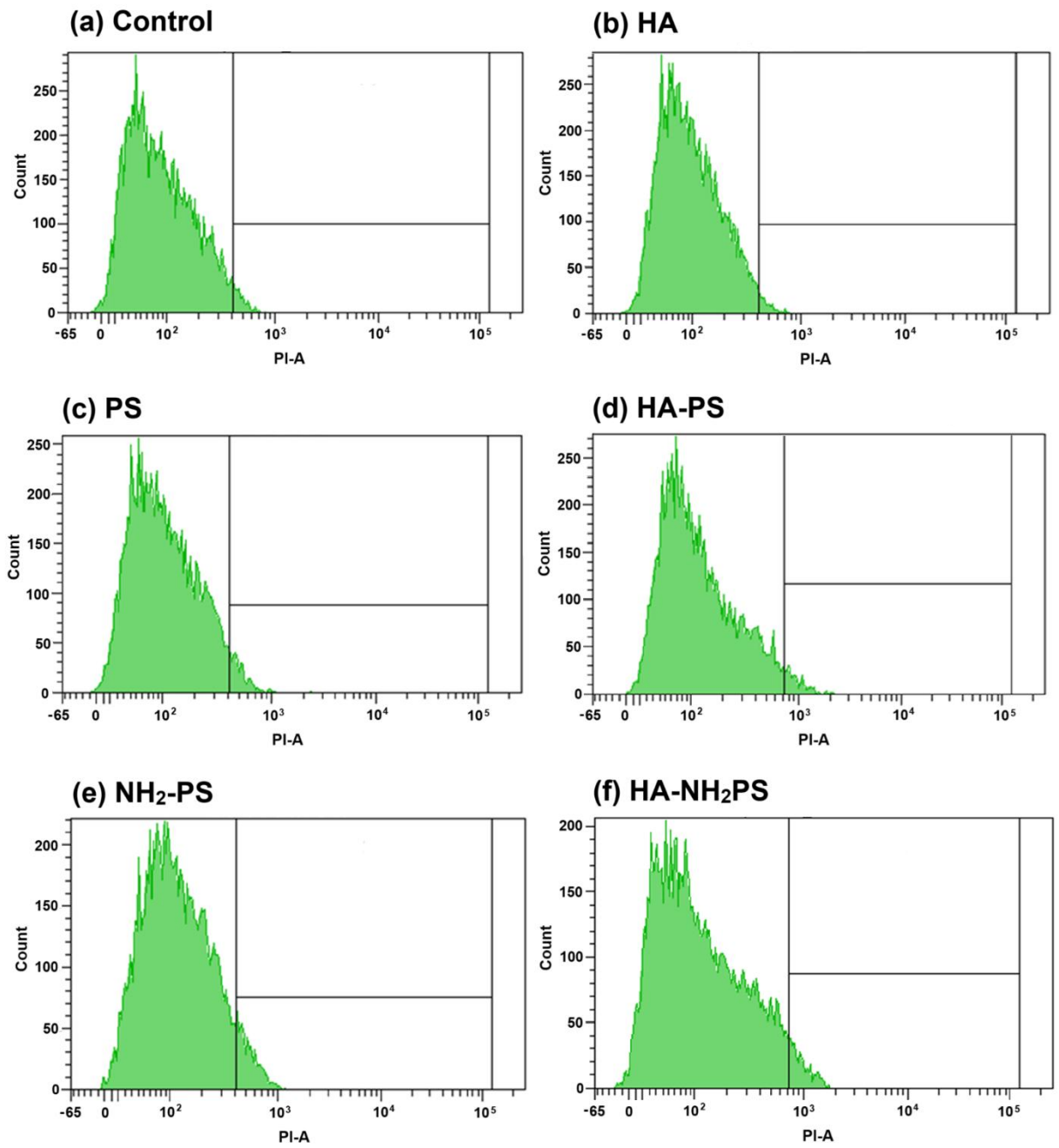

Fig. S3 Flow cytometry histograms showing the cell membrane integrity of microalgae exposed to nanoplastics (PS and NH_2PS) with or without HA for 96 h. X-axis of PI-A corresponds to propidium iodide fluorescence detected at 585 nm. The histograms in the left and right regions show the percentages of intact and membrane-damaged algal cells, respectively.

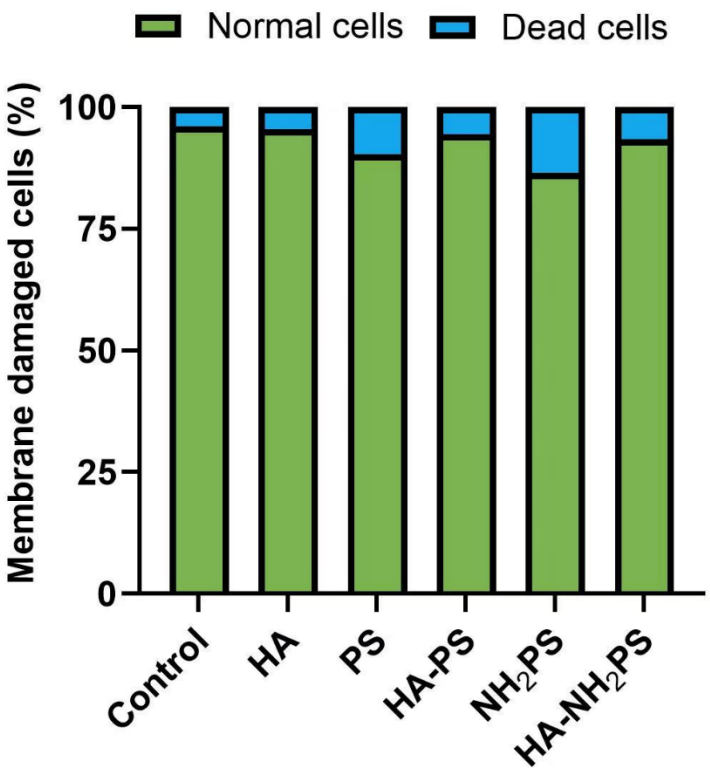

Fig. S4 Quantitative analysis of propidium iodide fluorescence intensity that indicates membrane damage in algal cells exposed to different combinations of PS, NH_2PS and HA for 96 h.

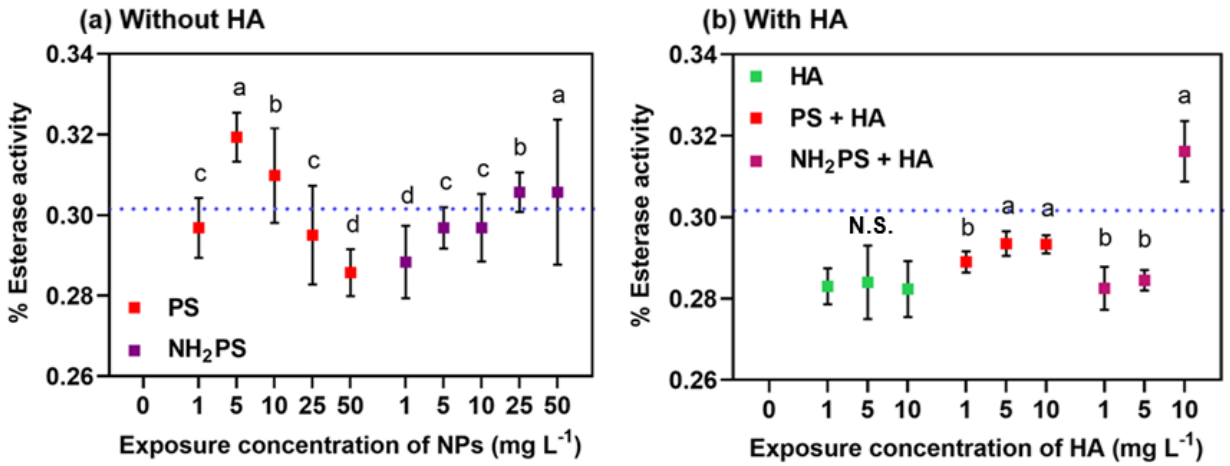

Fig. S5 Esterase activity in microalgae after exposure to NPs and HA for 96 h (mean ± S.D., n = 3). Exposure to PS caused a gradual decrease in esterase activity with increasing concentrations (1–50 mg L^{-1}), whereas exposure to NH_2PS resulted in an opposite trend of increased activity (1–50 mg L^{-1}). Exposure to HA alone or in combination with both NPs reduced esterase activity at concentrations between 1 and 10 mg L^{-1}. However, esterase activity was boosted when exposed to NH_2PS at 10 mg L^{-1} with HA. Different letters above error bars indicate significant difference across concentrations within a treatment group.

Table S1 The chemical composition of BG11 medium.

Chemical formula	Concentration (mg L^{-1})
$NaNO_3$	1500
$K_2HPO_4 \cdot 3H_2O$	40
$MgSO_4 \cdot 7H_2O$	75
$CaCl_2 \cdot 2H_2O$	36
$C_6H_8O_7$	6
$C_{12}H_{22}FeN_3O_{14}$	6
$C_{10}H_{14}N_2O_8$	1
Na_2CO_3	20
H_3BO_3	2.86
$MnCl_2 \cdot H_2O$	1.81
$ZnSO_4 \cdot 7H_2O$	0.222
$CuSO_4 \cdot 5H_2O$	0.079
$Na_2MoO_4 \cdot 2H_2O$	0.390
$Co(NO_3)_2 \cdot 6H_2O$	0.049

Table S2 Details of each experimental group based on the combinations of exposure to nanoplastics (NPs) and humic acid (HA). PS: polystyrene nanoplastics; NH_2PS: amine-modified polystyrene nanoplastics.

Treatment number	Treatment type	NP type	NP concentration (mg L^{-1})	HA concentration (mg L^{-1})
1	Control	-	-	-
2	NPs	PS	1	-
3	NPs	PS	5	-
4	NPs	PS	10	-
5	NPs	PS	25	-
6	NPs	PS	50	-
7	NPs	NH_2PS	1	-
8	NPs	NH_2PS	5	-
9	NPs	NH_2PS	10	-
10	NPs	NH_2PS	25	-
11	NPs	NH_2PS	50	-
12	HA	-	-	1
13	HA	-	-	5
14	HA	-	-	10
16	PS + HA	-	25	1
17	PS + HA	-	25	5
18	PS + HA	-	25	10
19	NH_2PS + HA	-	25	1
20	NH_2PS + HA	-	25	5
21	NH_2PS + HA	-	25	10

Table S3 Hydrodynamic diameters and zeta potentials of polystyrene nanoplastics (PS) and amine-modified polystyrene nanoplastics (NH_2PS) in Milli-Q water and BG11 medium, alone or combined with humic acid (HA).

Treatment	Hydrodynamic size (nm)		Zeta potential (mV)	
	Milli-Q water	BG11 medium	Milli-Q water	BG11 medium
PS	100 ± 4.84	329 ± 24.6	–17 ± 3.1	–23 ± 1.6
PS + HA	156 ± 4.02	386 ± 53.1	–31 ± 1.6	–24 ± 2.0
NH_2PS	100 ± 1.60	153 ± 2.40	44 ± 2.4	21 ± 1.1
NH_2PS + HA	1544 ± 66.7	1418 ± 12.8	–22 ± 0.1	–16 ± 0.7

Table S4 The exponential model-fitted parameters of the settling curves of the test substances and algae alone or their mixtures.

(1) PS with and without HA

Treatment number	Treatment type	OD Plateau	OD Reduced	Settling rate (ΔOD)	R^2
1	Algae	0.35	0.71	0.014	0.98
2	Algae + HA	0.34	0.73	0.014	0.98
3	Algae + PS	0.30	0.74	0.013	0.98
4	Algae + HA + PS	0.21	0.83	0.008	0.98
5	Algae | HA | PS	0.32	0.74	0.013	0.98

(2) NH_2PS with and without HA

Treatment number	Treatment type	OD Plateau	OD Reduced	Settling rate (ΔOD)	R^2
1	Algae	0.35	0.71	0.014	0.98
2	Algae + HA	0.34	0.73	0.014	0.98
3	Algae + NH_2PS	0.24	0.80	0.011	0.98
4	Algae + HA + NH_2PS	0.06	0.97	0.007	0.98
5	Algae | HA | NH_2PS	0.30	0.76	0.012	0.98

$OD_{plateau}$ refers to the optical density at the plateau of the settling curves; $OD_{reduced}$ refers to the reduced optical density from the initial time to the plateau.

Table S5 Assessment of the combined toxicity of nanoplastics and humic acid to microalgae using the independent action model. PS: polystyrene nanoplastics; NH_2PS: amine-modified polystyrene nanoplastics.

Treatment	Observed value (%)	Expected value (%)	Statistics	Interaction type
PS + HA1	14.0 ± 0.81	8.55 ± 0.26	$t = 20.5$, $p < 0.05$	Synergic
PS + HA5	11.1 ± 0.86	12.8 ± 1.22	$t = –5.38$, $p < 0.05$	Antagonistic
PS + HA10	2.78 ± 0.18	7.05 ± 0.69	$t = –17.1$, $p < 0.05$	Antagonistic
NH_2PS + HA1	10.5 ± 0.67	10.9 ± 0.73	$t = –1.19$, $p = 0.27$	Additive
NH_2PS + HA5	2.89 ± 0.16	11.3 ± 0.53	$t = –41.8$, $p < 0.05$	Antagonistic
NH_2PS + HA10	9.14 ± 0.45	7.08 ± 0.32	$t = –9.98$, $p < 0.05$	Antagonistic

Table S6 The results of two-way ANOVA showing the effects of different types of nanoplastics (NPs) and humic acid (HA) on various biomarkers of microalgae. Different superscript letters indicate significant difference between groups ($p < 0.05$). PS: polystyrene nanoplastics; NH_2PS: amine-modified polystyrene nanoplastics.

Source of variation	SS	*df*	MS	F	*p*	Comparison of means
Growth inhibition – Nanoplastic treatment						
NP type	0.969	10	9.69×10^{-2}	16.3	**< 0.0001**	PS: 1^{d} 5^{c} 10^{c} 25^{b} 50^{a}
NP concentration	0.691	5	0.138	23.3	**< 0.0001**	NH_2PS: 1^{e} 5^{d} 10^{c} 25^{b} 50^{a}
Interaction	3.01×10^{-2}	2	1.50×10^{-2}	2.53	8.53×10^{-2}	
Residual	0.534	90	5.94×10^{-3}			
Growth inhibition – Nanoplastic with humic acid treatment						
Treatment type	0.502	9	5.58×10^{-2}	3.84	**< 0.001**	Within HA: 10^{a} 5^{b} 1^{c}
HA concentration	0.597	3	0.199	13.7	**< 0.001**	Within PS+HA:10^{c} 5^{b} 1^{a}
Interaction	0.823	3	0.274	18.9	**< 0.001**	Within NH_2PS+HA: 10^{b} 5^{a} 1^{a}
Residual	1.16	80	1.45×10^{-2}			Within 1 mg/L: PS+HA^{a} NH_2PS+HA^{b} HA^{c}
						Within 5 mg/L: HA^{a} NH_2PS+HA ^{b} PS+HA ^{b}
						Within 10 mg/L: HA^{a} PS+HA^{b} NH_2PS+HA ^{b}
Reactive oxygen species – Nanoplastic treatment						
NP type	1.311	2	0.655	92.6	**<0.0001**	Within PS: 1^{c} 5^{c} 10^{c} 25^{b} 50^{a}
NP concentration	5.691	5	1.138	160.7	**<0.0001**	Within NH_2PS: 1^{c} 5^{c} 10^{c} 25^{b} 50^{a}
Interaction	3.688	10	0.368	52.1	**<0.0001**	Within 1 mg/L: NH_2PS^{a} PS^{a}
Residual	0.254	36	7.08×10^{-3}			Within 5 mg/L: NH_2PS^{a} PS^{a}
						Within 10 mg/L: NH_2PS^{a} PS^{a}
						Within 25 mg/L: NH_2PS^{a} PS^{b}
						Within 50 mg/L: NH_2PS^{a} PS^{b}
Reactive oxygen species – Nanoplastic with humic acid treatment						
Treatment type	1.421	3	0.473	84.44	**<0.0001**	Within HA: 10^{a} 5^{a} 1^{b}
HA concentration	3.457	3	1.152	205.4	**<0.0001**	Within PS+HA:10^{c} 5^{a} 1^{b}
Interaction	10.38	9	1.153	205.5	**<0.0001**	Within NH_2PS +HA: 10^{c} 5^{b} 1^{a}
Residual	0.179	32	5.61×10^{-3}			Within 1 mg/L: NH_2PS+HA^{a} PS+HA^{b} HA^{c}
						Within 5 mg/L: PS+HA^{a} HA^{b} NH_2PS+HA^{c}
						Within 10 mg/L: HA^{a} PS+HA ^{b} NH_2PS+HA ^{b}
Hydrogen peroxide – Nanoplastic treatment						
NP type	2.31×10^{-3}	2	1.11×10^{-4}	4.413	**1.38×10^{-2}**	Within PS: 1^{b} 5^{a} 10^{ab} 25^{ab} 50^{ab}

NP concentration	3.81×10^{-3}	5	7.65×10^{-5}	2.959	**1.42×10^{-2}**	Within NH_2PS: 1^{b} 5^{ab} 10^{a} 25^{ab} 50^{c}
Interaction	3.22×10^{-3}	10	3.21×10^{-4}	12.45	**<0.0001**	Within 1 mg/L: N.S
Residual	3.73×10^{-3}	144	2.58×10^{-5}			Within 5 mg/L: N.S
						Within 10 mg/L: PS ^{b} NH_2PS ^{a}
						Within 25 mg/L: PS ^{a} NH_2PS ^{b}
						Within 50 mg/L: PS ^{a} NH_2PS ^{b}
Hydrogen peroxide – Nanoplastic with humic acid treatment						
Treatment type	6.59×10^{-3}	3	2.21×10^{-3}	114.8	**<0.0001**	Within HA: 10^{a} 5^{a} 1^{b}
HA concentration	1.12×10^{-3}	3	3.71×10^{-3}	19.52	**<0.0001**	Within PS+HA:10^{c} 5^{b} 1^{a}
Interaction	4.45×10^{-3}	9	4.91×10^{-3}	25.82	**<0.0001**	Within NH_2PS+HA: 10^{b} 5^{b} 10^{a}
Residual	2.45×10^{-3}	128	1.91×10^{-4}			Within 1 mg/L: HA^{a} PS+HA ^{b} NH_2PS+HA ^{b}
						Within 5 mg/L: HA^{a} PS+HA^{b} NH_2PS+HA^{c}
						Within 10 mg/L: HA^{a} NH_2PS+HA ^{b} PS+HA ^{b}
Lipid peroxidation – Nanoplastic treatment						
NP type	7.51×10^{-4}	3	2.51×10^{-4}	0.089	9.64×10^{-1}	
NP concentration	4.61×10^{-4}	3	1.51×10^{-4}	0.054	9.82×10^{-2}	
Interaction	2.81×10^{-4}	9	3.12×10^{-4}	0.011	>0.9999	
Residual	4.47×10^{-2}	16	2.81×10^{-3}			
Lipid peroxidation – Nanoplastic with humic acid treatment						
Treatment type	4.61×10^{-4}	2	2.31×10^{-4}	0.087	9.16×10^{-1}	
HA concentration	6.61×10^{-4}	5	1.31×10^{-2}	0.049	9.98×10^{-1}	
Interaction	4.00×10^{-4}	10	4.02×10^{-4}	0.015	>0.9999	
Residual	4.76×10^{-2}	18	2.65×10^{-3}			
Superoxide dismutase – Nanoplastic treatment						
NP type	4.01×10^{-2}	2	2.01×10^{-2}	48.02	**<0.0001**	Within PS: 1^{ab} 5^{b} 10^{a} 25^{ab} 50^{b}
NP concentration	2.54×10^{-2}	5	5.09×10^{-3}	12.16	**<0.0001**	Within NH_2PS: 1^{c} 5^{a} 10^{b} 25^{c} 50^{d}
Interaction	2.75×10^{-2}	10	2.75×10^{-3}	6.58	**<0.0001**	Within 1 mg/L: PS ^{a} NH_2PS ^{b}
Residual	3.76×10^{-2}	90	4.21×10^{-4}			Within 5 mg/L: PS ^{b} NH_2PS ^{a}
						Within 10 mg/L: PS ^{a} NH_2PS ^{b}
						Within 25 mg/L: PS ^{a} NH_2PS ^{b}
						Within 50 mg/L: PS ^{a} NH_2PS ^{b}
Superoxide dismutase – Nanoplastic with humic acid treatment						
Treatment type	1.36×10^{-2}	3	4.56×10^{-3}	9.67	**<0.0001**	Within HA: 10^{c} 5^{a} 1^{b}
HA concentration	1.17×10^{-2}	3	3.93×10^{-3}	8.32	**<0.0001**	Within PS+HA:10^{b} 5^{b} 1^{a}
Interaction	3.10×10^{-2}	9	3.45×10^{-3}	7.31	**<0.0001**	Within NH_2PS+HA: 10^{a} 5^{b} 1^{c}

Residual	3.77×10^{-2}	80	4.70×10^{-4}			Within 1 mg/L: HA^{b} $PS+HA^{a}$ NH_2PS+HA^{c} Within 5 mg/L: HA^{a} $PS+HA^{b}$ NH_2PS+HA^{c} Within 10 mg/L: HA^{b} $PS+HA^{a}$ NH_2PS+HA^{a}
Catalase – Nanoplastic treatment						
NP type	1.97×10^{-2}	2	9.85×10^{-3}	32.9	**<0.0001**	Within PS: 1^{c} 5^{ab} 10^{b} 25^{a} 50^{b}
NP concentration	1.90×10^{-2}	5	3.81×10^{-3}	12.7	**<0.0001**	Within NH_2PS: 1^{b} 5^{ab} 10^{b} 25^{a} 50^{b}
Interaction	1.63×10^{-2}	10	1.63×10^{-3}	5.45	**<0.0001**	Within 1 mg/L: PS^{a} NH_2PS^{b}
Residual	2.69×10^{-2}	90	3.0×10^{-4}			Within 5 mg/L: PS^{a} NH_2PS^{b} Within 10 mg/L: PS^{a} NH_2PS^{b} Within 25 mg/L: PS^{a} NH_2PS^{b} Within 50 mg/L: PS^{a} NH_2PS^{b}
Catalase – Nanoplastic with humic acid treatment						
Treatment type	2.45×10^{-2}	3	8.17×10^{-3}	29.52	**<0.0001**	Within HA: 10^{b} 5^{a} 1^{ab}
HA concentration	2.87×10^{-2}	3	9.58×10^{-3}	34.63	**<0.0001**	Within PS+HA: N.S
Interaction	2.97×10^{-2}	9	3.30×10^{-3}	11.93	**<0.0001**	Within NH_2PS+HA: 10^{a} 5^{a} 1^{b}
Residual	2.21×10^{-2}	80	2.80×10^{-4}			Within 1 mg/L: N.S Within 5 mg/L: HA^{b} $PS+HA^{b}$ NH_2PS+HA^{a} Within 10 mg/L: HA^{c} $PS+HA^{b}$ NH_2PS+HA^{a}
Reduced glutathione – Nanoplastic treatment						
NP type	0.37	2	0.18	1678	**<0.0001**	Within PS: 1^{c} 5^{d} 10^{c} 25^{b} 50^{a}
NP concentration	0.24	5	4.87×10^{-2}	431.0	**<0.0001**	Within NH_2PS: 1^{b} 5^{b} 10^{a} 25^{b} 50^{b}
Interaction	0.48	10	4.82×10^{-2}	427.0	**<0.0001**	Within 1 mg/L: N.S
Residual	0.001	90	1.13×10^{-4}			Within 5 mg/L: N.S Within 10 mg/L: N.S Within 25 mg/L: PS^{a} NH_2PS^{b} Within 50 mg/L: PS^{a} NH_2PS^{b}
Reduced glutathione – Nanoplastic with humic acid treatment						
Treatment type	0.10	3	3.39×10^{-2}	533.9	**<0.0001**	Within HA: 10^{b} 5^{a} 1^{b}
HA concentration	9.06×10^{-2}	3	3.02×10^{-2}	475.9	**<0.0001**	Within PS+HA:10^{a} 5^{b} 1^{c}
Interaction	0.13	9	1.49×10^{-2}	235.6	**<0.0001**	Within NH_2PS+HA: 10^{a} 5^{b} 1^{c}
Residual	5.08×10^{-3}	80	6.35×10^{-4}			Within 1 mg/L: HA^{b} $PS+HA^{a}$ NH_2PS+HA^{a} Within 5 mg/L: HA^{a} NH_2PS+HA^{a} $PS+HA^{b}$ Within 10 mg/L: HA^{c} $PS+HA^{b}$ NH_2PS+HA^{a}
Esterase activity – Nanoplastic treatment						
NP type	6.01×10^{-4}	2	3.01×10^{-4}	0.03	9.69×10^{-1}	Within PS: 1^{c} 5^{a} 10^{b} 25^{c} 50^{d}

NP concentration	2.73×10^{-3}	5	5.50×10^{-4}	5.71	**0.0001**	Within NH_2PS: 1^{d} 5^{c} 10^{c} 25^{b} 50^{a}
Interaction	3.72×10^{-3}	10	3.70×10^{-4}	3.90	**0.0002**	Within 1 mg/L: PS ^{a} NH_2PS ^{b}
Residual	8.59×10^{-3}	90	9.54×10^{-4}			Within 5 mg/L: PS ^{a} NH_2PS ^{b}
						Within 10 mg/L: PS ^{a} NH_2PS ^{b}
						Within 25 mg/L: PS ^{b} NH_2PS ^{a}
						Within 50 mg/L: PS ^{b} NH_2PS ^{a}
Esterase activity – Nanoplastic with humic acid treatment						
Treatment type	2.41×10^{-3}	3	8.0×10^{-4}	15.65	**<0.0001**	Within HA: N.S
HA concentration	2.63×10^{-3}	3	8.80×10^{-4}	17.07	**<0.0001**	Within PS+HA:10^{a} 5^{a} 1^{b}
Interaction	4.0×10^{-3}	9	4.40×10^{-4}	8.650	**<0.0001**	Within NH_2PS+HA: 10^{a} 5^{b} 1^{b}
Residual	4.11×10^{-3}	80	5.14×10^{-4}			Within 1 mg/L: HA^{a} PS+HA^{b} NH_2PS+HA^{a}
						Within 5 mg/L: HA^{b} PS+HA^{b} NH_2PS+HA^{a}
						Within 10 mg/L: HA^{b} PS+HA^{b} NH_2PS+HA^{a}
Cell surface hydrophobicity – Nanoplastic treatment						
NP type	44.48	2	22.24	0.03	9.64×10^{-1}	
NP concentration	51.55	5	10.31	0.01	9.99×10^{-1}	
Interaction	401.2	10	40.12	0.06	>0.9999	
Residual	10880	18	604.4			
Cell surface hydrophobicity – Nanoplastic with humic acid treatment						
Treatment type	437.9	3	146	0.24	8.62×10^{-1}	
HA concentration	38.8	3	12.93	0.02	9.95×10^{-1}	
Interaction	746.9	9	82.99	0.14	9.97×10^{-1}	
Residual	9450	16	590.6			
Mitochondrial membrane damage – Nanoplastic treatment						
NP type	26.56	2	13.28	370.9	**<0.0001**	Within PS: 1^{c} 5^{c} 10^{c} 25^{b} 50^{a}
NP concentration	13.06	5	2.612	72.96	**<0.0001**	Within NH_2PS: 1^{d} 5^{d} 10^{c} 25^{b} 50^{a}
Interaction	9.022	10	9.02×10^{-1}	25.20	**<0.0001**	Within 1 mg/L: PS ^{b} NH_2PS ^{a}
Residual	1.289	36	3.58×10^{-2}			Within 5 mg/L: PS ^{b} NH_2PS ^{a}
						Within 10 mg/L: PS ^{b} NH_2PS ^{a}
						Within 25 mg/L: PS ^{b} NH_2PS ^{a}
						Within 50 mg/L: PS ^{b} NH_2PS ^{a}
Mitochondrial membrane damage – Nanoplastic with humic acid treatment						
Treatment type	6.984	3	2.328	97.68	**<0.0001**	Within HA: 10^{a} 5^{b} 1^{b}
HA concentration	7.173	3	2.391	100.3	**<0.0001**	Within PS+HA:10^{c} 5^{b} 1^{a}
Interaction	10.39	9	1.155	48.44	**<0.0001**	Within NH_2PS+HA: 10^{c} 5^{a} 1^{b}

Residual	7.62×10^{-1}	32	2.38×10^{-2}			Within 1 mg/L: HA^{c} $PS+HA^{b}$ NH_2PS+HA^{a} Within 5 mg/L: HA^{c} $PS+HA^{b}$ NH_2PS+HA^{a} Within 10 mg/L: HA^{a} $PS+HA^{b}$ NH_2PS+HA^{a}
Cell membrane damage – Nanoplastic treatment						
NP type	7.482	2	3.741	1.00	3.87×10^{-1}	
NP concentration	8.273	5	1.655	0.44	8.12×10^{-1}	
Interaction	5.471	10	5.47×10^{-1}	0.14	9.98×10^{-1}	
Residual	67.26	18	3.737			
Cell membrane damage – Nanoplastic with humic acid treatment						
Treatment type	2.756	3	0.918	0.41	7.47×10^{-1}	
HA concentration	1.473	3	0.491	0.21	8.81×10^{-1}	
Interaction	3.34	9	0.371	0.16	9.95×10^{-1}	
Residual	35.77	16	2.235			
Chlorophyll *a* – Nanoplastic treatment						
NP type	0.04	2	0.024	11.08	**<0.0001**	Within PS: N.S
NP concentration	0.43	5	0.086	39.86	**<0.0001**	Within NH_2PS: 1^{a} 5^{a} 10^{a} 25^{a} 50^{b}
Interaction	0.34	10	0.034	16.05	**<0.0001**	Within 1 mg/L: N.S
Residual	0.19	90	0.0021			Within 5 mg/L: N.S Within 10 mg/L: N.S Within 25 mg/L: N.S Within 50 mg/L: N.S
Chlorophyll *a* – Nanoplastic with humic acid treatment						
Treatment type	0.82	3	0.275	146.7	**<0.0001**	Within HA: N.S
HA concentration	0.49	3	0.163	87.06	**<0.0001**	Within PS+HA: N.S
Interaction	0.35	9	0.039	21.07	**<0.0001**	Within NH_2PS+HA: 10^{b} 5^{a} 1^{a}
Residual	0.15	80	0.0018			Within 1 mg/L: HA^{a} $PS+HA^{a}$ NH_2PS+HA^{b} Within 5 mg/L: N.S Within 10 mg/L: N.S
Chlorophyll *b* – Nanoplastic treatment						
NP type	0.028	2	0.014	9.516	**0.0002**	Within PS: 1^{a} 5^{b} 10^{b} 25^{c} 50^{c}
NP concentration	0.304	5	0.060	41.29	**<0.0001**	Within NH_2PS: 1^{a} 5^{a} 10^{b} 25^{b} 50^{c}
Interaction	0.244	10	0.024	16.61	**<0.0001**	Within 1 mg/L: N.S
Residual	0.132	90	0.001			Within 5 mg/L: N.S Within 10 mg/L: PS ^{a} NH_2PS ^{b} Within 25 mg/L: N.S

						Within 50 mg/L: PS ^{a} NH_2PS ^{b}
Chlorophyll *b* – Nanoplastic with humic acid treatment						
Treatment type	0.55	3	0.185	110.0	**<0.0001**	Within HA: 10^{a} 5^{b} 1^{b}
HA concentration	0.35	3	0.119	70.92	**<0.0001**	Within PS+HA: N.S
Interaction	0.25	9	0.028	16.63	**<0.0001**	Within NH_2PS+HA: 10^{b} 5^{a} 1^{a}
Residual	0.13	80	0.0016			Within 1 mg/L: HA^{a} PS+HA^{b} NH_2PS+HA^{c}
						Within 5 mg/L: HA^{a} PS+HA^{b} NH_2PS+HA^{b}
						Within 10 mg/L: HA^{a} PS+HA^{b} NH_2PS+HA^{b}
Carotenoid – Nanoplastic treatment						
NP type	0.051	2	0.025	9.380	**0.0002**	Within PS: 1^{a} 5^{a} 10^{b} 25^{c} 50^{c}
NP concentration	0.415	5	0.083	30.31	**<0.0001**	Within NH_2PS: 1^{a} 5^{a} 10^{b} 25^{c} 50^{d}
Interaction	0.345	10	0.034	12.60	**<0.0001**	Within 1 mg/L: N.S
Residual	0.246	90	0.0027			Within 5 mg/L: N.S
						Within 10 mg/L: N.S
						Within 25 mg/L: N.S
						Within 50 mg/L: PS ^{a} NH_2PS ^{b}
Carotenoid – Nanoplastic with humic acid treatment						
Treatment type	0.83	3	0.27	85.75	**<0.0001**	Within HA: 10^{b} 5^{b} 1^{a}
HA concentration	0.53	3	0.17	54.56	**<0.0001**	Within PS+HA:10^{b} 5^{b} 1^{a}
Interaction	0.34	9	0.038	11.90	**<0.0001**	Within NH_2PS+HA: 10^{c} 5^{b} 1^{a}
Residual	0.26	80	0.003			Within 1 mg/L: HA^{a} PS+HA^{b} NH_2PS+HA^{c}
						Within 5 mg/L: HA^{a} PS+HA^{b} NH_2PS+HA^{c}
						Within 10 mg/L: HA^{a} PS+HA^{b} NH_2PS+HA^{b}