Electronic Supplementary Material (ESI) for Environmental Science: Processes & Impacts.

Supplementary Information

Polystyrene nanoplastics alter the ecotoxicological effects of Diclofenac on freshwater microalgae *Scenedesmus obliquus*

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Methods:

Cell viability assessment

After the incubation period, the samples were examined using the MTT dye to determine the cell viability. In sterile PBS buffer (pH 7.4), freshly prepared dye at a concentration of 5 mg/ml was created. 500 µl of the cells were taken from the interacting samples, and 20 µl of the dye was added. This mixture was then incubated in the dark for 4 hours. The samples were centrifuged at 8000 rpm after the incubation period, and the supernatant was discarded. Then, the pellet was then dispersed in 200 µl DMSO. In ELISA plate reader (xMARK microplate absorbance spectrophotometer, BIO-RAD), this was evaluated for absorbance at 570 nm. There was no observed inference from DCF and FNPs when the samples were analyzed using MTT dye at 570 nm because the absorbance wavelength of DCF and FNPs were 275 nm and 547 nm, respectively. The cell viability (%) in the treated samples was determined using the following formula and then subtracted from 100 and compared with the control.

$$Cell\ viability\ (\%) = \frac{OD_{570}\ at\ Control - OD_{570}\ at\ Test}{OD_{570}\ at\ Control} \times 100$$

Total ROS production

The samples of interacting algae were incubated with 100 µl of DCFH-DA (100 µM) solution, and the mixture was left in a static state for 30 min in the dark. Using a fluorescence spectrophotometer (Cary Eclipse, G9800A; Agilent Technologies, USA), the fluorescent intensity of the treated samples was measured at an excitation and emission wavelength of 485 nm and 530 nm, respectively, after 30 minutes of incubation. Plots of the data were made with respect to the ROS generated in the control algal cells. The fluorescence intensity of a sample devoid of algal cells (an abiotic condition) was evaluated to rule out the interference of FNPs. There was no observed interference from FNPs when the samples were analyzed for ROS at

an excitation and emission wavelength of 485 and 530 nm, respectively. This is because the excitation and emission wavelength of FNPs are 552 and 636 nm, respectively.

Lipid peroxidation

After being incubated, the algal cells were centrifuged for 10 minutes at 7000 rpm (4 °C). After that, the algal pellet was dispersed in 2 mL of a TBA-TCA mixture solution (0.25% w/v TBA and 10% w/v TCA), placed in a water bath for 30 minutes at 95°C, and then the mixture was allowed to cool down. After cooling the mixture, an ELISA plate reader (xMARK microplate absorbance spectrophotometer, BIO-RAD) was utilized to measure the supernatant at 532 nm and 600 nm.

Antioxidant enzyme assessment

For the superoxide dismutase (SOD) assay, the 72 h interacted samples were centrifuged to get the pellet of cells. Then, it was homogenized in 0.5 M phosphate buffer (pH 7.5) using an ultrasonicator (Sonics, USA; 130 W, 20 kHz). The collected homogenate was then centrifuged at 13,000 rpm (4 °C) and the supernatant was collected. 100 µl of supernatant was mixed with 2 ml of the reaction mixture (50 mM Na_2CO_3 buffer at pH 10, 96 mM nitro tetrazolium blue chloride (NBT), 0.6 % triton X-100, and 20 mM hydroxylamine hydrochloride) and placed at 37 °C under light for 20 minutes. The absorbance at 560 nm was taken using an ELISA plate reader (xMARK microplate absorbance spectrophotometer, BIO-RAD).

After centrifugation of the same homogenate as mentioned in the SOD assay, the supernatant (2 ml) was collected and treated with 10 mM H_2O_2 solution (1 mL) and the mixture was taken to perform the CAT assay. Using a UV-Vis spectrophotometer (Evolution 220, Thermo Fischer Scientific, USA), the absorbance of the treated solution was determined immediately at 240 nm for 3 min. The mixture without H_2O_2 was regarded as being “blank”.

Photosynthetic pigments assessment

After incubating for 72 h, the algal sample of 4.5 ml was centrifuged at 4000 rpm for 15 min to remove the supernatant, and the pellet was then given in 3 ml of 95% ethanol with glass beads. The suspension was then vortexed before being centrifuged for three minutes at a speed of 4000 rpm. Then the absorbance was measured at the wavelengths 665, 649, and 470 nm. The following formulae were used to determine the pigment concentrations.

$$Chlorophyll\ A\ (CHL\ A)\ =\ 13.95\ A665–6.88\ A649;$$

$$Chlorophyll\ B\ (CHL\ B)\ =\ 24.96\ A649–7.32\ A665;$$

$$Carotenoids\ (CAR)\ =\ \frac{(1000\ A470–2.05\ Chl\ a–114.8\ Chl\ b)}{245}.$$

Figures:

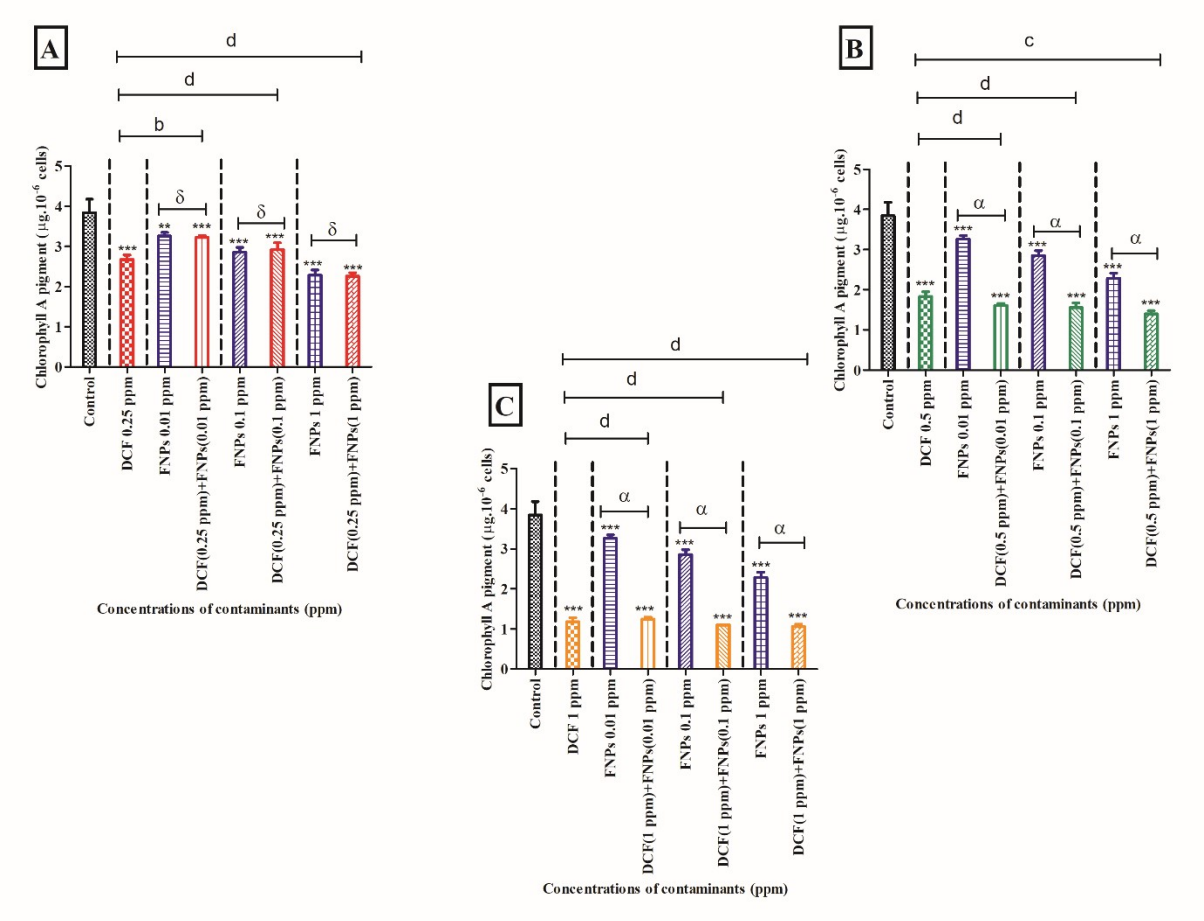


Figure S1: The comparison of chlorophyll A pigment content for DCF and FNPs for both pristine and combined forms (A) the comparison for the pristine and combinations of 0.25 mg/L of DCF + (0.01, 0.1, 1 mg/L) FNPs (B) the comparison for the pristine and combinations of 0.5 mg/L of DCF + (0.01, 0.1, 1 mg/L) FNPs (C) the comparison for the pristine and combinations of 1 mg/L of DCF + (0.01, 0.1, 1 mg/L) FNPs. The level of significance for algal cells treated with pristine DCF and FNPs with respect to control is marked with '***' ($p < 0.001$) and '**' ($p < 0.01$), 'α and δ' indicate significant difference between pristine and DCF combined FNPs treatment groups (α = $p < 0.001$ and δ = no significance), 'b, c, and d' indicates a significant difference between pristine DCF and DCF combined FNPs treatment groups (b = $p < 0.01$, c = $p < 0.05$ and d = no significance).

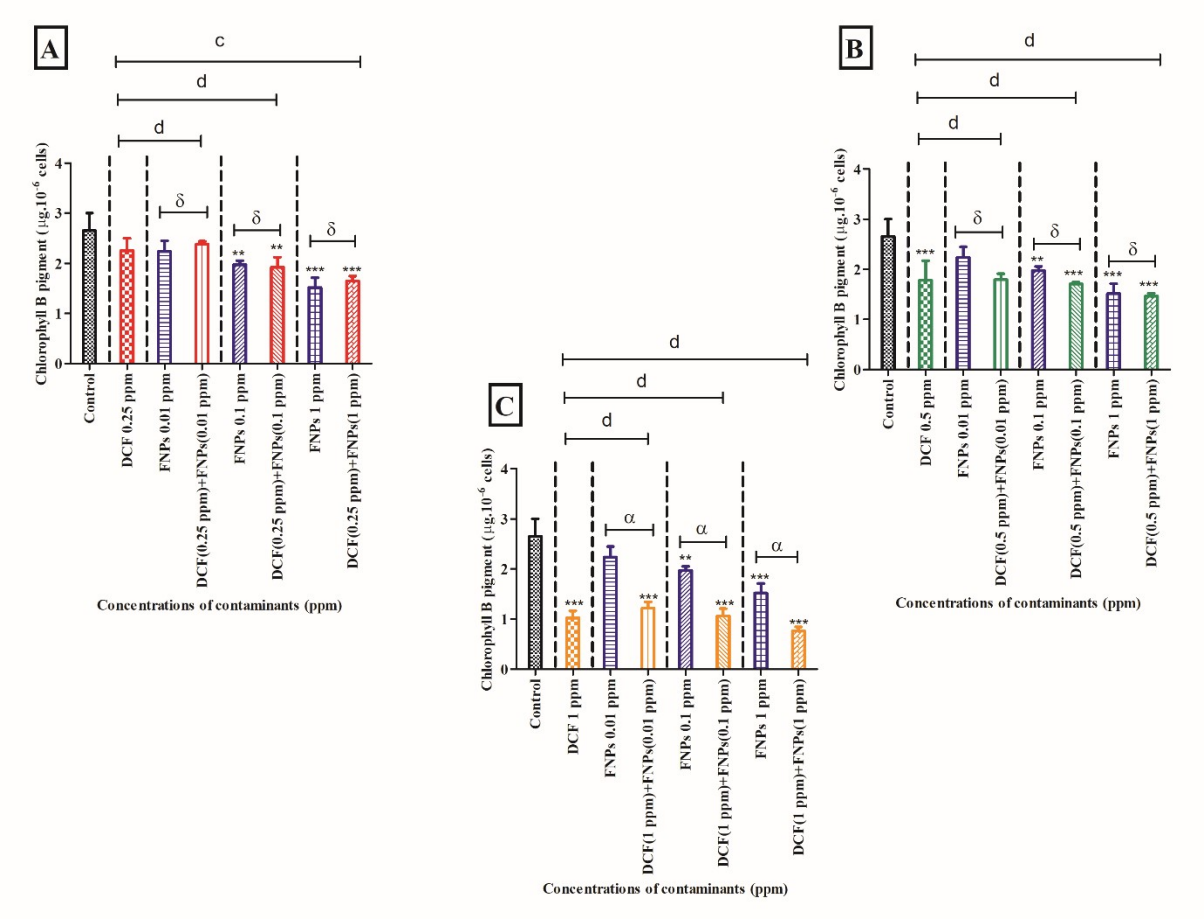


Figure S2: The comparison of chlorophyll B pigment content for DCF and FNPs for both pristine and combined forms (A) the comparison for the pristine and combinations of 0.25 mg/L of DCF + (0.01, 0.1, 1 mg/L) FNPs (B) the comparison for the pristine and combinations of 0.5 mg/L of DCF + (0.01, 0.1, 1 mg/L) FNPs (C) the comparison for the pristine and combinations of 1 mg/L of DCF + (0.01, 0.1, 1 mg/L) FNPs. The level of significance for algal cells treated with pristine DCF and FNPs with respect to control is marked with '***' ($p < 0.001$) and '**' ($p < 0.01$), 'α and δ' indicate significant difference between pristine and DCF combined FNPs treatment groups (α = $p < 0.001$ and δ = no significance), 'b, c, and d' indicates a significant difference between pristine DCF and DCF combined FNPs treatment groups (b = $p < 0.01$, c = $p < 0.05$ and d = no significance).

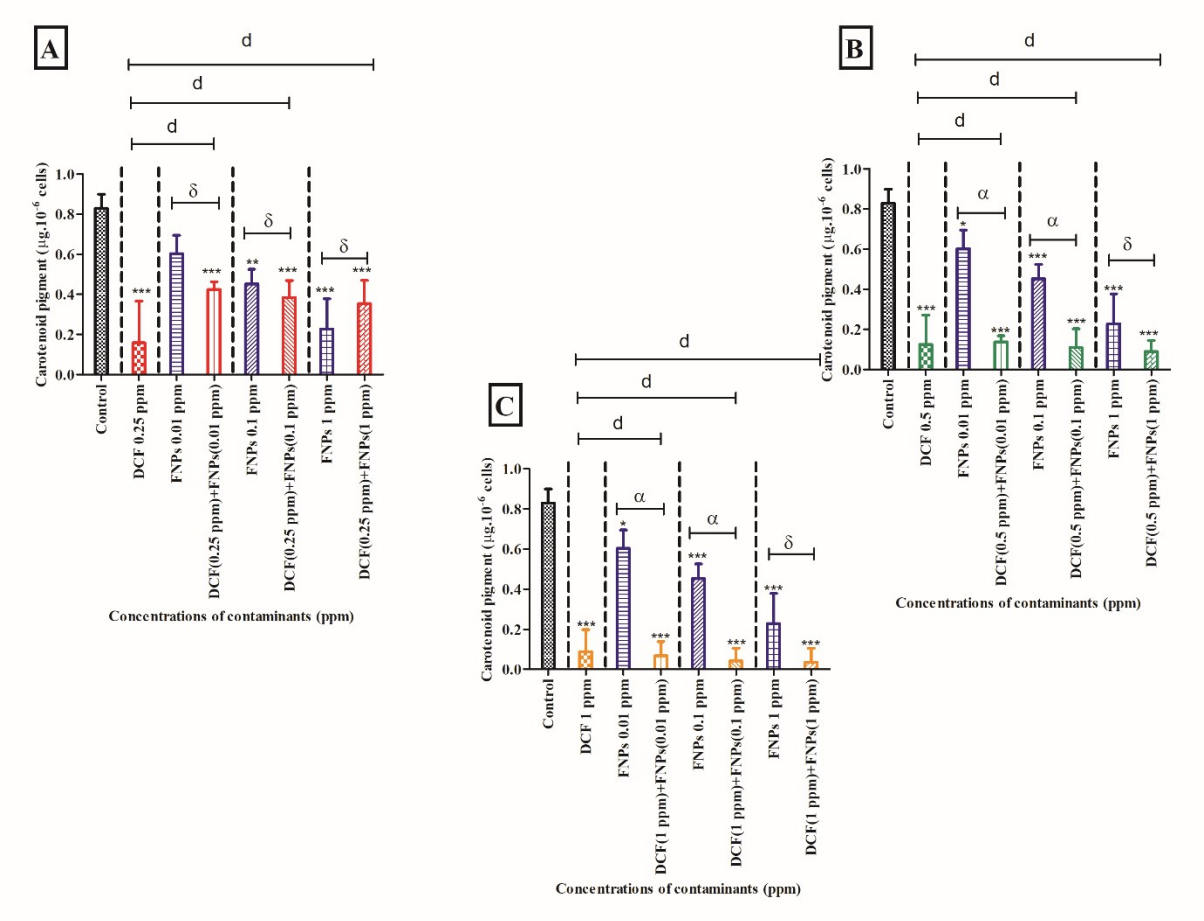
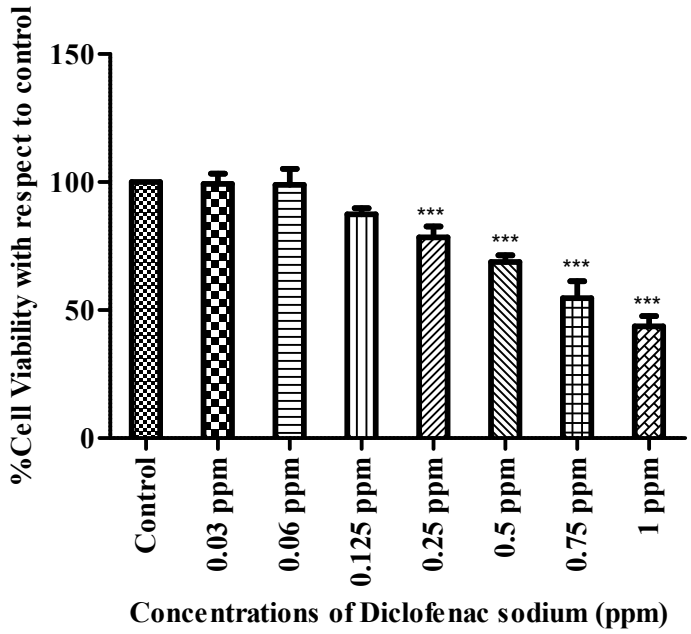
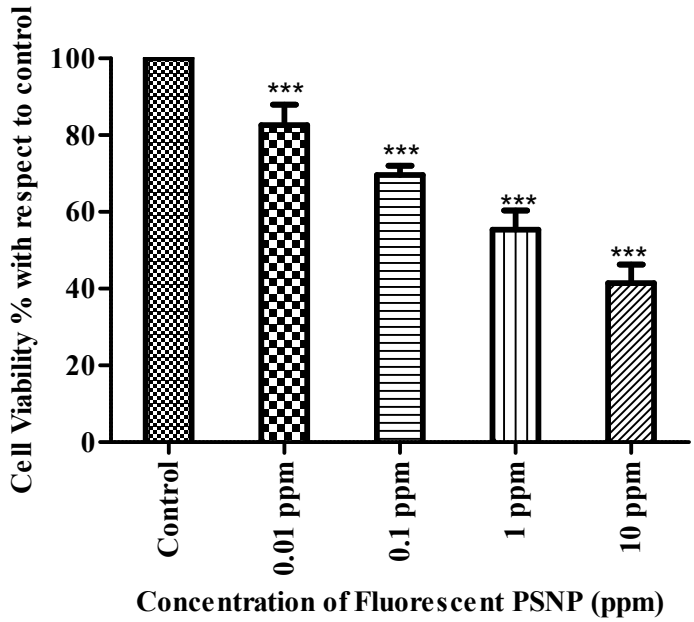


Figure S3: The comparison of carotenoids pigment content for DCF and FNPs for both pristine and combined forms (A) the comparison for the pristine and combinations of 0.25 mg/L of DCF + (0.01, 0.1, 1 mg/L) FNPs (B) the comparison for the pristine and combinations of 0.5 mg/L of DCF + (0.01, 0.1, 1 mg/L) FNPs (C) the comparison for the pristine and combinations of 1 mg/L of DCF + (0.01, 0.1, 1 mg/L) FNPs. The level of significance for algal cells treated with pristine DCF and FNPs with respect to control is marked with '***' ($p < 0.001$), '**' ($p < 0.01$), and '*' ($p < 0.05$), 'α and δ' indicate significant difference between pristine and DCF combined FNPs treatment groups ($\alpha = p < 0.001$ and δ = no significance), 'd' indicates a significant difference between pristine DCF and DCF combined FNPs treatment groups (d = no significance).

EC50 determination for DCF and FNPs



The calculated EC50 through probit analysis is 0.85 ppm of DCF.



The calculated EC50 through probit analysis is 1.72 ppm of FNPs.