Electronic Supplementary Material (ESI) for Environmental Science: Nano.

Toxicity of mixture of nanoparticles on algae-bacteria consortia in OECD media

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Appendix A: Previous studies

Table A.1: Various studies showing algae-bacteria consortia at different water matrix.

Reference	Algae/bacteria Information	Matrix	Findings	Toxicants	Parameters observed	Comparison with the present study	Remarks/ Limitations
Han et al. (2016)	***Bacteria: Muricauda sp.*** **Axenic microalga:** ***Tetraselmis chuii, Cylindrotheca fusiformis & Nannochloropsis gaditana***	Wastewater	Microalgae-bacteria co-cultures effective strategy for microalgal cultivation under mixotrophic conditions. Algal cell density increases with bacteria.	Not present	Growth curve, algae-bacteria ratio, plating	Growth profile, chl a, chl b, biomass, lipid, protein, carbohydrate, EPS, FTIR, TEM etc.	No data of Toxicity of NPs; Only growth studies done.
Berthold et al. (2019)	**Algae:** ***Characium sp.*** **Bacteria:** ***Pseudomonas composti***	BG-11 media	bacteria release of unidentified extracellular compounds which might affect the growth rate and lipid metabolism of algae.	Not present	Biomass, Lipid, FAME, Molecular phylogenetic analyses	Growth profile, chl a and b, biomass, lipid, protein, carbohydrate, EPS, FTIR, etc	No data of toxicity of NPs; Mechanism unexplored.

Ferro et al. (2019)	**algal strain: *Chlorella vulgaris* Bacteria*: Rhizobium sp.***	Synthetic Municipal Wastewater	Culture stability along with high nutrient removal capacity even at HRTs of 5 and 3 days	No	Biomass, Specific growth rate, O_2, CO_2, N_2, Lipid, Protein Carbohydrate, FTIR	Present study has NPs and TEM was performed along apart from other parameters done in the study.	No data of Toxicity of NPs.
Thøgersen et al. (2018)	**Alga *Emiliania huxleyi* bacterium *Phaeobacter inhibens* DSM17395**	Growth Media	The presence of the alga facilitated the attachment of the bacterium to a surface	No	DNA isolation, PCR, Fluorescence tagging	Growth profile, chl a, chl b, biomass, lipid, protein, carbohydrate, EPS, FTIR, TEM etc.	No data of Toxicity of NPs; Only growth study
Ashok et al. (2014)	**Algal-bacterial consortia. *Chlorella vulgaris. Chlamydomonas reinhardtii***	Synthetic wastewater	Almost 90 % removal of Nitrogen and Phosphorus and 80% of COD (2-day HRT)	No	Temp., pH, chl a, biomass, nitrogen, phosphorus, polysaccharides, alkalinity.	Growth profile, chl a, chl b, biomass, lipid, protein, carbohydrate, EPS, FTIR, TEM etc.	condition optimum for OECD condition or mixture of NPs
Holmes et al. (2019)	**Bacteria-** ***Escherichia coli*** **Algae-** ***Auxenochlorella protothecoides***	Simulated wastewater	In cocultures with algae, minimal or no acetate was observed; COD removal up to 66% faster than *E. coli* in co-culture.	No	Culture growth, qPCR, organic acid analysis	Growth profile, chl a and b, biomass, lipid, protein, carbohydrate, EPS, FTIR	No data on toxicity of NPs; no consideration for mixture of NPs
Cao et al.	**Algae: *Isochrysis***	NMB3	Decrease in the	No	Growth, chl via	Growth profile, chl a	No NPs toxicity; no information at

(2019)	***galbana*** **Bacteria*:*** ***Pseudomonas stutzeri***	media	chlorophyll contents by 23–74% in co-culture as compared with the axenic culture in the period of 6 days.		fluorescence, DNA extraction, PCR,	and b, biomass, lipid, protein, carbohydrate, EPS, FTIR	cellular levels (EC_{50}, etc).
Segev et al (2016)	**Algae:** ***Emiliania huxleyi*** **Bacteria*:*** ***Phaeobacter Inhibens***	Growth media	Naked algal cells covered by bacteria attached via their poles; Over time more attachment of algae with bacteria in co-culture conditions	No	Growth profile, flow cytometry, fluorescence, SEM, LC-MS, chl a, cell analysis.	Growth profile, chl a and b, biomass, lipid, protein, carbohydrate, EPS, FTIR	No toxicological studies on co-culture; no toxicity study due to NPs
Fie et al (2019)	**Bacteria:** ***R. radiobacter*** **Algae:** ***C. variabilis.***	*Growth media*	*R. radiobacter*-derived nitrogen stimulates fatty-acid oxidation in *C. variabilis* and promotes its growth	No	Growth profile, nitrogen, carbon, FAMEs.	Growth profile, chl a and b, biomass, lipid, protein, carbohydrate, EPS, FTIR, TEM.	No toxicity studies; no consideration for OECD conditions
Lepine et al. (2018)	***Chlorella spp.***	Industrial wastewater	A microalgae-bacteria consortium was grown in a mixture of industrial wastewater; Profitable process from reduced	No	Cell count, pH, growth, Lipid, FAME,	Growth profile, chl a and b, biomass, lipid, protein, carbohydrate, EPS, FTIR, TEM.	No NPs related toxicity study

			wastewater treatment costs & no added nutrients.				
Grover et al. (2020)	***C. vulgaris with Nitrobacter***	Growth media	Co-culturing enhanced growth (w/ increased cellular composition and biomass content)	No	Growth profile, cell count. biomass	Growth profile, chl a and b, biomass, lipid, protein, carbohydrate, EPS, FTIR	No NPs related toxicity study
Contreras-Angulo et al. (2019)	***Co-culture of Azospirillum brasilense and Scenedesmus sp.***	Growth media	Symbiotic co-culturing of microalgae-bacteria on nitrogen-deficient media for enhancing microalgae colony size and the fatty acid content of biomass for biofuels.	No	Biomass, cell size, protein carbohydrate, fatty acids, nitrogen	Growth profile, chl a and b, biomass, lipid, protein, carbohydrate, EPS, FTIR, TEM, etc	No NPs related toxicity study
Zhou et al, (2020)	***Chlorella pyrenoidosa; Bacteria: High-efficient ammonia-oxidizing strain FN5***	Antibiotic containing Wastewater	FN5 enhanced biomass concentration and lipid content of microalga *Chlorella pyrenoidosa; Chlorella pyrenoidosa-FN5* culture removed NH_3–N and accumulated	No	Enzyme activity (SOD, MDA, CAT), SEM, EPS, IAA, Nitrogen removal, Phosphate, COD removal	Growth profile, chl a and b, biomass, lipid, protein, carbohydrate, EPS, FTIR, TEM, CAT, etc.	No NPs related toxicity study

			lipid				
Verma et al, (2020)	***Algae: Chlorella sp.*** ***Activated sludge***	Lakewater	Removals of 93% nitrates, 99% phosphates and 73% COD; maximum biomass content =7.8g/L	No	Microalgal growth, biomass, SEM, COD, FTIR, nutrient removal	Growth profile, chl a, b, biomass, lipid, protein, carbohydrate, EPS, FTIR, TEM, CAT, etc.	No NP related toxicity studies
Xie et al. (2020)	***Microalgal strain C. sacchrarophila*** ***bacterium C. pealriver***	*Growth media*	*Chlorella sacchrarophila* was grown in bioreactor while a xylanolytic bacterium *Cellvibrio pealriver;* During the CTS strategy, the co-cultivation using xylan as feedstock promotes the microalgal growth.	No	Microbial growth, SEM, total nitrogen concentration, lipid.	Growth profile, chl a and b, biomass, lipid, protein, carbohydrate, EPS, FTIR, TEM, etc	No NPs related toxicity study
Xu et al. (2021)	***C. vulgaris, S. obliquus, Spirulina platensis*** ***Aerobic activated sludge***	Raw municipal wastewater	Nutrient removal was increased; season-dependent nutrient removal; Aeration helps in the removal efficiency.	No	Wastewater characteristic, biomass, nutrient removal, pH, DO, Nitrogen, Phosphorous, plate	Growth profile, chl a and b, biomass, lipid, protein, carbohydrate, EPS, FTIR, TEM, CAT, etc	No NPs related toxicity study

					count, TSS, Growth kinetics.		
Loria et al. (2021)	***C. vulgaris, C. sorokiniana, S. dimorphus, Neochloris oleoabundans; Activated sludge (AS)***	Growth media, sludge	Several microalgal taxa bio flocculated with AS within 2 h; extent of bio-flocculation varied between species of microalgae & P removal was inconsistent in *C. vulgaris* and *N. oleoabundans* reactors, but stable and high in *S. dimorphus* in SBR reactors, though S. dimorphus reactors also exhibited the poorest settleability	No	Biomass, lipid, TSS, growth profile, nitrogen, phosphorous, DO	Growth profile, chl a and b, biomass, lipid, protein, carbohydrate, EPS, FTIR, TEM, CAT, etc.	No NPs related toxicity study
Huo et al. (2020)	***Algae Chlorella sp. Bacteria: Bacillus***	Vinegar production	Nutrient removal rates were significantly	No	TN, TFA, Cell count, TN, TP,	Growth profile, chl a and b, biomass, lipid,	No NPs related toxicity study

	firmus and Beijerinckia fluminensis	Wastewater	increased after adding bacteria cultures; Mean growth rate of *Chlorella* was decreased slightly after co-cultures with bacteria; *B. fluminensis* enhanced the pigment content of *Chlorella sp.;* Co-culturing had more notable effect on fatty acid composition rather than oil content.		COD, Lipid, Fatty acid	protein, carbohydrate, EPS, FTIR, TEM, CAT, etc.	
Mujtaba et al. (2017)	***microalga Chlorella vulgaris; bacterium Pseudomonas putida.***	Municipal wastewater	higher removal of both nutrients and COD in coculture than each axenic culture; the best removal performance with suspended *P. putida* and immobilized *C. vulgaris*	No	TP, TN, COD, TOC, TSS, Cell growth, wastewater characterisation	Growth profile, chl a and b, biomass, lipid, protein, carbohydrate, EPS, FTIR, TEM, CAT, etc.	No NPs related toxicity study

Xu et al. (2021 b)	***Chlorella vulgaris, Scenedesmus obliquus, Spirulina platensis***	Municipal wastewater	In summer & autumn seasons high removal rates and biomass production percentages. In summer season with aeration, highest specific growth rate was 0.46 d−1; the highest TN removal rate was 2.34 d^{-1}; and the highest TSS removal efficiency was 96.3 ± 2.1%. In autumn season with aeration highest TP removal rate was 1.67 d^{-1}. An overall analysis indicated that *Chlorella vulgaris* and *Scenedesmus obliquus*, combined with bacteria (*Proteobacteria, Firmicutes,*	No	Biomass, plate count, COD, pH, DO, TP, TN, nutrient removal kinetics, growth kinetocs	Growth profile, chl a and b, biomass, lipid, protein, carbohydrate, EPS, FTIR, TEM, CAT, etc.	No NPs related study

			Bacteroidetes, Chloroflexi) can effectively use different carbon, nitrogen, and phosphorus sources from wastewater in different seasons.				
Xu et al, (2021 a)	***Algae: Chlorella vulgaris*** ***Bacteria: Bacillus.***	Growth media	Two bacterial strains of different genera were isolated from *Chlorella vulgaris;* Bacillus strain improved algae growth, photosynthesis, and nutrient removal; 7-day optimal co-culturing conditions with 10:1 bacteria-to-microalgae ratio	No	Biomass, cell count. Nutrient removal, growth kinetics, chl a	Growth profile, chl a and b, biomass, lipid, protein, carbohydrate, EPS, FTIR, TEM, CAT, etc.	No NPs related toxicity study
Tao et al. (2020)	***Chlorella sp. and Bacillus simplex***	Growth media	Consortium improved phenol degradation efficiency and *Chlorella*	No	Cell count, growth kinetics, phenol degradation	Growth profile, chl a and b, biomass, lipid, protein, carbohydrate,	No NPs related toxicity study

			sp. Growth			EPS, FTIR, TEM, CAT, etc.	
Li et al. (2021)	***Scenedesmus obliquus and Bacillus megaterium***		Co-culture was found more efficient in treating high concentration biogas slurry compared with the pure microalgae culture. Co-culture could efficiently reduce various nutrients in biogas slurry and simultaneously accumulate biomass with higher biofuel characteristics.	No	Biomass, cell growth, chl a, chl b, lipid, TP, TN, COD, etc.	Growth profile, chl a and b, biomass, lipid, protein, carbohydrate, EPS, FTIR, TEM, CAT, etc.	No NPs related toxicity studies
Wang et al. (2021)	***Monoculture and co-culture of algae (Chlorella vulgaris) and bacteria (activated sludge)***	Swine manure	When co-cultivated, the algal growth and precipitation (harvest) were promoted, while aerobic bacteria growth was initially promoted, and then inhibited.	No	Biomass, pH, TN, COD, DO, cell count, SEM, DNA, Biomass settling efficiency	Growth profile, chl a and b, biomass, lipid, protein, carbohydrate, EPS, FTIR, TEM, CAT, etc.	NPs toxicity was not studied.

Wang et al., 2022	***Algae-bacteria consortia (ABC) in activated sludge***	Cooking wastewater	ABC reactors show satisfactory removal ability. ABC can secrete large EPS to protect themselves and form flocs with good sedimentation performance under toxic and refractory organic wastewater stress.	No	COD, TN, pH, SS, biomass, EPS, BOD, chl a	Growth profile, chl a and b, biomass, lipid, protein, carbohydrate, EPS, FTIR, TEM, CAT, etc.	NPs toxicity not discussed
Rossi et al., 2022	*Chlorellaceae (1·10^6 cell·mL^{-1}), Scenedesmaceae (0.2·10^6 cell·mL^{-1}) and Chlamydomonadaceae (0.2·10^6 cell·mL^{-1}); bacterial culture: heterotrophs and nitrifiers.*	Piggery wastewater	Removal of NH_4^+, PO_4^{3-} (90%) and COD (59%), with 10.7 g/m^2/d biomass productivity. The process allowed to reduce the nitrogen spread to arable land by 77%, by increasing the nitrogen valorised as biofertilizers/ bio stimulants and the	No	TSS, TAN, COD, Biomass, chl a, FDA, SEM.	Growth profile, chl a and b, biomass, lipid, protein, carbohydrate, EPS, FTIR, TEM, CAT, etc.	NPs were not present in this study.

			nitrogen released to the atmosphere.				
Xu et al., 2023	*Cladophora, activated sludge*	Wastewater	The addition of microorganisms increases the removal efficiency of TN in atrazine-containing wastewater by 43.70%, and the addition of Cladophora further increased by 3.82%. The protein signal produced by the microbial release of EPS triggered the algal resistance mechanism, and the conversion and electron transfer between humic acid and fulvic acid constituted the synergistic effect. Proteobacteria was the	Atrazine	DO, biomass, TN, TP, COD, chl a, chl b, SOD, POD, MDA, EPS.	Growth profile, chl a and b, biomass, lipid, protein, carbohydrate, EPS, FTIR, TEM, CAT, etc.	NPs not studied.

			dominant phylum under atrazine stress, accounting for 56.70%–59.67% in the single bacteria groups, whereas it accounted for approximately 3% more in the algae–bacteria consortia.				
Cheng et al., 2022	*Laboratory grown algae and bacteria from activated sludge*	Municipal wastewater	A new type of algae-bacteria biofilm reactor (ABBR) was designed. ABBR allowed a marked improvement on the removals of IMI, TDN, TDP and cod during the 16-day operation. Meanwhile, more IMI degradation products were found in PBR while lower biological toxicity	No	pH, TDS, TN, ICP-MS, biomass,	Growth profile, chl a and b, biomass, lipid, protein, carbohydrate, EPS, FTIR, TEM, CAT, etc.	NPs presence was absent.

			was detected in ABBR. Furthermore, it's also proved that light played an important role on the performance of ABBR, and a much higher removal efficiency was achieved under 80 μmol m−2·s−1.				
Wang et al., 2023	*Trebouxiophyceae,* *Saccharimonadales*, *Propionibacteriaceae*, *Propionicivicla*, and *Micropruina*	Municipal wastewater	The addition of algae led to an increase in sedimentation performance, biomass, and EPS. The AEBC had a maximum 77.15 % removal rate of C, 63.22 % of N, and 82.54 % of P, respectively. The effluent of algae enhanced reactors suggested that algae had significant	NPs, abiotic stress	Total DNA, PCR, SEM, biomass, chl a, chl b, carotenoid, PCA.	Growth profile, chl a and b, biomass, lipid, protein, carbohydrate, EPS, FTIR, TEM, CAT, etc.	No NPs were used in this study.

			effects on pollutant removal.				

Table A. 2: Concentrations of NP used in previous toxicity studies.

S.No.	Nanoparticle used	Algae used	NPs conc.	Time duration	References
1.	ZnO	*Chlorella vulgaris*	0.5, 1, 2 mg/L	72 h	Ko et al., 2018
	CuO		8, 16, 33 mg/L		
2.	ZnO	*Chlamydomonas reinhardtii*	1-100 mg/L	8 days	Gunawan et al., 2013
3.	ZnO	*Skeletonema costatum*	5-50 mg/L	96 hrs.	Zhang et al., 2016
4.	ZnO	*Phaeodactylum tricornutum* (marine diatom)	0.1-10 mg/L	72 hrs.	Li et al., 2017
5.	ZnO	*Scenedesmus obliquus*	0.01 -50 mg/L	72 hrs.	Ye et al., 2018
6.	ZnO and CuO	*Scenedesmus obliquus*	1 mg/L	96 hrs.	Ye et al., 2017
7.	ZnO and CuO	*Scenedesmus obliquus*	1 and 10 mg/L	35 days	Rana and Kumar, 2023
8.	ZnO and CuO	*Scenedesmus obliquus*	0.1, 1, 10 and 100 mg/L	96 hrs.	Rana and Kumar, 2023
9.	MWCNTs and CuO	*Tetradesmus obliquus*	0.01-200 mg/L	96 hrs.	Fang et al., 2022
10.	$nTiO_2$ and BPA	*Scenedesmus obliquus*	0.4-3.2 mg/L	72 hrs.	Das et al., 2024

Appendix B: Methodology

B.1 Estimation of different parameters using analysis data

B.1.1. Growth Profile and cell number

Growth curve or growth profiling shows the number of cells growing in a population against time for various phases of growth (such as lag phase, log or exponential phase, stationary phase and decline phase). Absorbance at 750 nm was taken after every alternate day till t=96 hours as per the method given in the (Trenkenshu et al., 2009) study. Absorbance was adjusted for NP presence by subtracting OD of NP only suspension from OD of suspension containing both NP and algae. Cell number was counted every alternate day and cell density was calculated using the equation A.1 (Guillard, 1973):

$$Cell\ Density\ (per\ ml) = Total\ number\ of\ cells * 25000 * Dilution\ Factor \quad \textbf{(A.1)}$$

B.1.2 Dry cell weight

Dry cell weight (dcw) of the microalgal strain was determined gravimetrically according to Rai et al. (1991). Briefly, the dry weight of the microalgae biomass with a known volume i.e., 5 ml was centrifuged at 5000 rpm (REMI C-24) _ for 10 min. The harvested biomass was dried at 105℃ until the weight was invariant. The microalgae dry weight was obtained by subtracting the blank biomass. The OD_{680} value was converted to biomass concentration via appropriate calibration between OD_{680} and dry cell weight as per the following obtained Eq. (B.2):

$$Dry\ weight\ of\ biomass = \frac{Final\ weight(g) - initial\ weight(g)}{initial\ weight\ (g)} \quad \textbf{(B.2)}$$

B.1.3 Specific growth rate

Specific growth rate can be defined as the rate of increase of biomass of a cell per unit of biomass concentration with respect to time. The specific growth rate (μ: day^{-1}) was determined

at different intervals from day 0 to 96 hours based on the values obtained from the cell concentration using the equation (B.3) proposed by Arredondo et al., 2017:

$$\mu = \frac{ln\,N2 - ln\,N1}{t2 - t1} \quad \text{.....}$$

(B.3)

where N1 and N2 are the cell density values at times t1 and t2, respectively. Cell densities were calculated using the formula mentioned above in equation 1 at different time intervals from day 0 to day 96 hours.

Maximum specific growth (μ_{max}) was calculated using the different μ values calculated for all the samples. Cell doubling time was also calculated using the following equation (B.4) (Sankar et al., 2011):

$$t_d = \frac{ln2}{\mu_{max}} \quad \text{.....}$$

(B.4)

B.1.4 Determination of Chlorophyll a & b and Carotenoid

Chlorophyll a and b, colourful pigments, are found in plants and algal cells. Chlorophyll a (green pigment) plays an important role in the photosynthesis process. Chlorophyll b (also a green pigment) absorbs blue-violet wavelength light. Carotenoid, pigments help in the process of photosynthesis. At t=96 hours, 10 mL algae suspension was collected and centrifuged at 4,000 ×g for 10 min. After the removal of the supernatant, 2.5 mL of 80% (V/V) acetone was added and extracted. The extracts were then centrifuged at 10,000 ×g for 10 min. The supernatant was analyzed for optical density at 350–700 nm light wavelength continuously (UV/Visible spectrophotometer, Hitachi). The contents of chl-a and chl -b, and carotenoids were calculated according to equations (B.5-B.7) given in the Xiong et al. (2005) study:

$$Chlorophyll\ a, C_A = \left(12.7\ OD_{663} - 2.69\ OD_{645}\right) \text{..... (B.5)}$$

$$Chlorophyll\ b, C_B = \left(22.9\ OD_{645} - 4.68\ OD_{633}\right) \text{..... (B.6)}$$

$$Carotenoid, C_k = \left(1000\ OD_{440} - 1.9\ C_A - 63.14\ C_B\right) \div 214 \text{..... (B.7)}$$

where, C_A (µg/ml) and C_B (µg/ml) are contents of chl-a and chl-b, and C_K (µg/ml) is content of carotenoids.

B.1.5 Protein content

Algal cells are said to have high amount of proteins, amino acids and lipids. For protein content determination, the Bradford assay was followed (Bradford, 1975). Bradford reagent was made using 50 mg of Coomassie Brilliant Blue G-250 in 50 mL methanol. Then, 100 mL of 85 % phosphoric acid was taken and transferred to make the volume 1 L by using 850 mL H_2O. 10 dilutions of BSA (Bovine Serum Albumin) were prepared and standard curve was made up. Absorbance was measured at 595 nm using spectrophotometer.

B.1.6 Lipid content

Total lipid content was calculated using the Bligh and Dyer method (1959) gravimetrically. A know volume of dried biomass was taken and was washed with (2:1 v/v) Chloroform-methanol solution. The extract was then dissolved in 1 ml of chloroform and transferred into glass vial of 15 ml (pre-weighted). The extract was dried and kept in desiccator containing silica gel for 24 hours and then weighed. The value of lipid content was calculated using equation (B.8).

$$Lipid\ Content = \frac{Weight\ of\ lipid + Vial\ (final\ weight\ of\ vial) - weight\ of\ empty\ vial}{dry\ weight\ biomass} X\ 100\% \quad (B.8)$$

B.1.7 Microscopic Characterization

For the structural analysis of different samples, microscopic characterization of all the samples at t=0 and t=96 hours was done using Transmission Electron Microscopy (TEM) (instrument: JEOL 2100F). Microscopic characterisation was done to see the changes and aggregation formation inside and outside the algal cells and in the suspension. The size of NPs was determined using TEM, and the observed size of standard suspension of NPs was < 50 nm for CuO NPs and < 40 nm for ZnO NPs. Also, at 10X and 40X, the microscopic images were also taken using the table-top microscope (Olympus CX21i).

B.1.8 FTIR (Fourier Transmittance Infrared Spectroscopy)

FTIR was done to see the presence of bio component in the algal biomass. Samples (1.5 mL) were collected at t=0 and t=96 hours and centrifuged for 5 min at 10,000 rpm, followed by discarding of supernatant. 10 µl of the sample was used for the FTIR analysis (Instrument: NICOLET - IS-50, Thermo). IR spectra were recorded with transmission mode in the spectral range of 4000-800 cm^{-1}.

B.1.9 Metal and ion Content

Metal and ion content in suspension was calculated by taking sample from the flask at t=0 and t=96 hrs., digesting it with acid (3050G method (APHA, 1998)) and analysing it with ICP-MS (ICP-MS Agilent 7900). Using obtained values at T=0 and t=96 hours for all the samples, change in metal contents of suspension was calculated.

B.1.10 Measurement of hydrodynamic diameter of particles and zeta potential

For particle size measurement using DLS, the suspension was ultrasonicated (100 W, 33 ± 3 kHz) for 30 min. The suspension was then transferred in cuvettes for size determination. The DLS size (hydrodynamic diameter) was observed to be in a range of 90–400 nm for standard NPs. The variation of HDD at t=0 and t=96 hours was studied to observe change in size of nanoparticles over a period in the algal suspension. Values were measured using the dynamic

light scattering particle sizer (Nicomp Zetasizer ZLS380; wavelength= 633 nm; detector angle=173^0.

B.1.11 CAT assay

CAT assay was analysed for all the samples using the CAT assay analysing kit obtained from Sigma Aldrich (CAT-100). All the steps which were performed was as per the instructions of the kit.

B.1.12 EPS estimation and quantification

EPS extraction was done by the using ethanol method as mentioned by Gong et al., 2009, Jolanta Jaroszuk-Ściseł et al., 2020. EPS lipid, EPS protein and EPS carbohydrate was estimated using the procedure as described in above sections.

Table B.1: Composition of the OECD TG 201 media. (pH-8.1)

Component	Concentration (mg/L)
$NaHCO_3$	50.00
NH_4Cl	15.00
$MgCl_2.6H_2O$	12.00
$CaCl_2.2H_2O$	18.00
$MgSO_4.7H_2O$	15.00
KH_2PO_4	1.60
$FeCl_3.6H_2O$	0.0640
$Na_2EDTA.2H_2O$	0.100
H_3BO_3	0.185
$MnCl_2.4H_2O$	0.415
$ZnCl_2$	0.00300
$CoCl_2.6H_2O$	0.00150
$Na_2MnO_4.2H_2O$	0.00700
$CuCl_2.\ 2H_2O$	0.00001

Table B.2: Following is the BG-11 media composition. (Hong et al., 2016)

BG-11 media composition

A) Stock solutions for BG-11

Stock solution -1

Na_2MG EDTA	0.1 gm/L
Ferric ammonium citrate	0.6 gm/L
Citric acid. 1 H_2O	0.6 gm/L
$CaCl_2$. 2 H_2O	3.6 gm/L

Autoclave the solution.

Stock solution -2

$MgSO_4$. 7 H_2O	7.5 gm/L

Autoclave the solution.

Stock solution-3

K_2HPO_4	3.05 gm/L

Autoclave the solution.

Stock solution – 4

H_3BO_3	2.86 gm/L
$MnCl_2$. $4H_2O$	1.81 gm/L

$ZnSO_4$. $7H_2O$	0.22 gm/L
$CuSO_4$. $5H_2O$	0.079 gm/L
$CoCl_2$. $6H_2O$	0.050 gm/L
$NaMoO_4$. $2H_2O$	0.391 gm/L

Autoclave the solution.

As per the method of paper Hong et al., 2016 and Pandey et al., 2023; Air or carbon dioxide has been provided to the algal culture externally.

Appendix C: Results

C.1 Effect on algae pigments

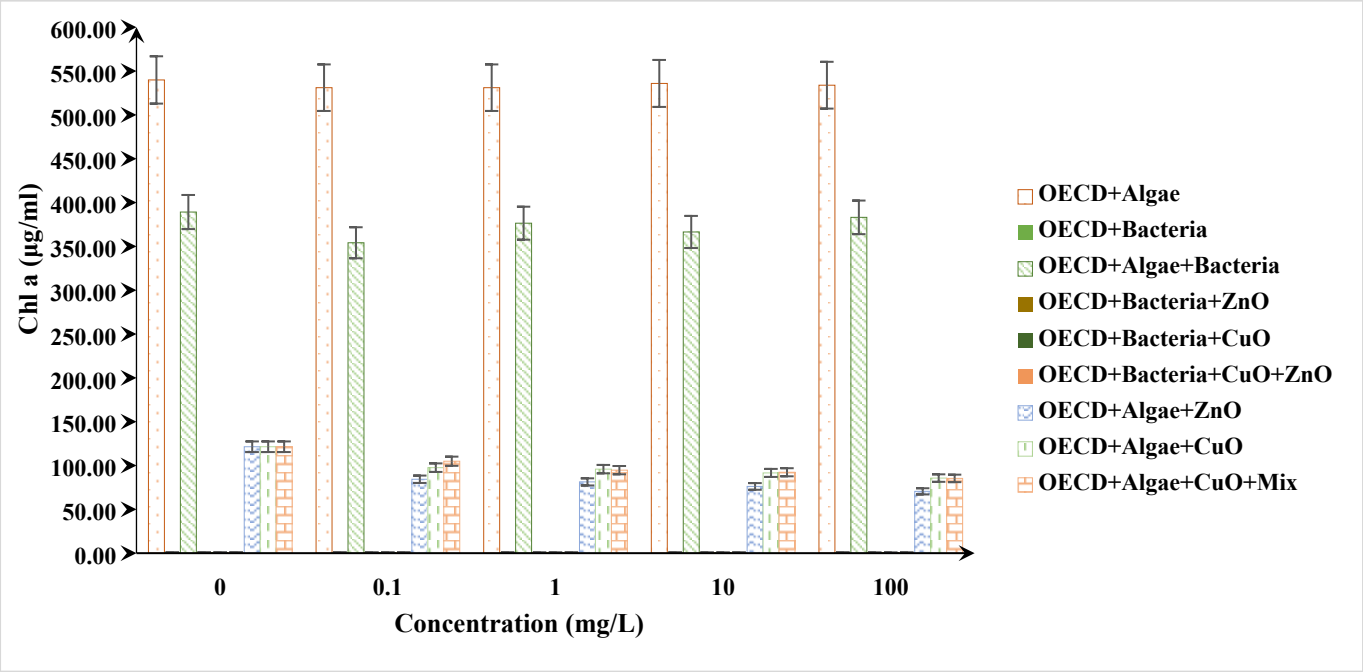

Figure C.1.1: Chl a content in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:100).

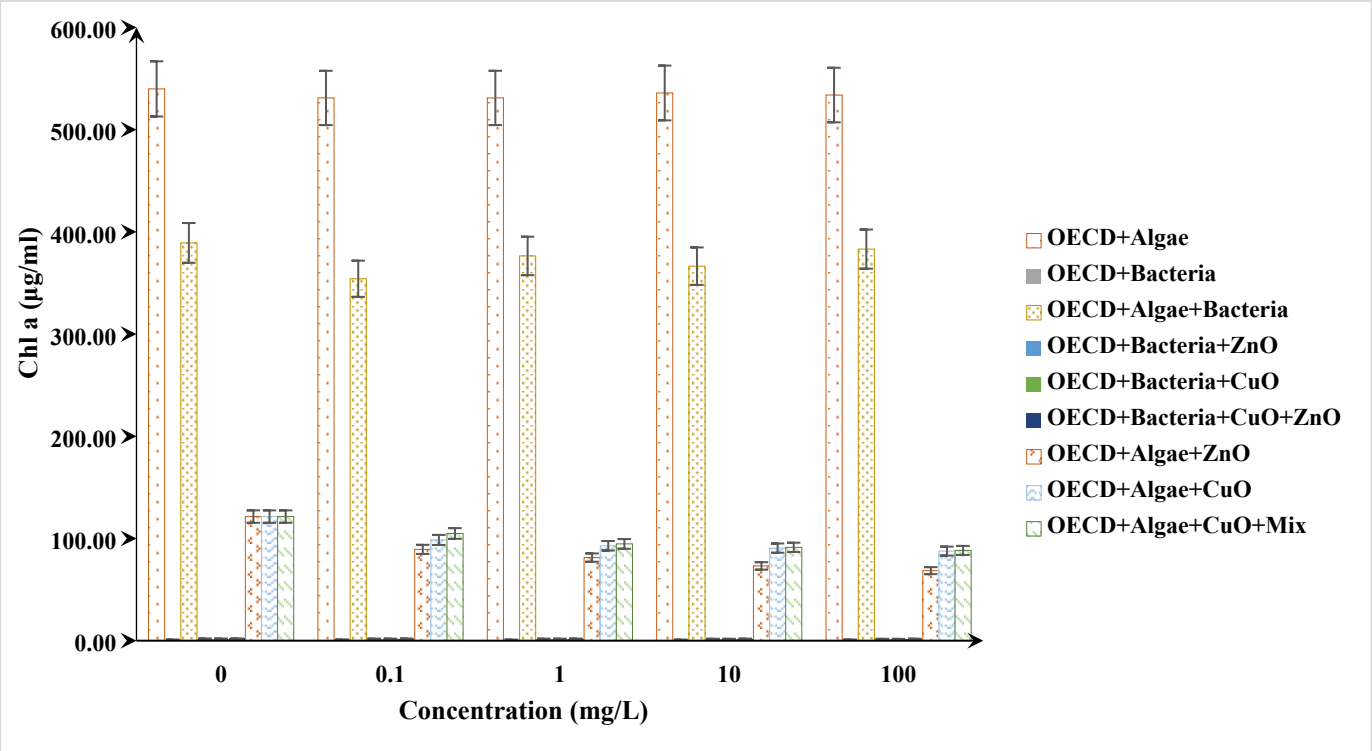

Figure C.1.2: Chl a content in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:1).

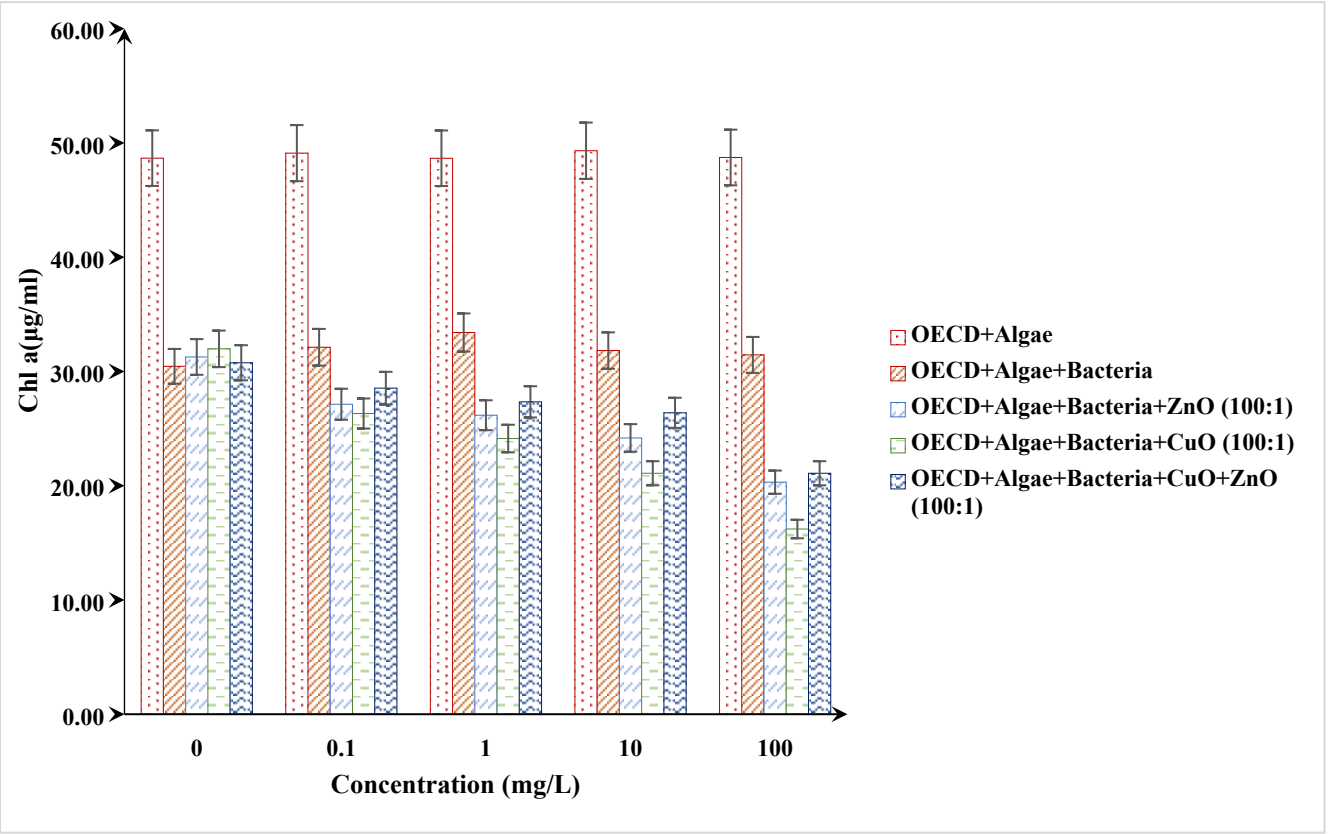

Figure C.1.3: Chl b content in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (100:1).

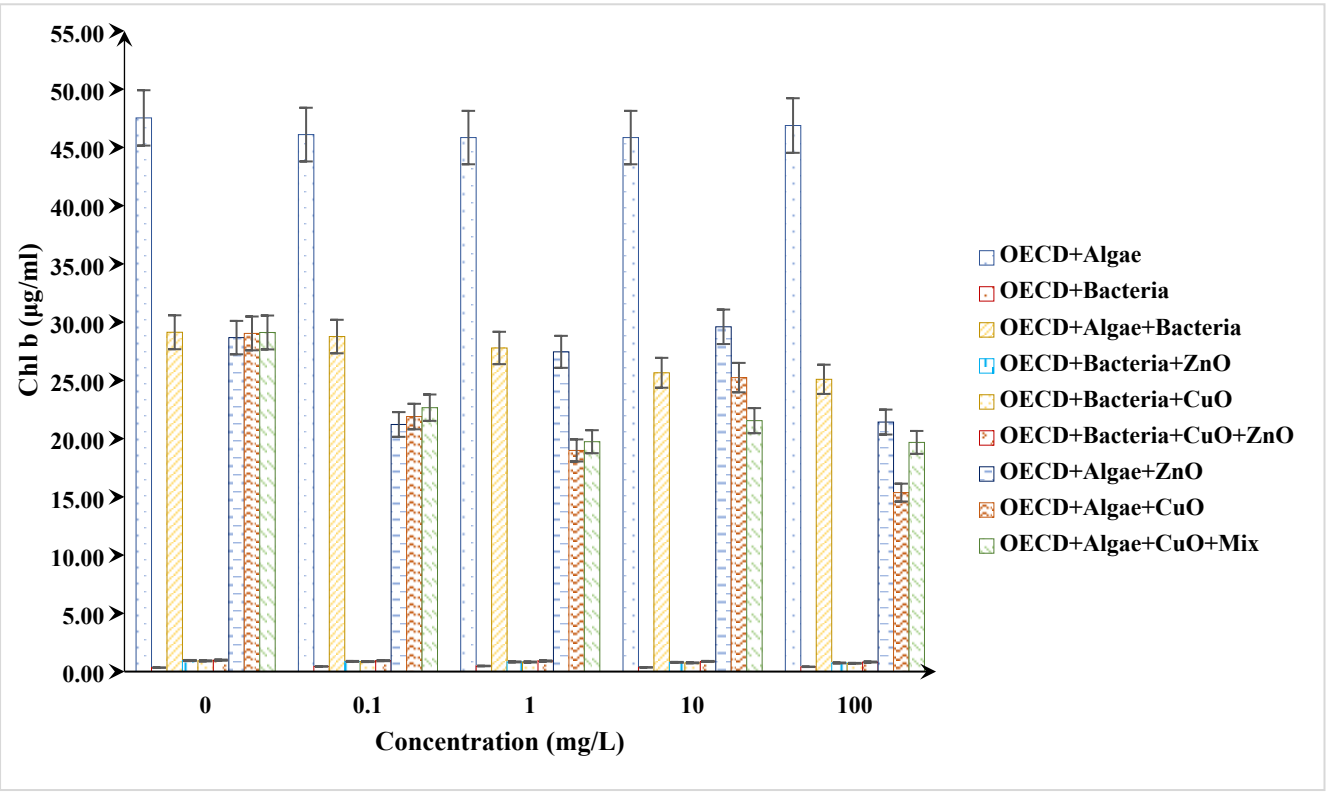

Figure C.1.4: Chl b content in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:100).

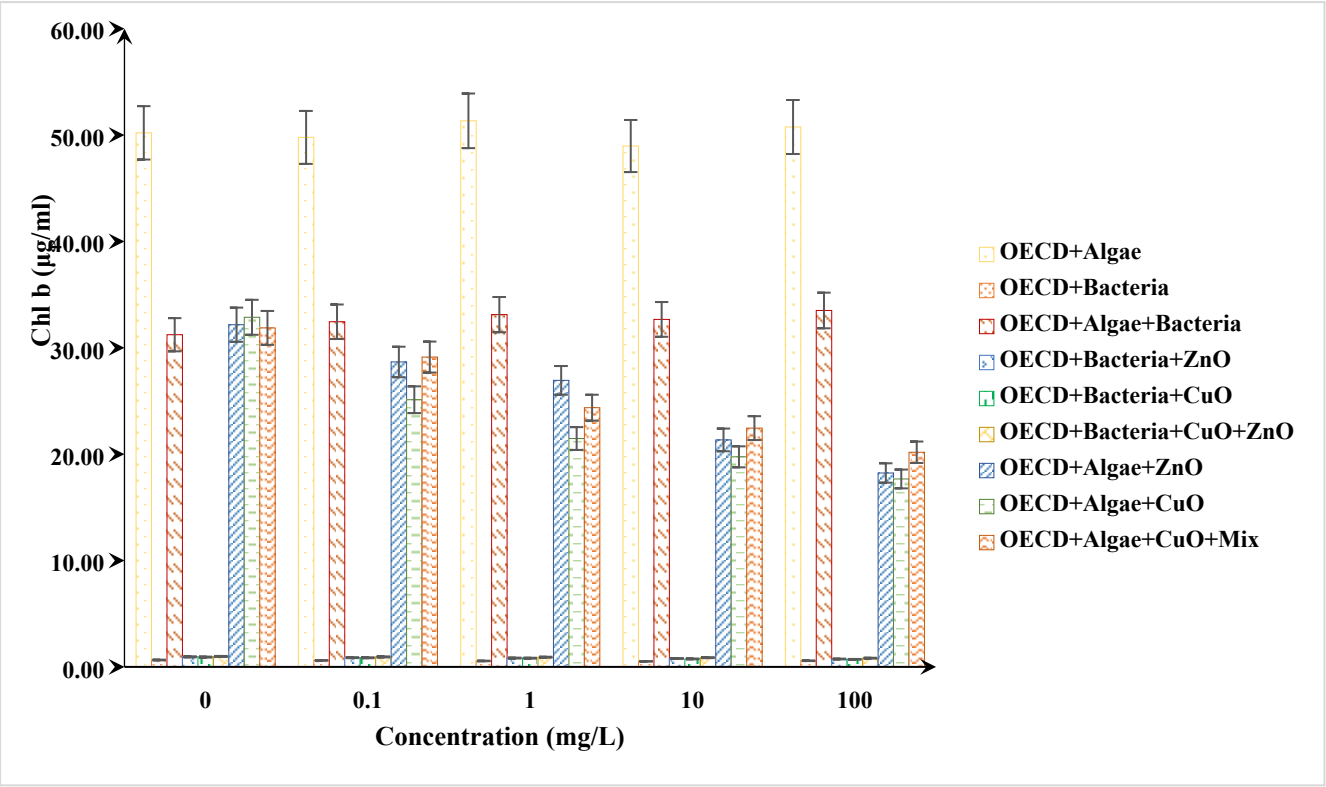

Figure C.1.5: Chl b content in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:1).

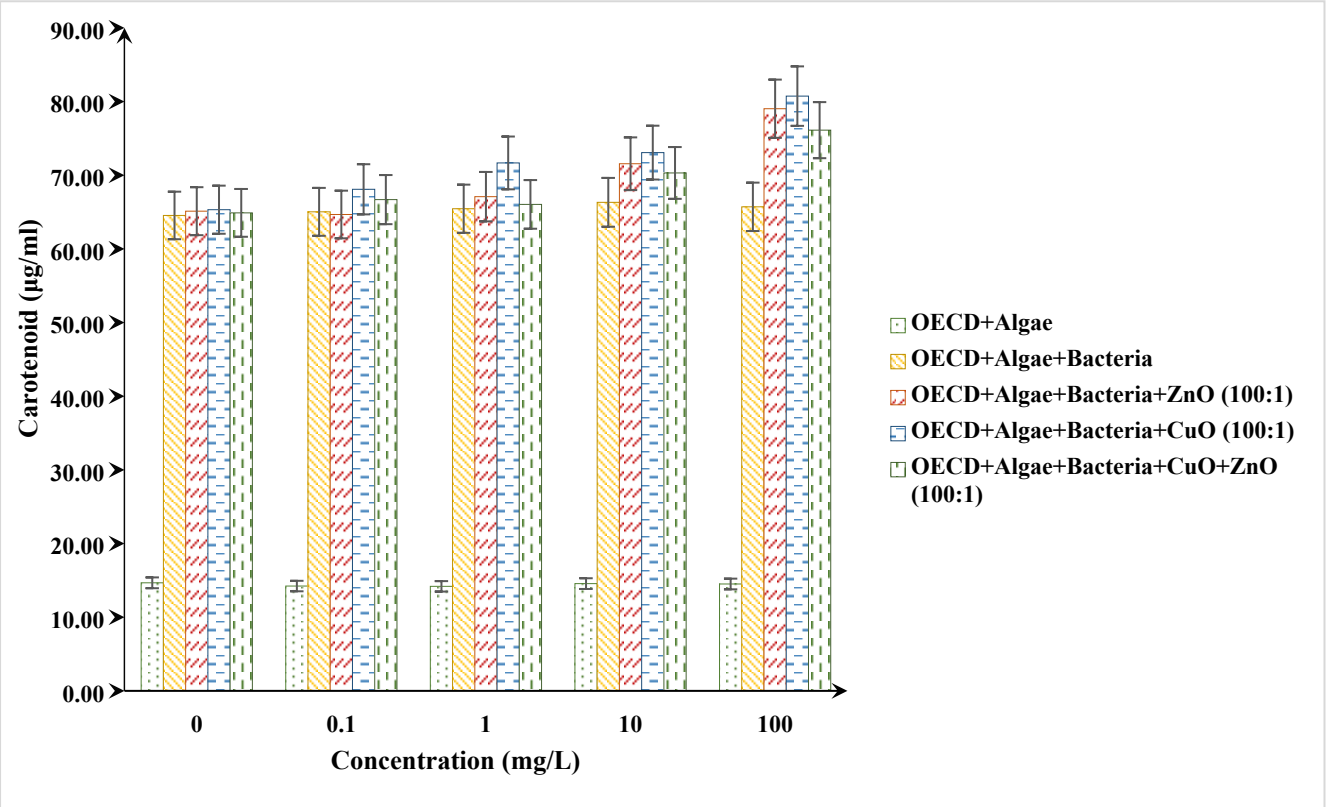

Figure C.1.6: Carotenoid content in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:100).

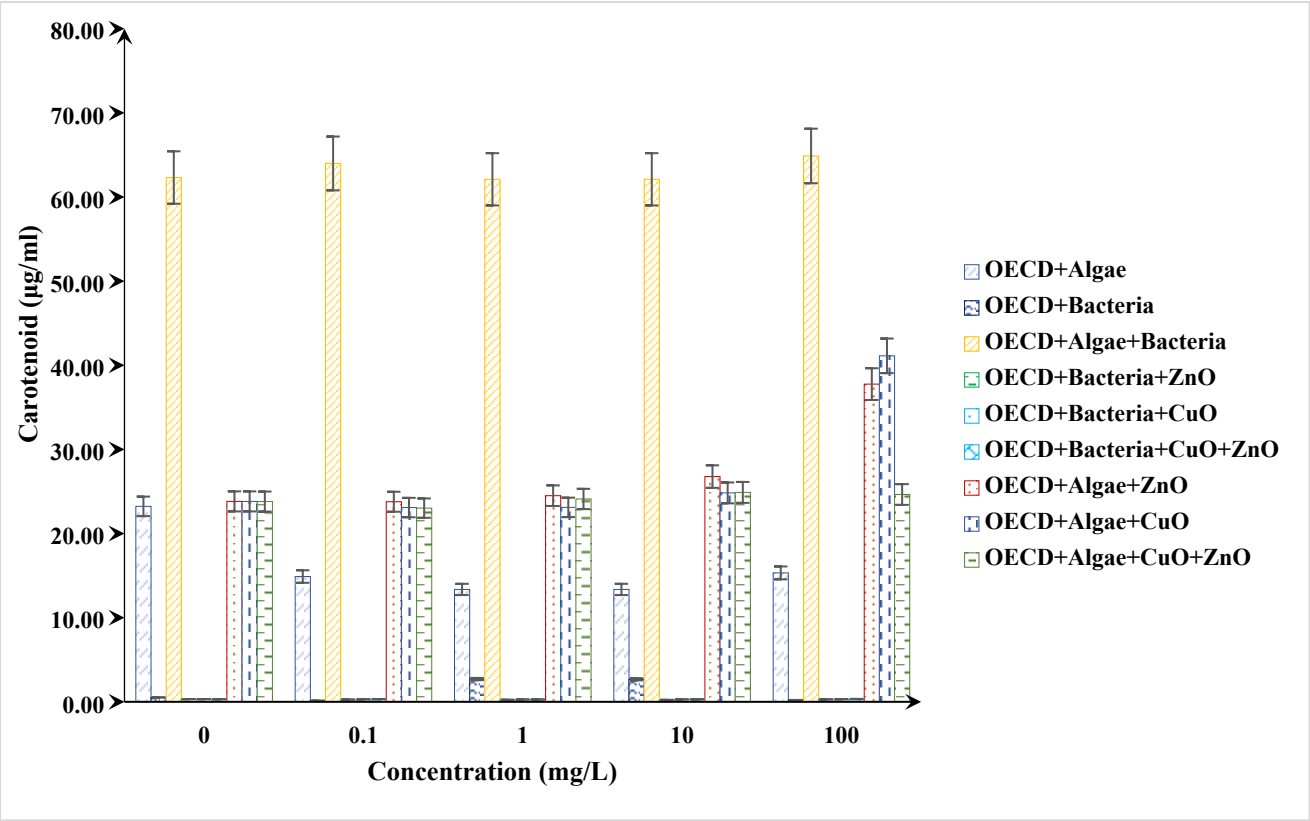

Figure C.1.7: Carotenoid content in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:100).

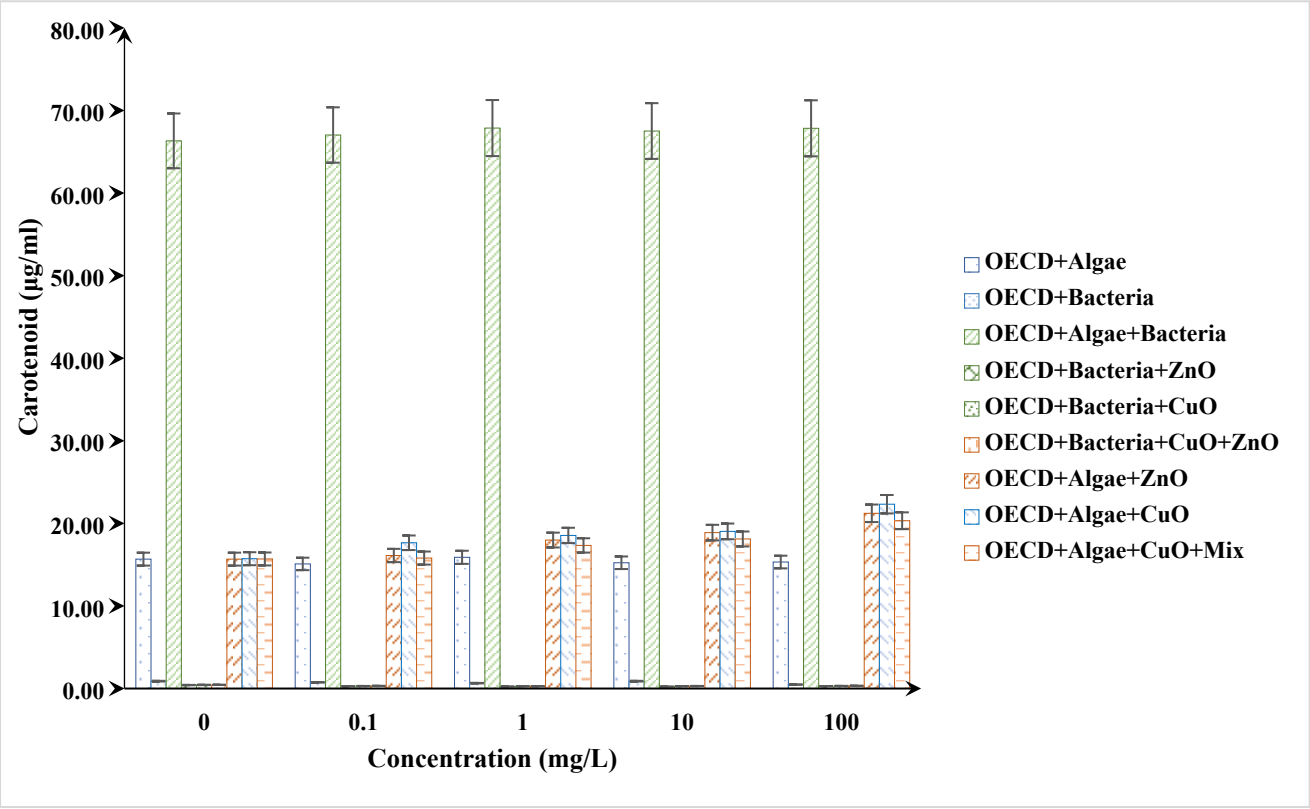

Figure C.1.8: Carotenoid content in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:1).

C.2 Effect on algal and bacterial cell count and biomass

Table C.1. Calculation of % growth reduction difference for algal cell count for 100:1 ratio.

Conc. (mg/L)	%GR ZnO	%GR CuO	%GR Dual	% $(GR)_{cal}$	% $(GR)_{obs}$	%GR $(GR)_{diff}$	Remark
0.1+0.1	35.05	38.64	27.27	73.69	27.27	46.42	Antagonistic
1+1	41.08	45.76	32.65	86.84	32.65	54.19	Antagonistic
10+10	51.09	55.76	42.93	106.85	42.93	63.92	Antagonistic
100+100	66.23	69.63	61.90	135.86	61.90	73.96	Antagonistic

(Antagonistic as $\%(GR)_{diff}$=Positive)

Table C.2: Calculation of Standard Error Difference for algal cell count.

Conc. (mg/L)	SE ZnO	SE CuO	SE Dual	$(SE)_{cal}$	$(SE)_{obs}$	$(SE)_{diff}$
0.1+0.1	2.43	4.52	3.67	6.95	3.67	7.859
1+1	3.56	5.43	4.02	8.99	4.02	9.848
10+10	3.99	6.43	4.87	10.42	4.87	11.502
100+100	5.78	8.34	6.88	14.12	6.88	15.707

Table C.3: Calculation of t-statistics for algal cell count.

Conc. (mg/L)	% $(GR)_{cal}$	% $(GR)_{obs}$	$(SE)_{diff}$	t_{cal}	t_{obs}	Remark
0.1+0.1	73.69	27.27	7.859	9.377	3.47	Significant
1+1	86.84	32.65	9.848	8.818	3.32	Significant
10+10	106.85	42.93	11.502	9.290	3.73	Significant
100+100	135.86	61.90	15.707	8.650	3.94	Significant

(Significant as $t_{cal} > t_{obs}$)

Table C.4. Calculation of % growth reduction difference for bacterial cell count.

Conc. (mg/L)	%GR ZnO	%GR CuO	%GR Dual	% $(GR)_{cal}$	% $(GR)_{obs}$	%GR $(GR)_{diff}$	Remark
0.1+0.1	16.33	18.42	10.53	34.75	10.53	24.22	Antagonistic
1+1	18.65	23.45	13.64	42.10	13.64	28.46	Antagonistic
10+10	23.65	27.04	16.92	50.69	16.92	33.77	Antagonistic
100+100	26.09	30.89	18.67	56.98	18.67	38.31	Antagonistic

(Antagonistic as $\%(GR)_{diff}$=Positive)

Table C.5: Calculation of Standard Error Difference for bacterial cell count.

Conc. (mg/L)	SE ZnO	SE CuO	SE Dual	$(SE)_{cal}$	$(SE)_{obs}$	$(SE)_{diff}$
0.1+0.1	3.45	4.06	5.43	7.51	5.43	9.27
1+1	4.98	6.56	6.83	11.54	6.83	13.41
10+10	6.09	9.45	7.98	15.54	7.98	17.47
100+100	8.72	11.23	9.32	19.95	9.32	22.02

Table C.6: Calculation of t-statistics for bacterial cell count.

Conc. (mg/L)	% $(GR)_{cal}$	% $(GR)_{obs}$	$(SE)_{diff}$	t_{cal}	t_{obs}	Remark
0.1+0.1	34.75	10.53	9.27	3.75	1.14	Significant
1+1	42.10	13.64	13.41	3.14	1.07	Significant
10+10	50.69	16.92	17.47	2.90	0.968	Significant
100+100	56.98	18.67	22.02	2.59	0.848	Significant

(Significant as $t_{cal} > t_{obs}$)

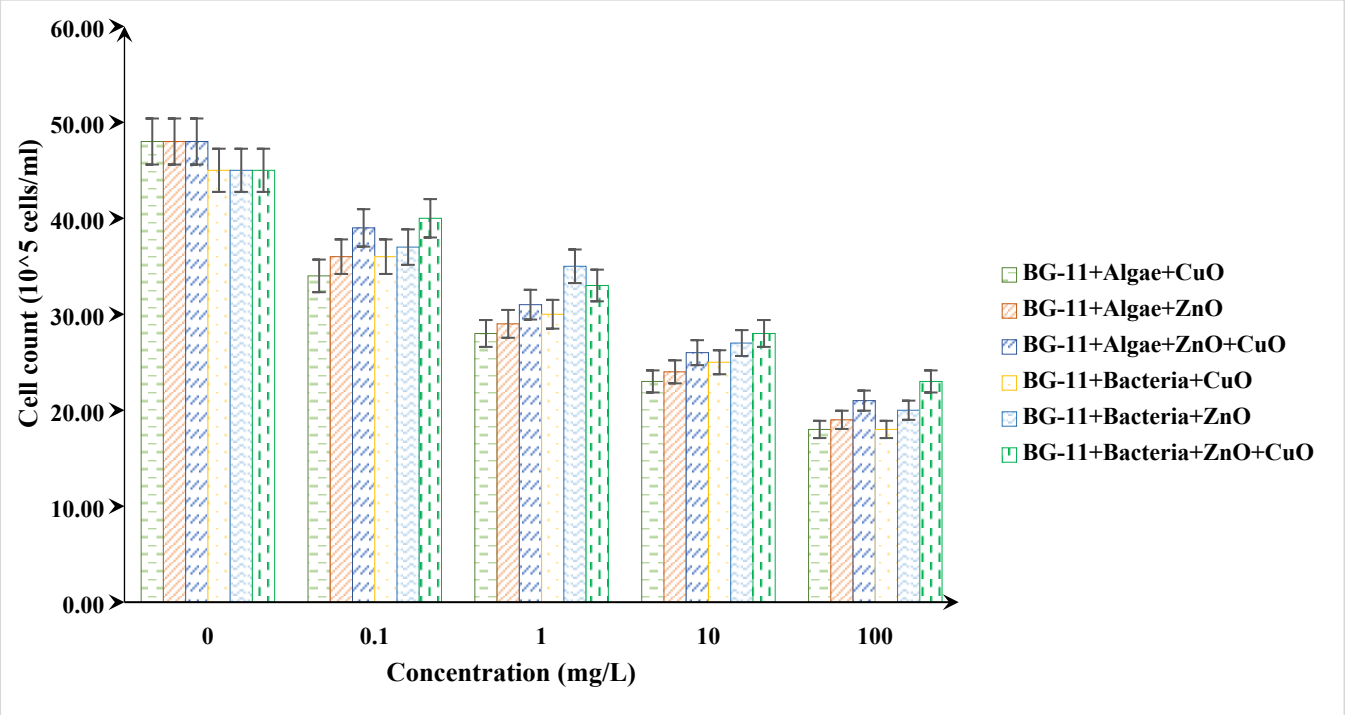

Figure C.2.1: Cell count in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (100:1).

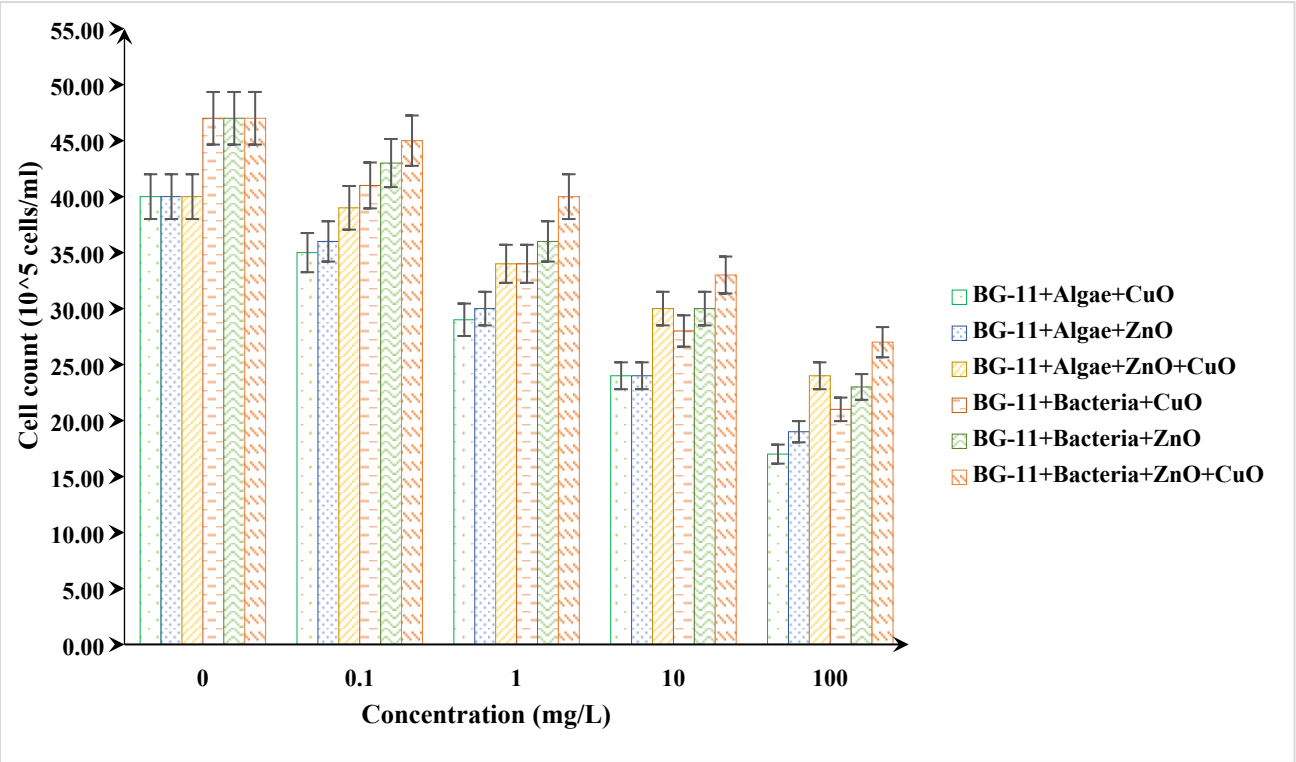

Figure C.2.2: Cell count in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:100).

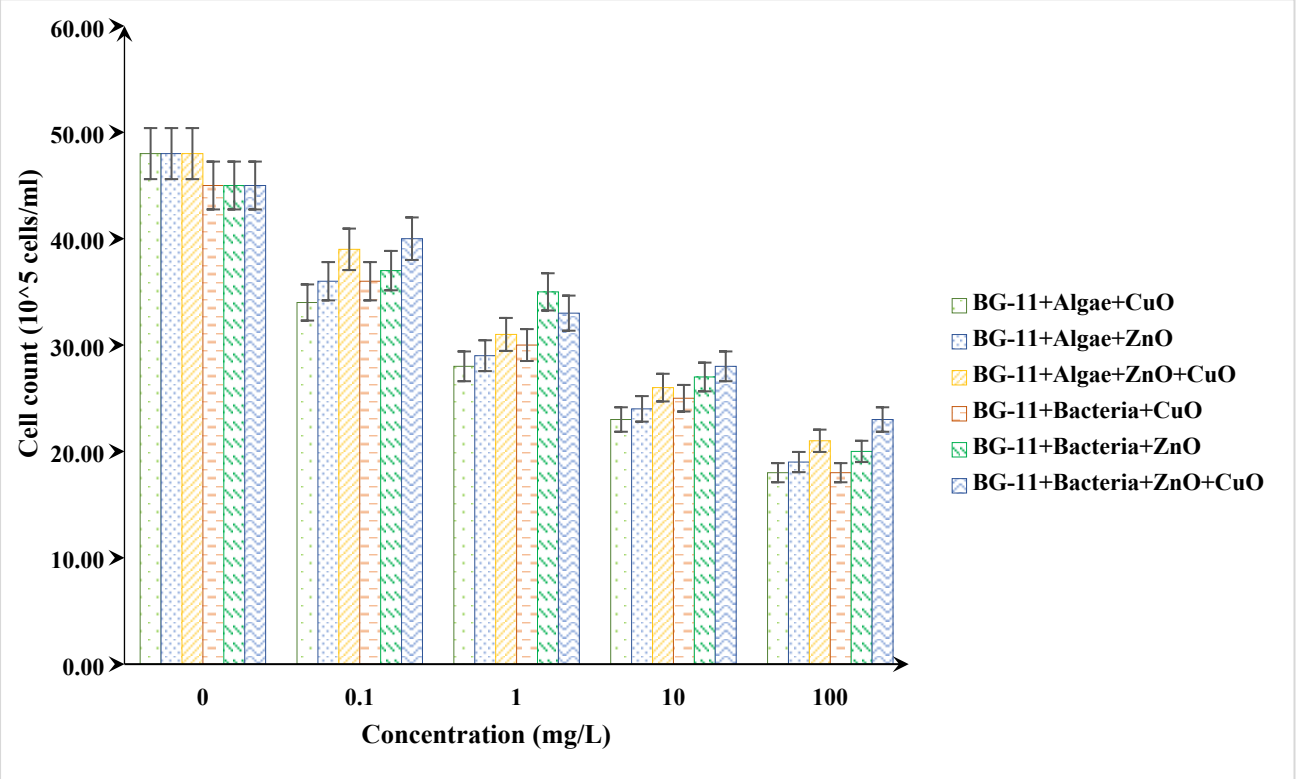

Figure C.2.3: Cell count in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:1).

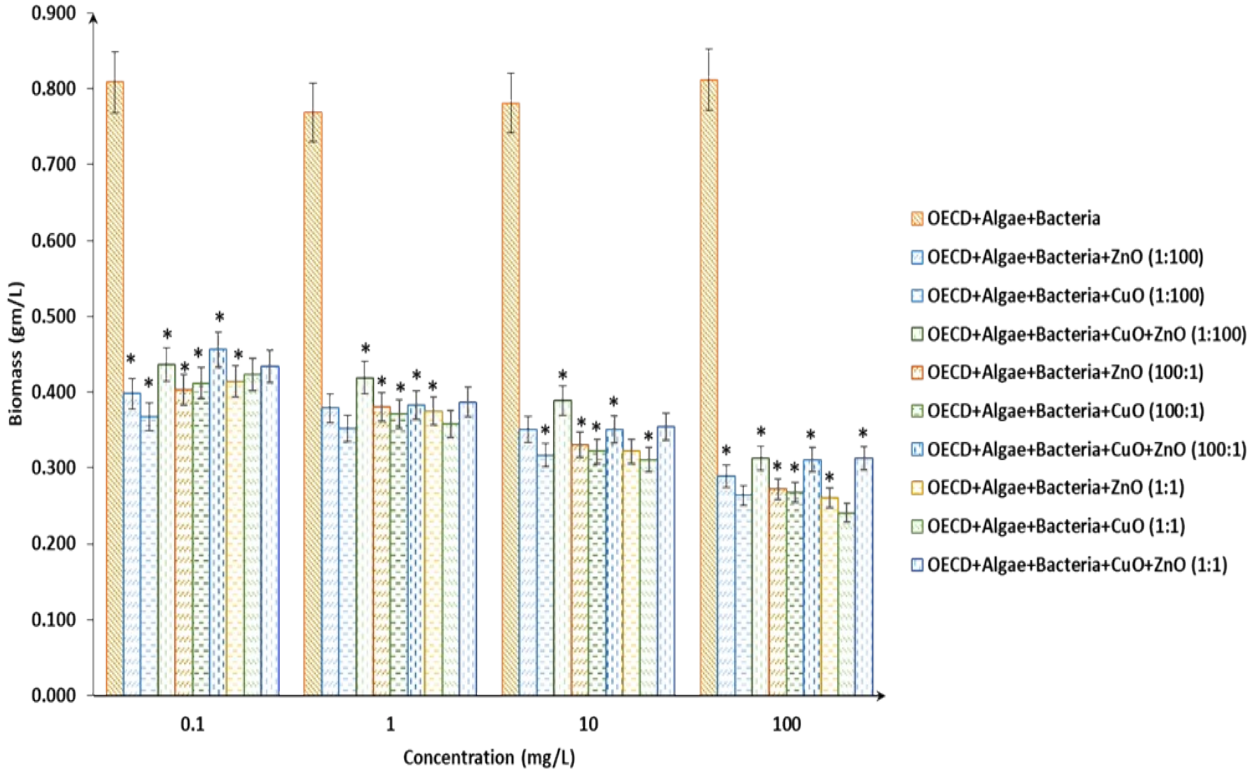

Figure C.2.4: Biomass in the various samples containing algae+bacteria at different concentration (0, 0.1, 1, 10 and 100 mg/L) of ZnO, CuO and ZnO+CuO NPs in the OECD media at after 96 hours at different ratios. *Error bars indicate one standard deviation value of three replicates around average values;* ***:** *significance ($p < 0.05$).*

Figure C.3 shows the total biomass of various samples at different concentrations of ZnO, CuO and CuO+ZnO NPs after 96 hours at different ratios. At 0.1 mg/L concentration of NPs in the mixture samples, the decrease in the total biomass after 96 hours in OECD media at different ratio of algae-bacteria (1:100, 100:1, 1:1) was found to be 44.74±1.31%, 43.91±0.67% and 46.85±1.11% respectively. At 1 mg/L concentration of NPs in the mixture samples, the reduction in the total biomass after 96 hours in OECD media at different ratio of algae-bacteria (1:100, 100:1, 1:1) was found to be 45.51±1.23%, 53.12±1.73% and 51.99±0.93% respectively. At 100 mg/L concentration of NPs in the mixture samples, decrease in the total biomass after 96 hours in OECD media at different ratio of algae-bacteria (1:100, 100:1, 1:1) was found to be 61.45±1.83%, 62.67±0.63% and 61.51±0.62% respectively. For the samples containing OECD media, one-way ANOVA result shows effect of ratio on mixture of NPs ($p<0.05$) indicating effect of various concentration on total biomass having in all the three ratios in OECD media. 3-way ANOVA shows that, there was a significant effect of concentration, nanoparticles type and ratio on total biomass.

For the single NPs (CuO NPs and ZnO NPs) in algae-bacteria samples at different ratios (0, 0.1, 1, 10 and 100 mg/L) at different concentrations after 96 hours, total biomass shown in Figure 5. For 0.1 mg/L concentration, decrease in the total biomass in the samples containing CuO NPs in OECD media having algae-bacteria at different concentration (1:100, 100:1, 1:1) was observed to be 54.51±1.63%, 49.32±0.34% and 47.97±1.37% respectively. For 0.1 mg/L of ZnO NPs, the reduction in total biomass was found to be 50.80±1.18%, 50.06±2.13% and 49.57±1.12% respectively. For 100 mg/L concentration, the decrease in the total biomass in the samples containing CuO NPs in OECD media having algae-bacteria at different concentration (1:100, 100:1, 1:1) was observed to be 67.49±1.06%, 67.83±1.93% and 70.36±1.18% respectively. For 100 mg/L concentration, the reduction in the total biomass in the samples containing ZnO NPs in OECD media having algae-bacteria at different concentration (1:100, 100:1, 1:1) was observed to be 64.41±2.13%, 67.35±1.19% and 67.89±1.59% respectively. For the samples containing algae-bacteria in the OECD media, one-way ANOVA shows the significant effect on the total biomass on all the concentrations at different ratios. 3-way ANOVA results shows that there was significant effect on total biomass w.r.t concentration, ratio, and nanoparticles. The maximum decrease in the sample in total biomass was observed to be in the ratio 1:1 followed by 100:1 and 1:100 in all the samples containing single and mixture of NPs.

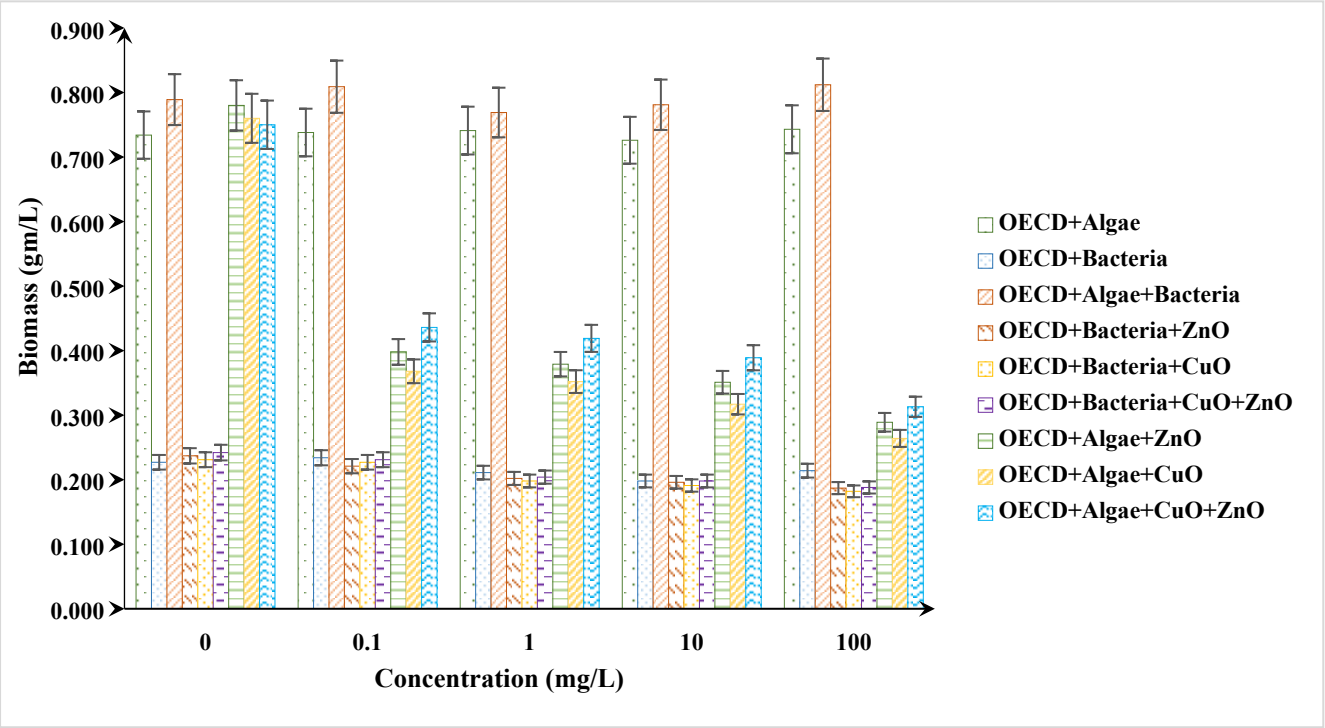

Figure C.2.5: Biomass in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:100).

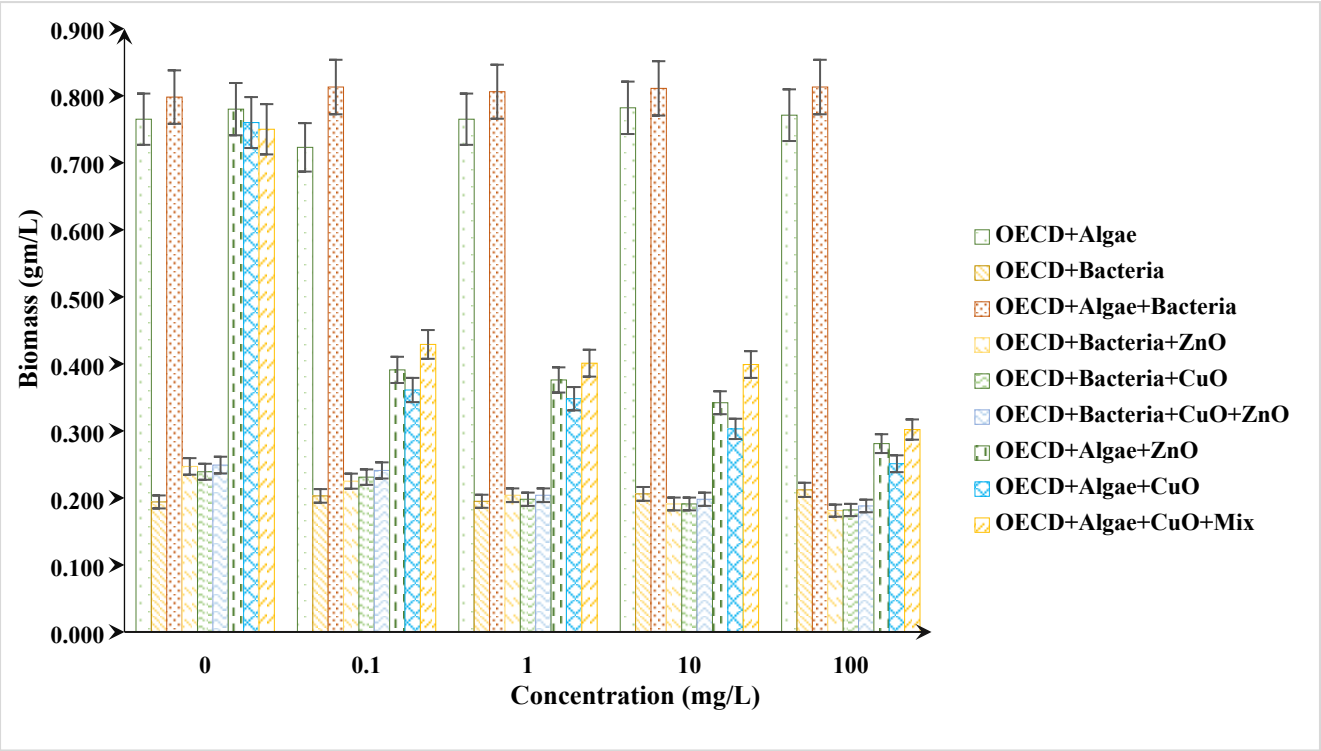

Figure C.2.6: Biomass in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:1).

3.3. Effect on algae-bacteria consortia

Lipid and Protein content

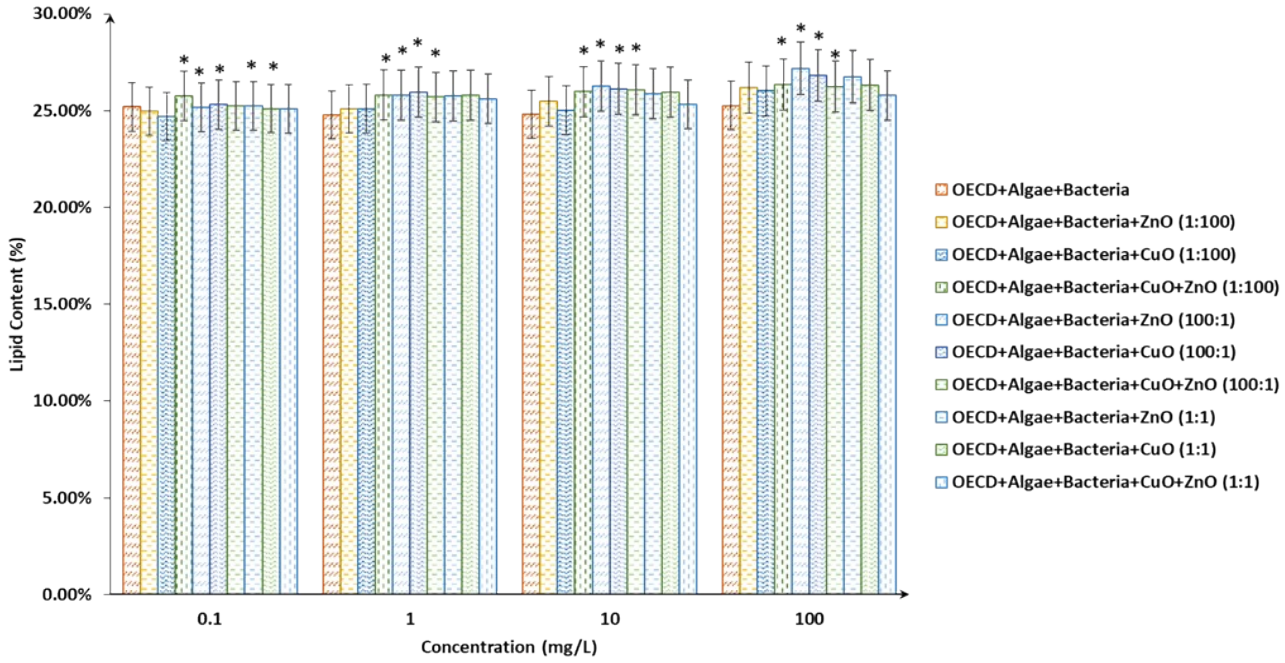

Figure C.3.1: Lipid content (%) in the various samples containing algae+bacteria at different concentration (0, 0.1, 1, 10 and 100 mg/L) of ZnO, CuO and ZnO+CuO NPs in the OECD media at after 96 hours at different ratios. *Error bars indicate one standard deviation value of three replicates around average values;* ***:** *significance ($p < 0.05$).*

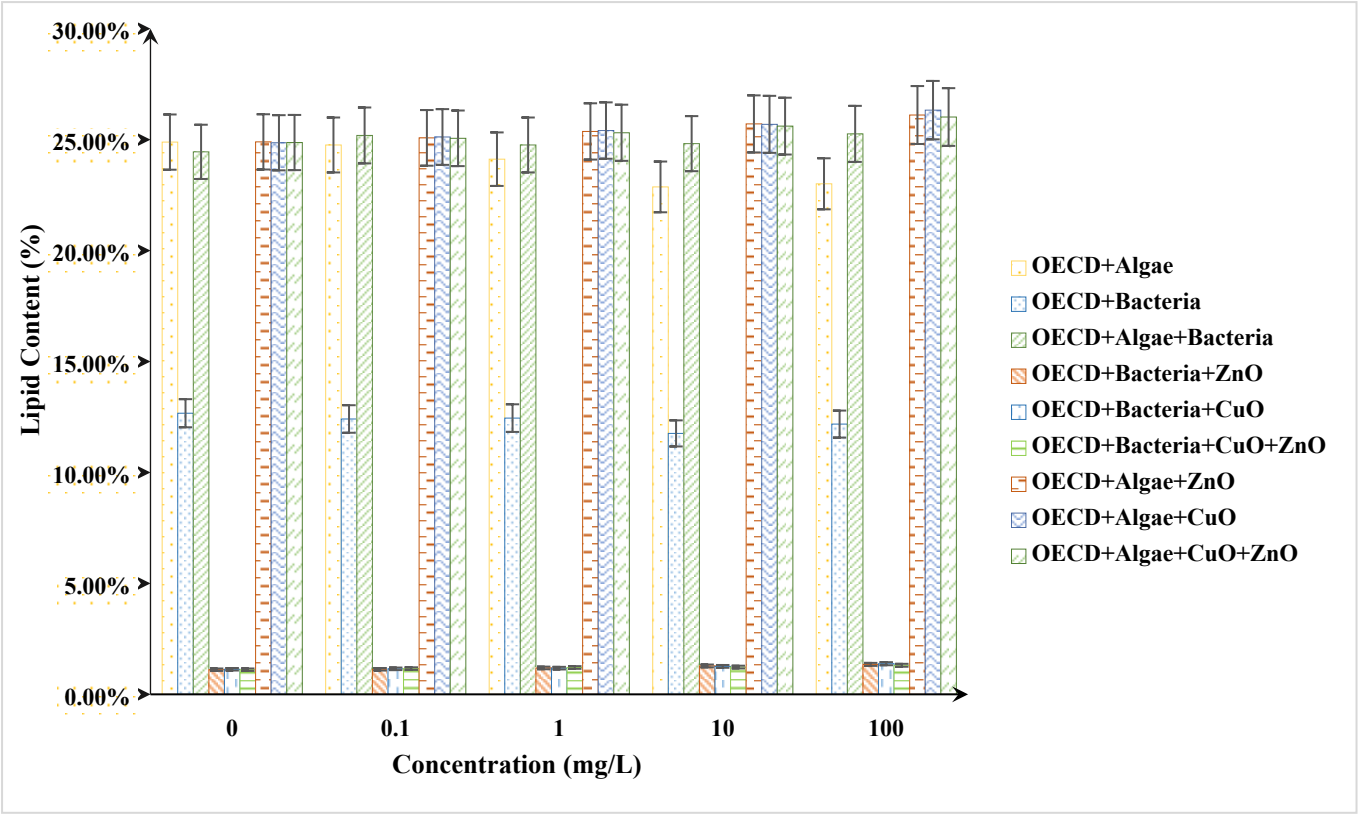

Figure C.3.2: Lipid content in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:100).

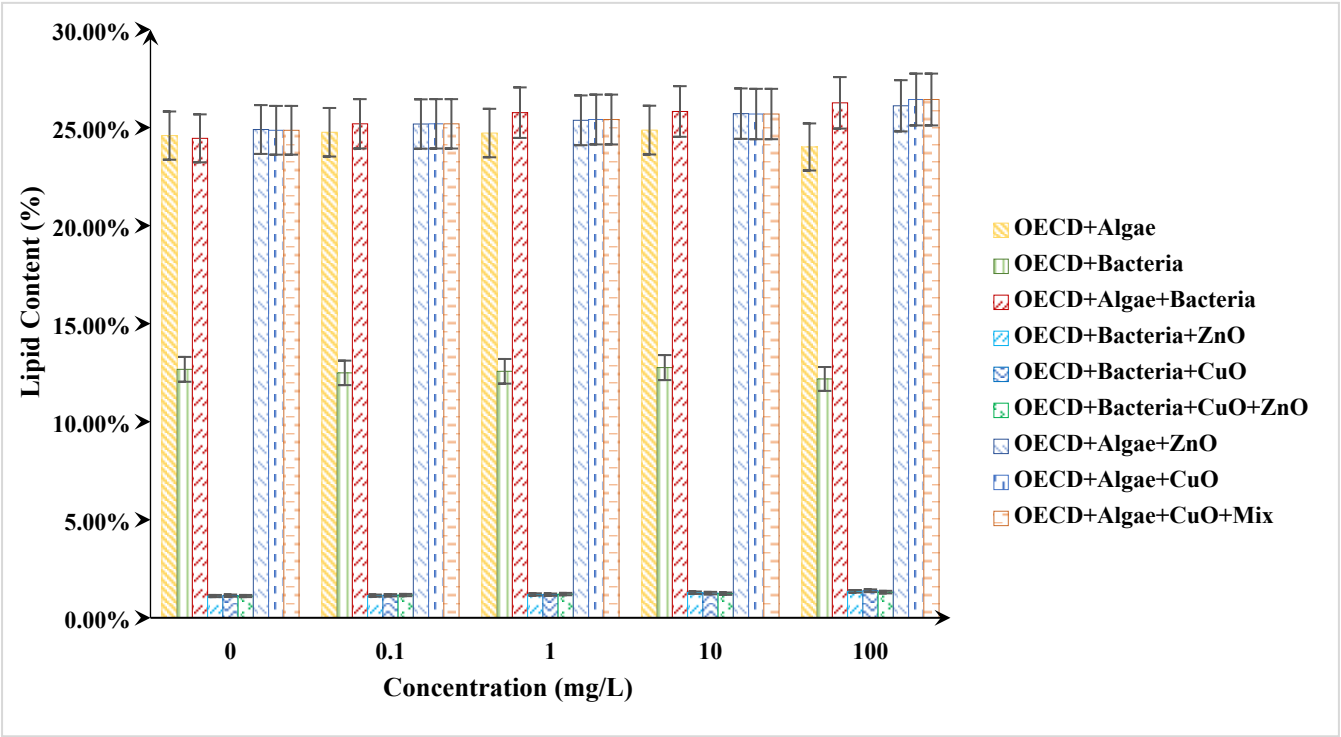

Figure C.3.3: Lipid content in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:1).

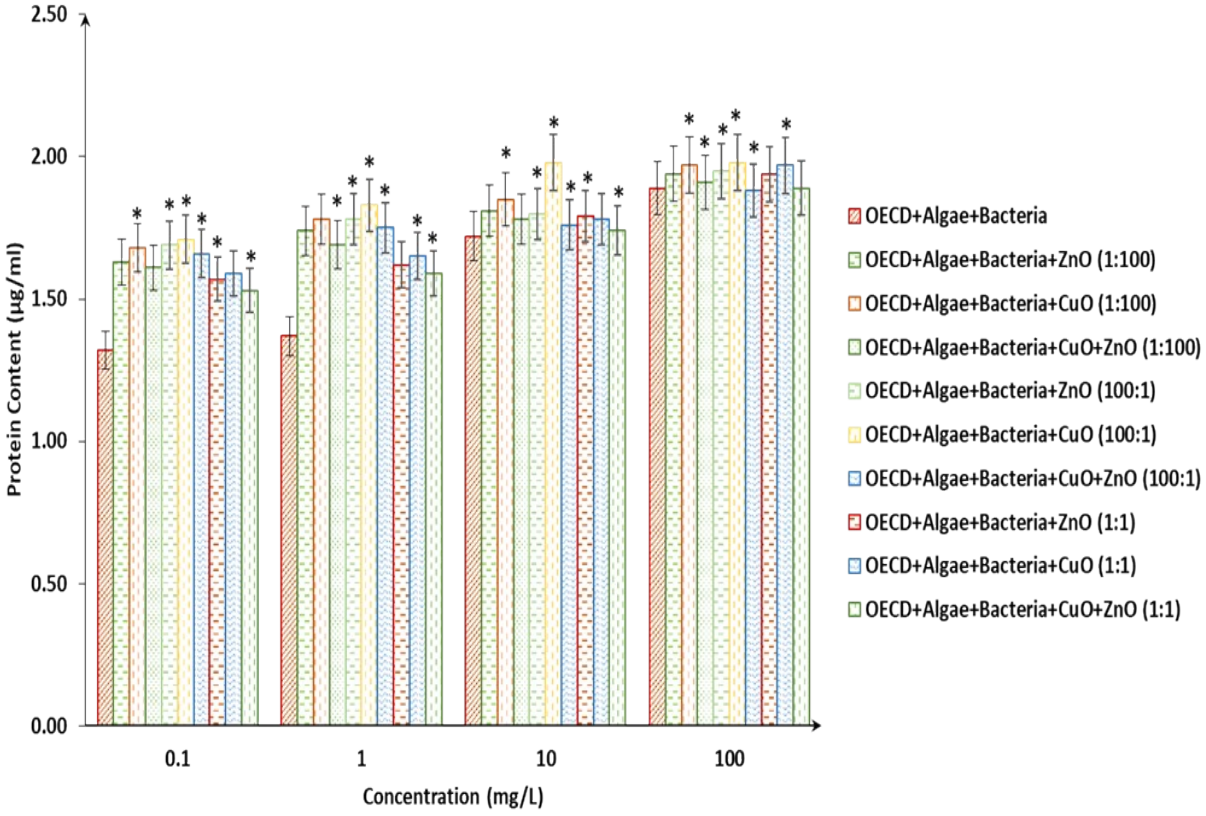

Figure C.3.4: Protein content in the various samples containing algae+bacteria at different concentration (0, 0.1, 1, 10 and 100 mg/L) of ZnO, CuO and ZnO+CuO NPs in the OECD media at after 96 hours at different ratios. *Error bars indicate one standard deviation value of three replicates around average values;* ***:** *significance ($p < 0.05$).*

Figure C.4 shows the protein content in all the samples at different ratios. At 0.1 mg/L concentration of NPs in the mixture samples, the increase in the protein content after 96 hours in OECD media at different ratio of algae-bacteria (1:100, 100:1, 1:1) was found to be 18.02±1.63%, 23.49±1.15% and 7.19±1.73% respectively. At 1 mg/L concentration of NPs in the mixture samples, the increase in the protein content after 96 hours in OECD media at different ratio of algae-bacteria (1:100, 100:1, 1:1) was found to be 18.94±1.64%, 21.14±1.53% and 10.06±2.09% respectively. At 100 mg/L concentration of NPs in the mixture samples, increase in the protein content after 96 hours in OECD media at different ratio of algae-bacteria (1:100, 100:1, 1:1) was found to be 1.05±1.49%, 22.34±1.54% and 23.28±1.47% respectively. For the samples containing OECD media, one-way ANOVA result shows effect of ratio on mixture of NPs ($p<0.05$) indicating effect of various concentration on protein content having in all the three ratios in OECD media. 3-way ANOVA shows that, there was a significant effect of concentration, nanoparticles type and ratio on protein content.

For the single NPs (CuO NPs and ZnO NPs) in algae-bacteria samples at different ratios (0, 0.1, 1, 10 and 100 mg/L) at different concentrations after 96 hours, protein content shown in Figure 7. For 0.1 mg/L concentration, increase in the protein content in the samples containing CuO NPs in OECD media having algae-bacteria at different concentration (1:100, 100:1, 1:1) was observed to be 21.43±1.74%, 25.73±1.37% and 10.69±1.39% respectively. For 0.1 mg/L of ZnO NPs, the increase in the protein content was found to be 19.02±1.41%, 25.85±2.21% and 9.55±2.31% respectively. For 100 mg/L concentration, the increase in the protein content in the samples containing CuO NPs in OECD media having algae-bacteria at different concentration (1:100, 100:1, 1:1) was observed to be 4.06±1.59%, 26.26±1.73% and 26.39±2.03% respectively. For 100 mg/L concentration, the increase in the protein content in the samples containing ZnO NPs in OECD media having algae-bacteria at different concentration (1:100, 100:1, 1:1) was observed to be 2.63±0.82%, 25.13±0.99% and 25.13±1.04% respectively. For the samples containing algae-bacteria in the OECD media, one-way ANOVA shows the significant effect on the protein content on all the concentrations at different ratios. 3-way ANOVA results shows that there was significant effect on protein content w.r.t concentration, ratio, and nanoparticles.

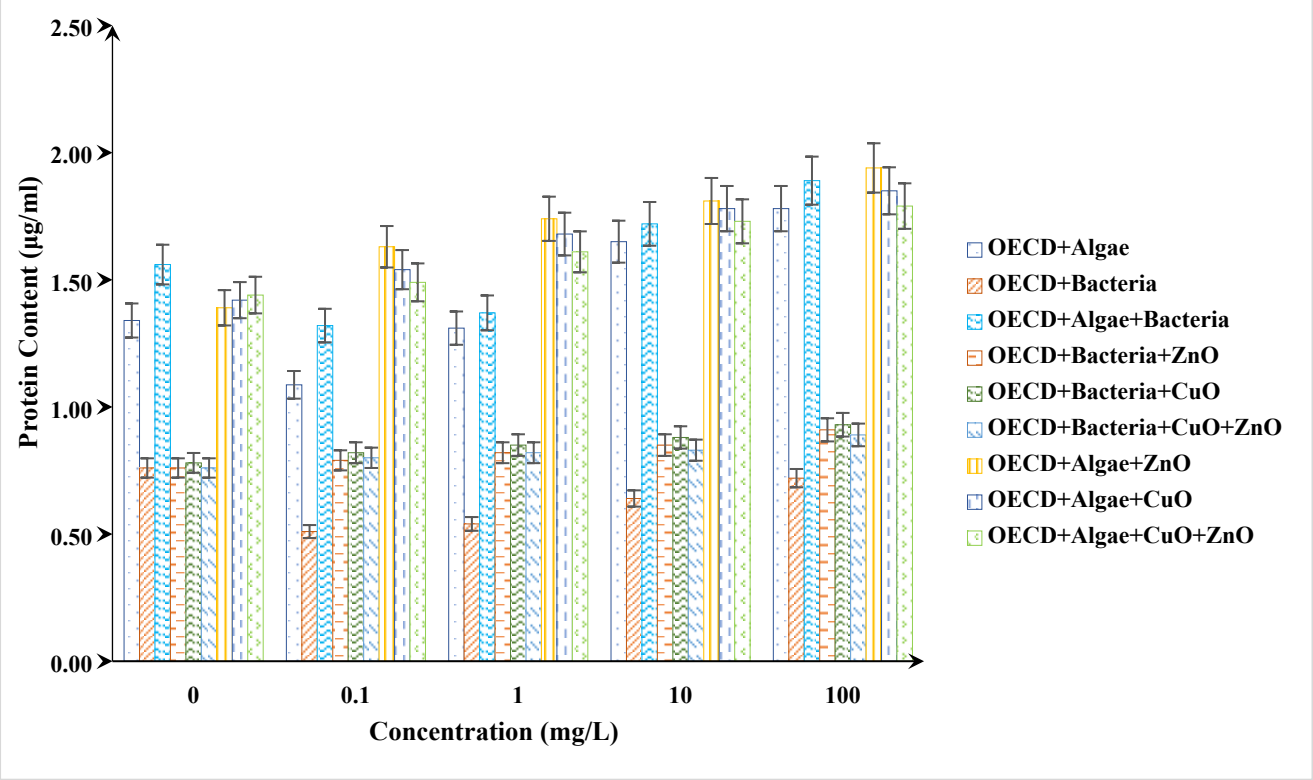

Figure C.3.5: Protein content in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:100).

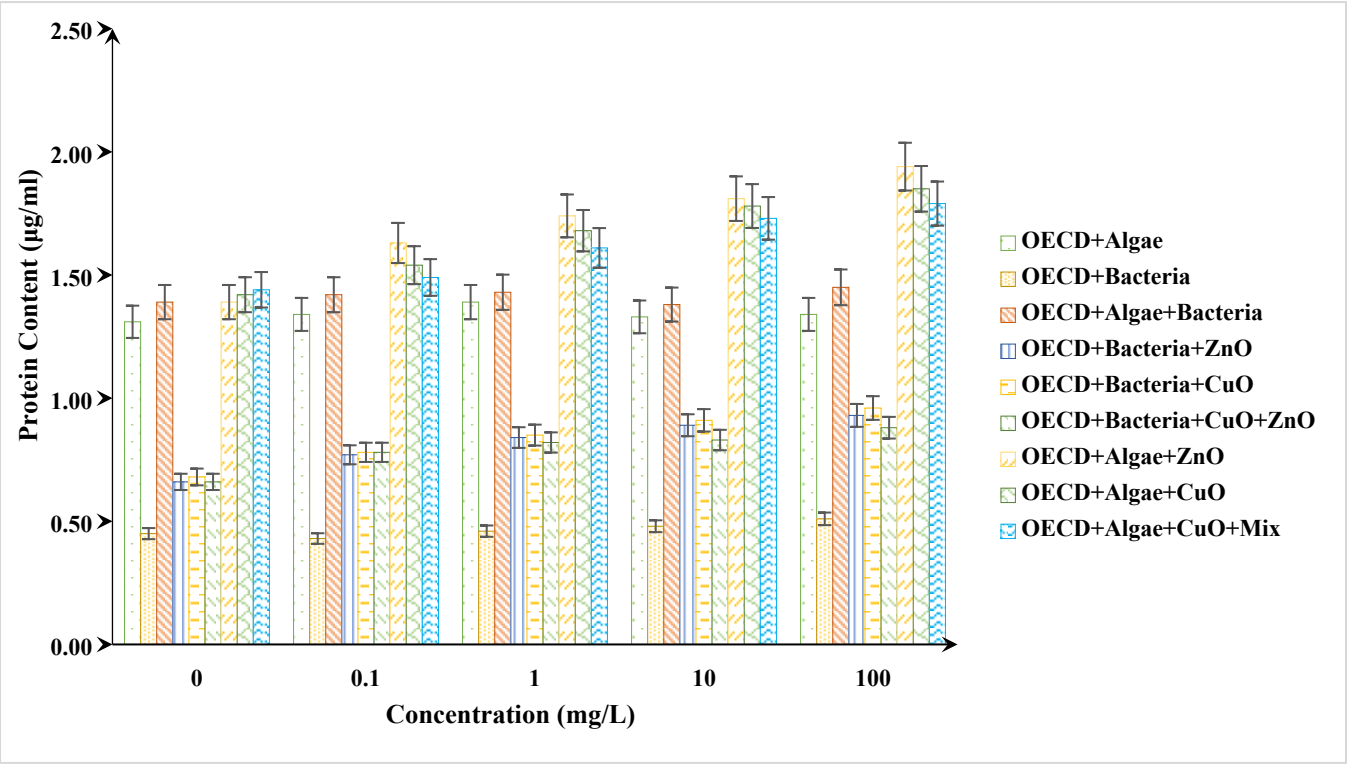

Figure C.3.6: Protein content in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:1).

3.4 EPS Constituents

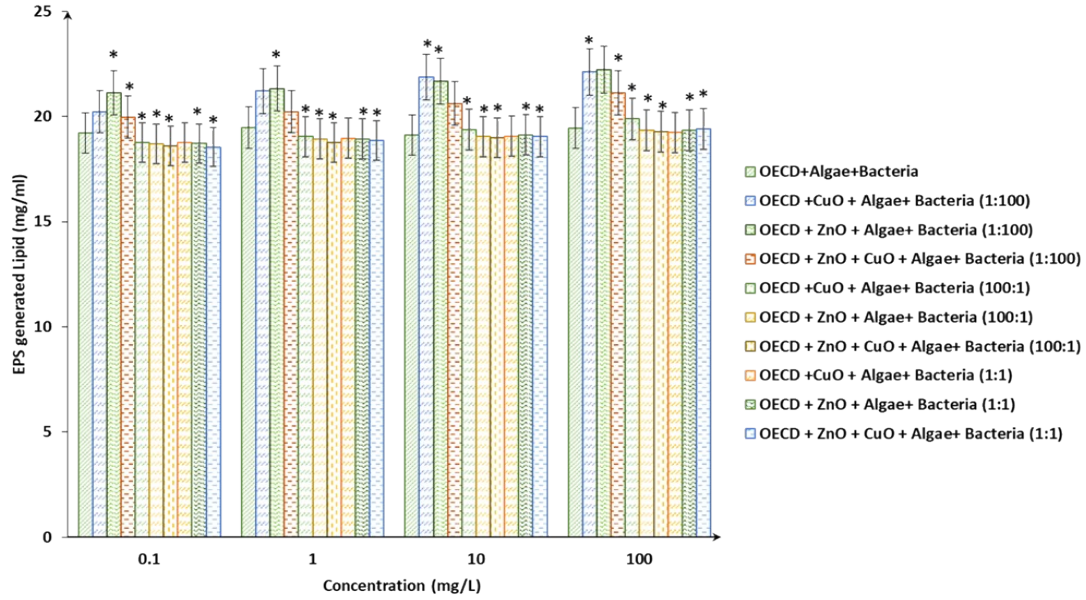

Figure C.4.1: EPS-related lipid in the various samples containing algae+bacteria at different concentration (0, 0.1, 1, 10 and 100 mg/L) of ZnO, CuO and ZnO+CuO NPs in the OECD media at after 96 hours at different ratios. *Error bars indicate one standard deviation value of three replicates around average values;* ***:** *significance ($p < 0.05$).*

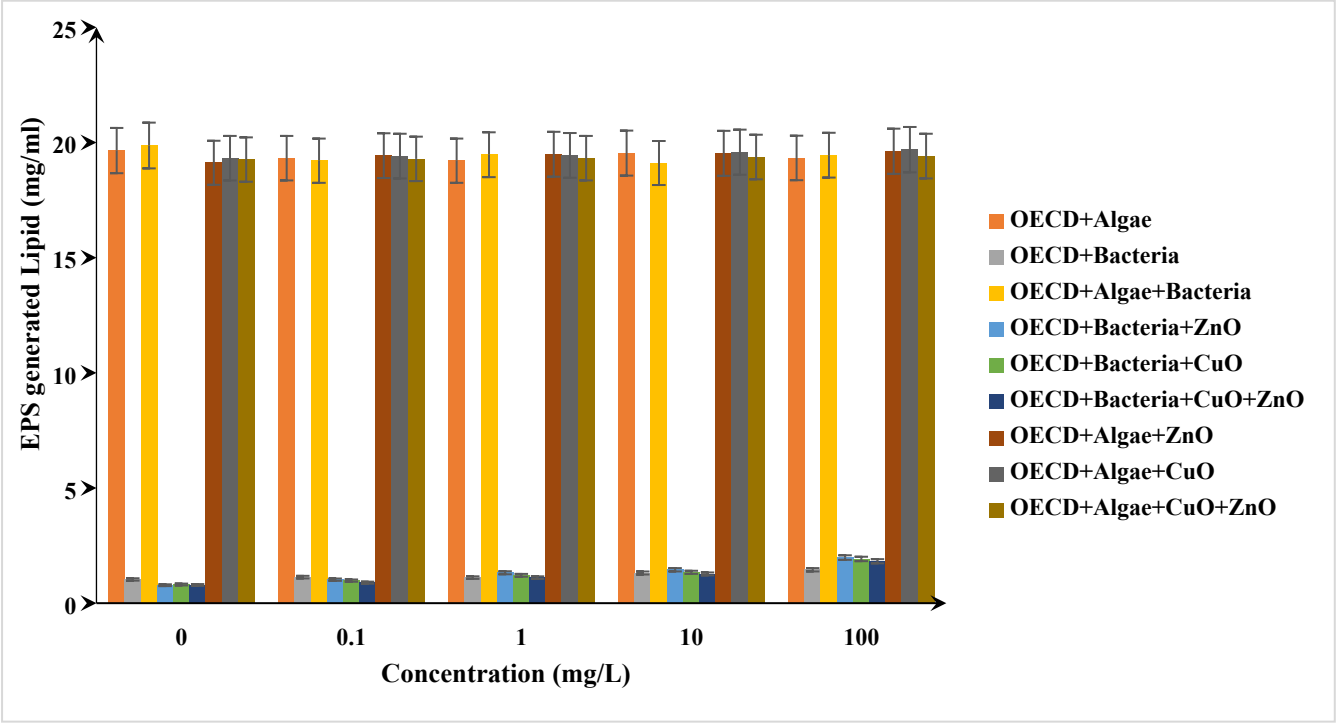

Figure C.4.2: EPS generated lipid in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:100).

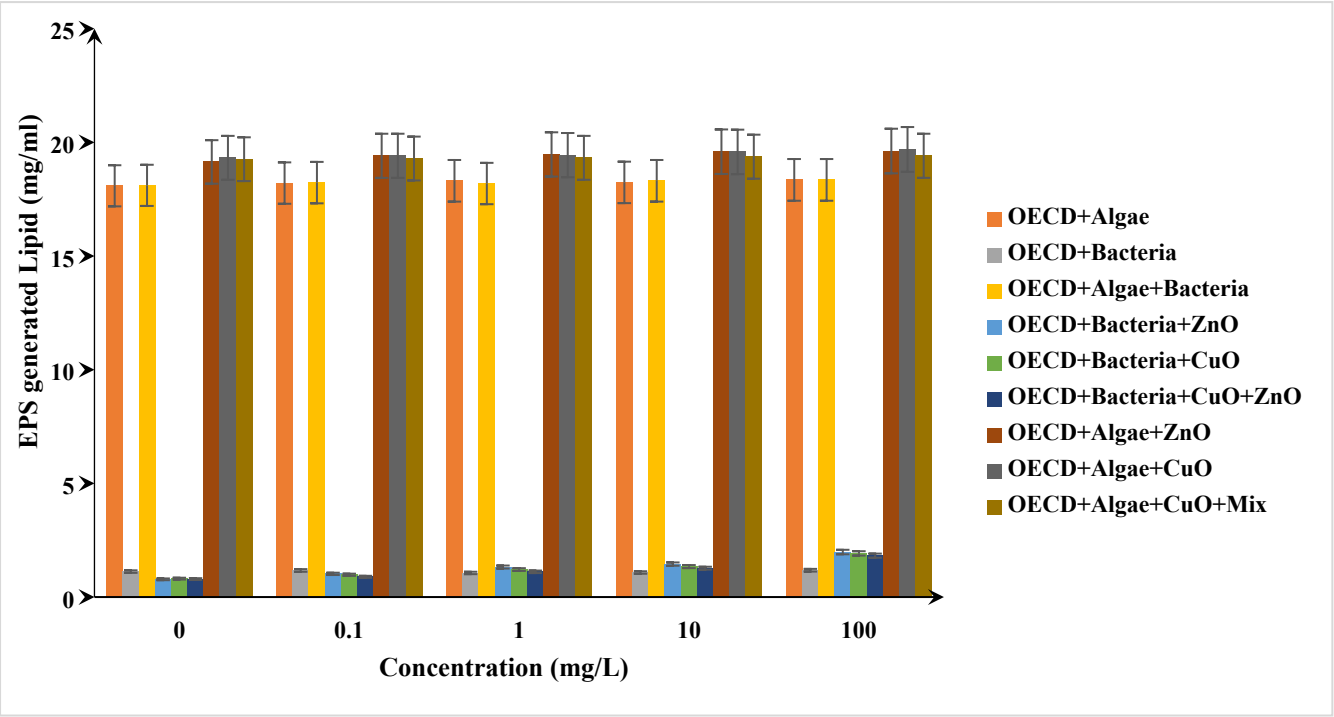

Figure C.4.3: EPS generated lipid in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:1).

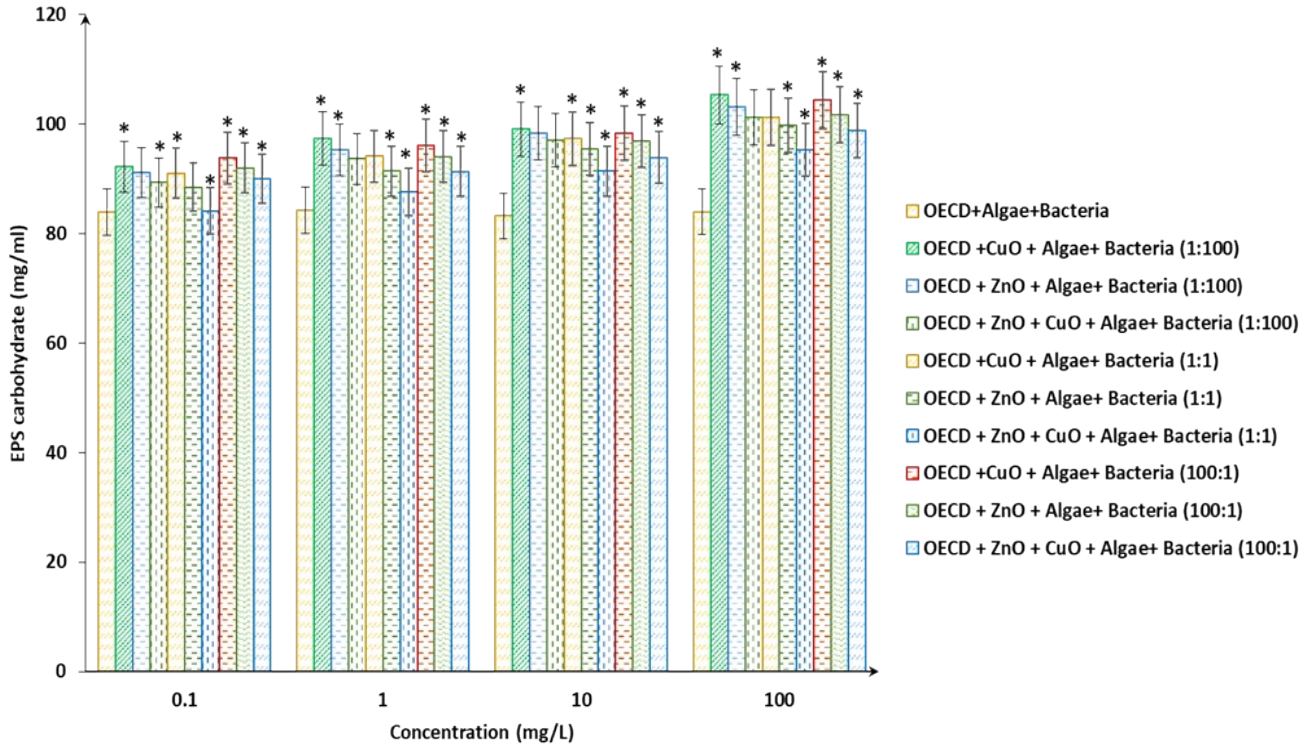

Figure C.4.4: EPS-related carbohydrate in the various samples containing algae+bacteria at different concentration (0, 0.1, 1, 10 and 100 mg/L) of ZnO, CuO and ZnO+CuO NPs in the OECD media at after 96 hours at different ratios. *Error bars indicate one standard deviation value of three replicates around average values;* ***:** *significance ($p < 0.05$).*

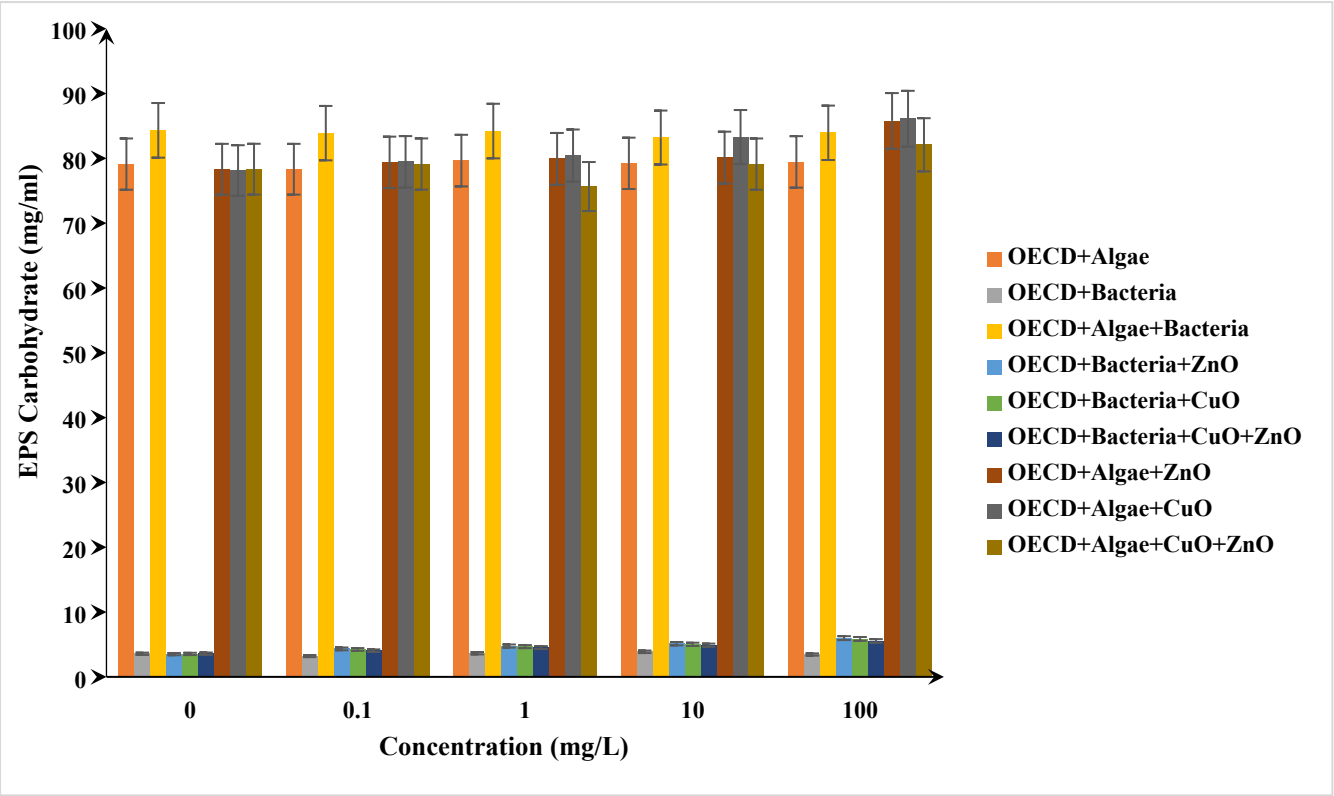

Figure C.4.5: EPS generated carbohydrate in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:100).

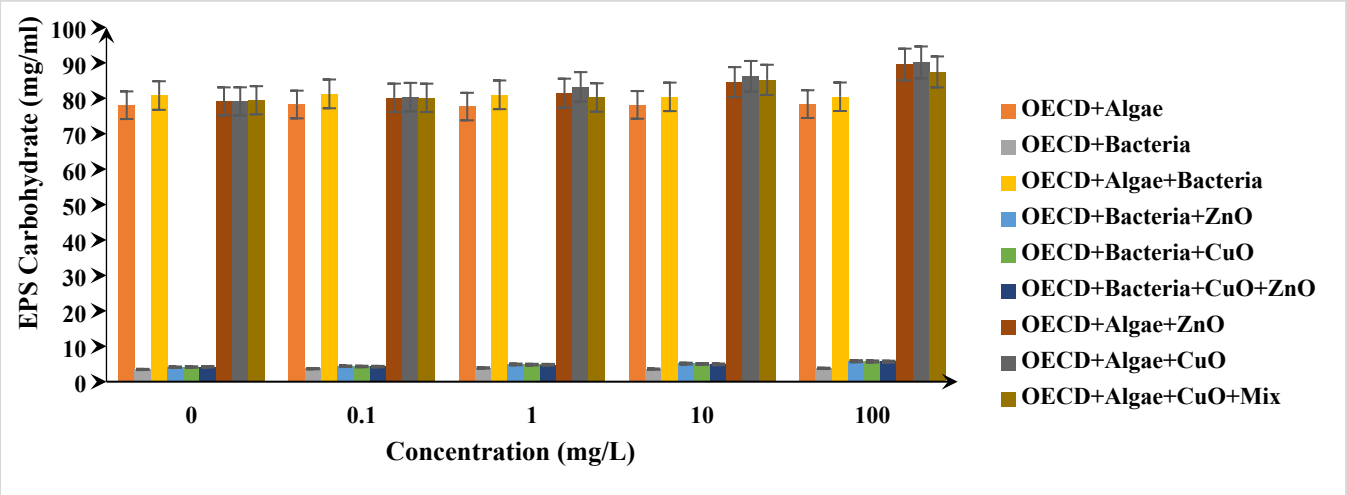

Figure C.4.6: EPS generated carbohydrate in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:1).

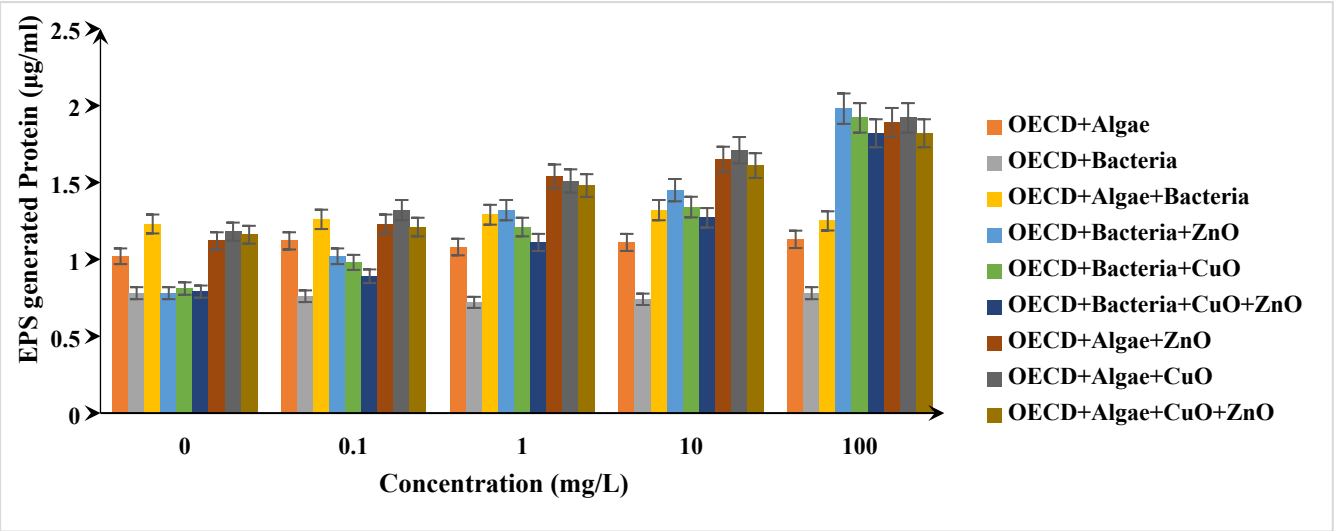

Figure C.4.7: EPS generated protein in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:100).

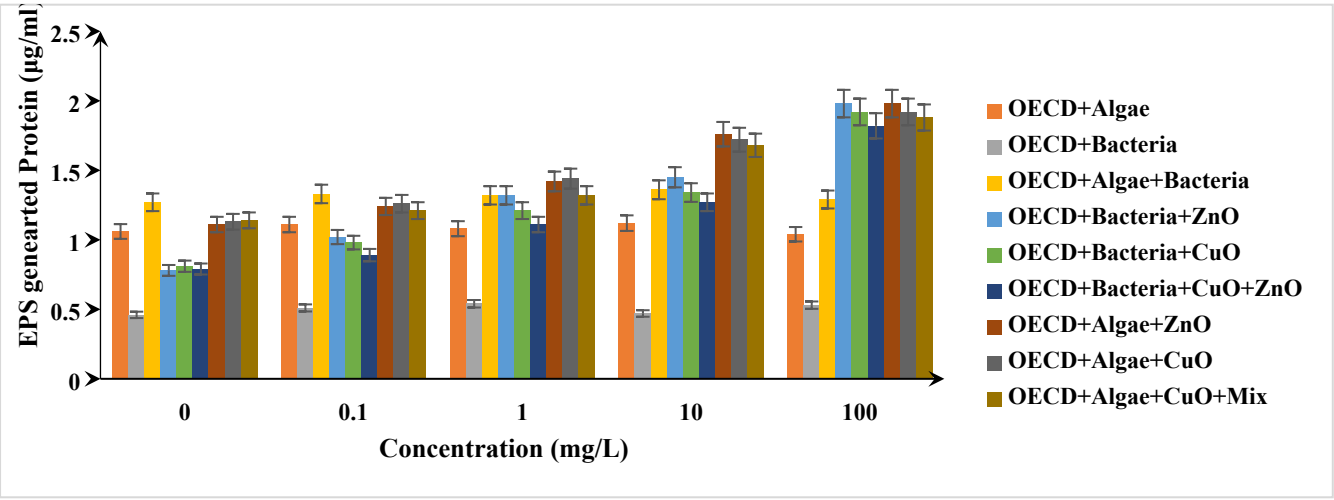

Figure C.4.8: EPS generated protein in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:1).

3.5 Metal and ion release

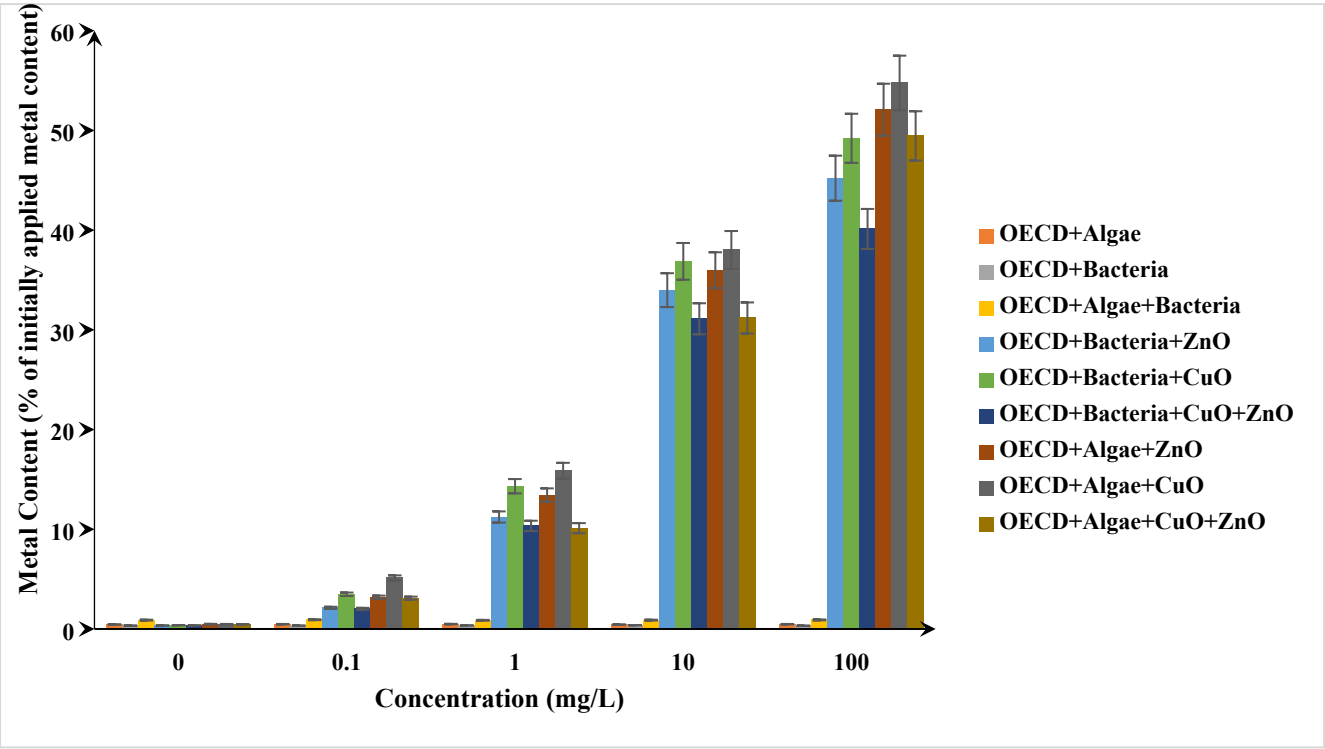

Figure C.5.1: Metal content (% of initially applied metal content) in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:100).

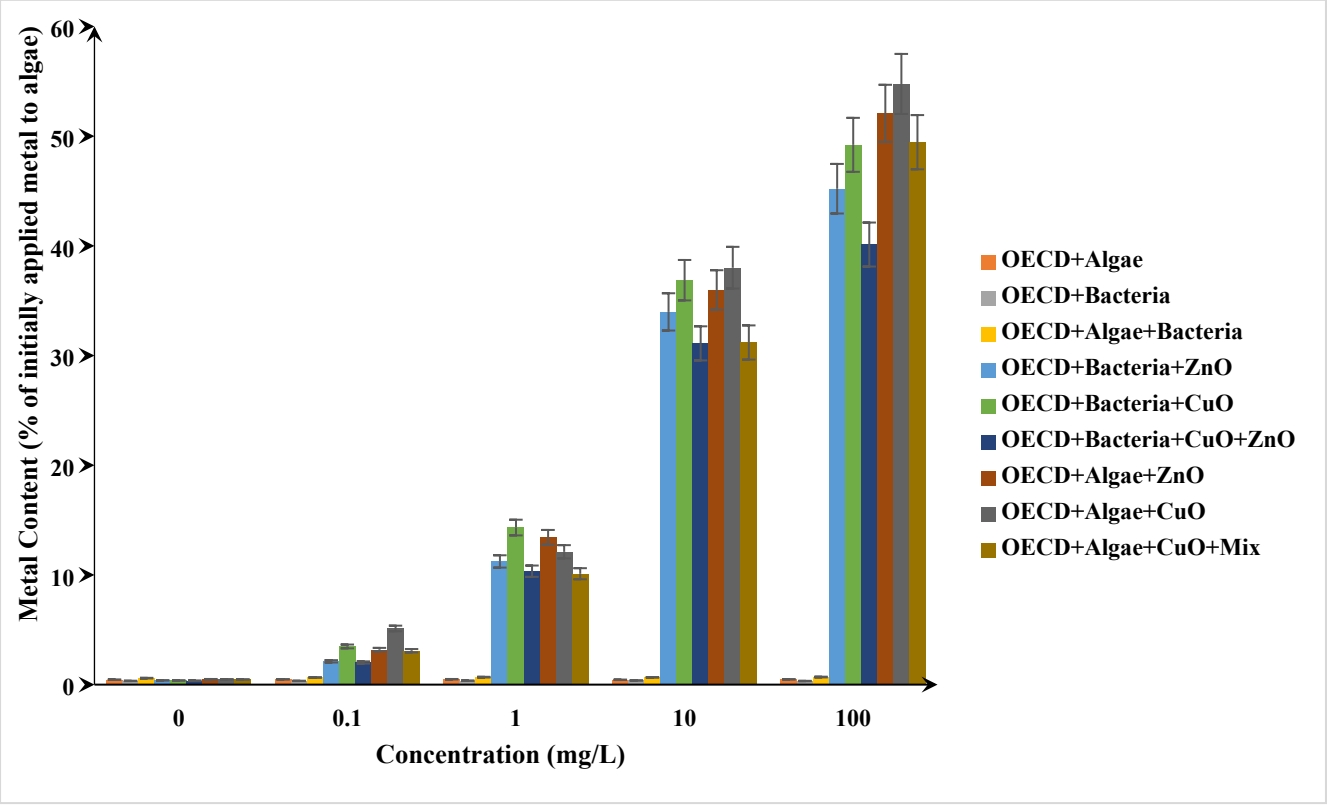

Figure C.5.2: Metal content (% of initially applied metal content) in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:1).

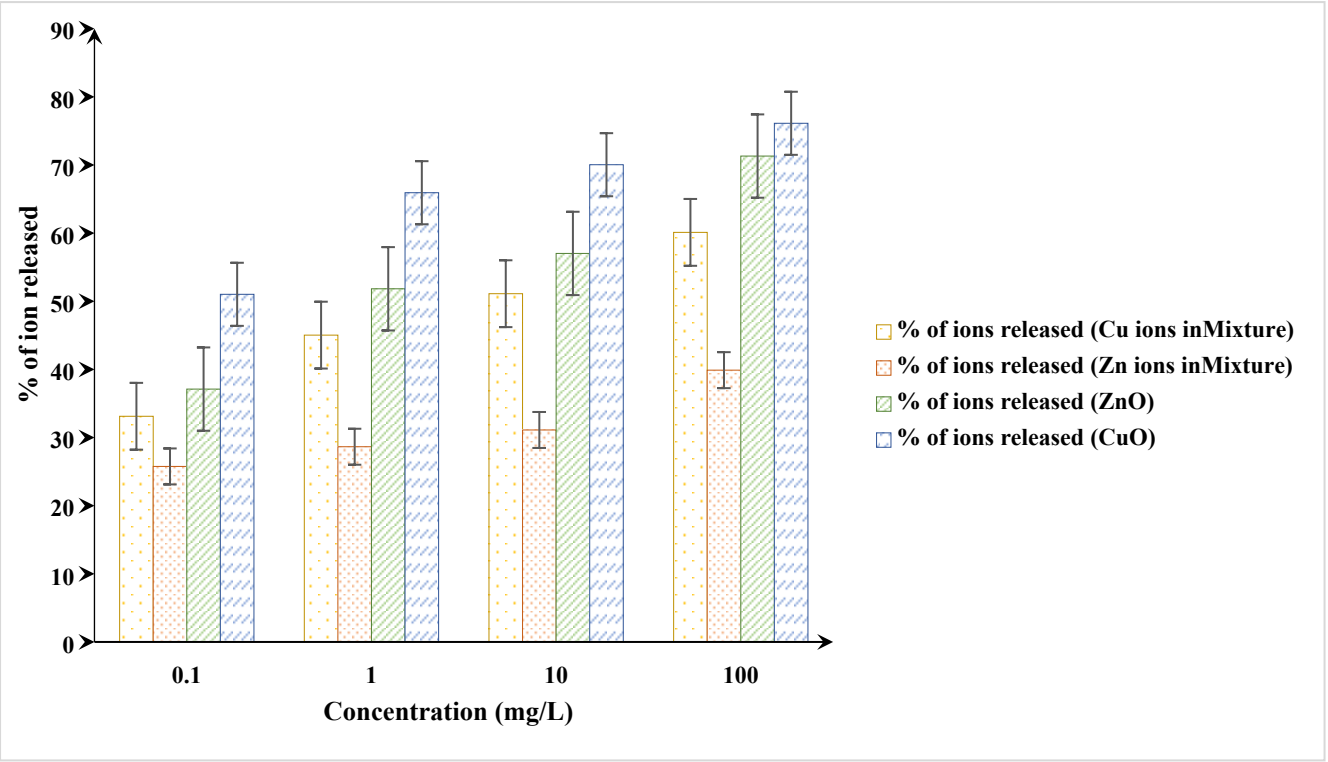

Figure C.5.3: % ion release (% of initially applied metal content) in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:100).

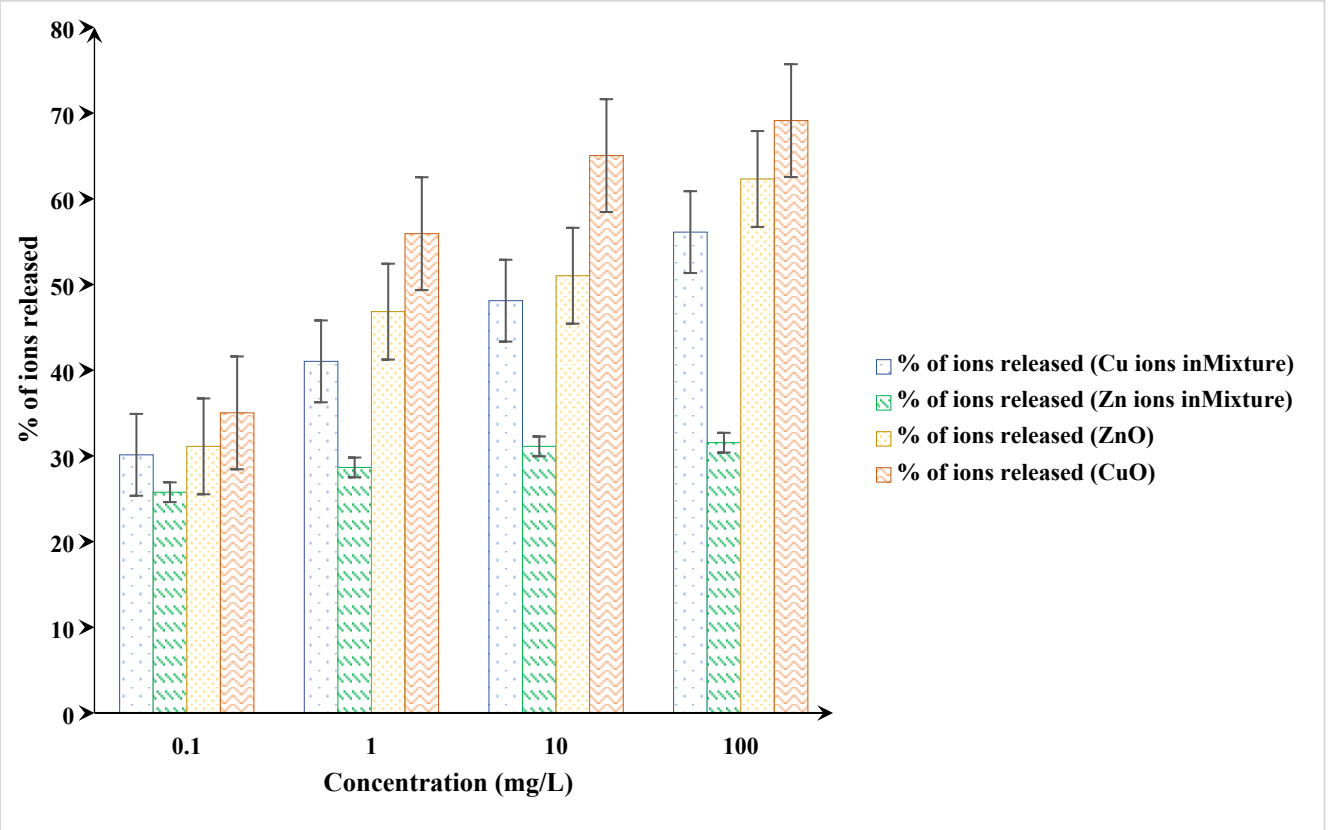

Figure C.5.4: % ion release (% of initially applied metal content) in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:1).

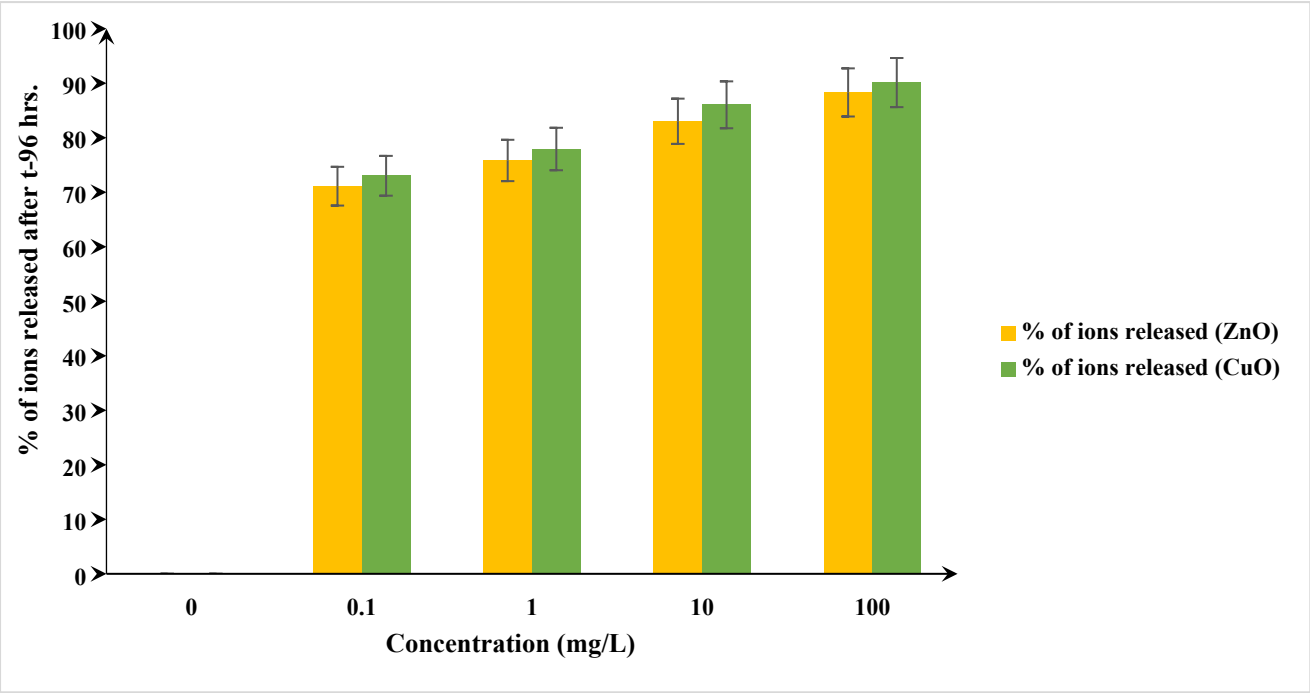

Figure C.5.5: % of ions released after t=96 hours vs. NPs concentrations (mixture as well as single) (0 mg/L, 0.1 mg/L, 1 mg/L, 10 mg/L and 100 mg/L) at different ratio of algae-bacteria consortia (1:100, 100:1 and 1:1). * Shows the significance among the control samples and samples containing ZnO NPs and CuO alone at different concentrations.

3.6 Interrelationship of different parameters

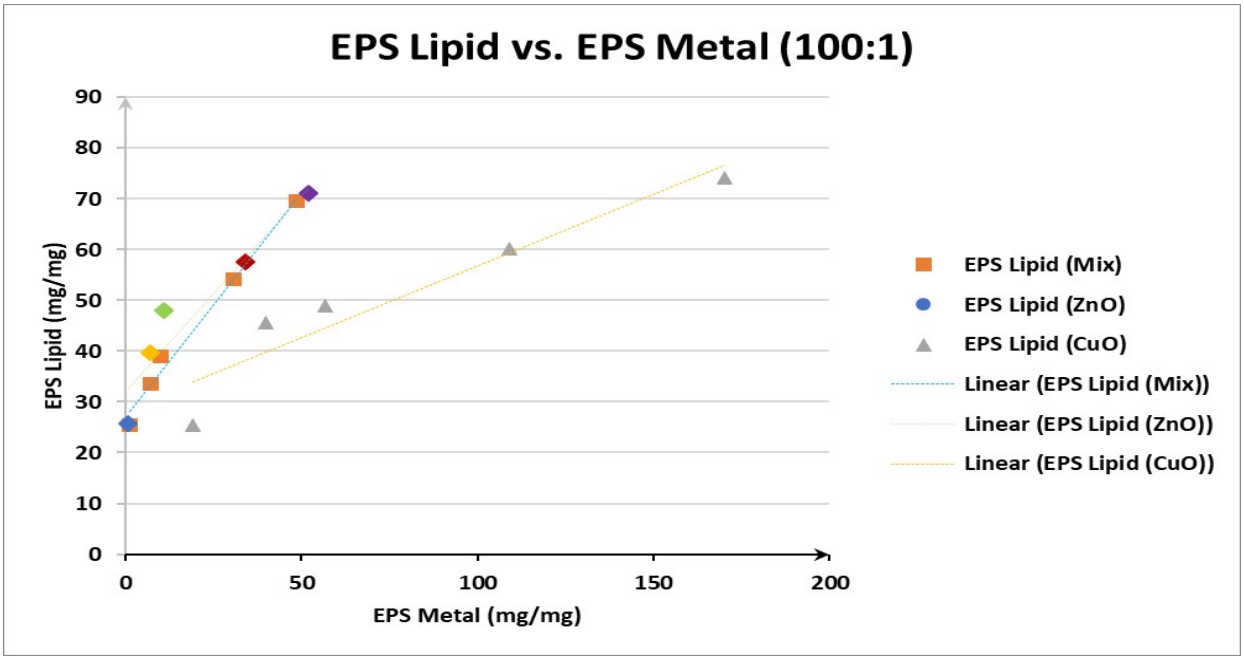

Figure 3.6.1: EPS Lipid (normalised with biomass) (mg/mg) vs. EPS Metal (mg/mg) after t=96 hours at different concentrations (0, 0.1 mg/L, 1 mg/L, 10 mg/L, and 100 mg/L) at 100:1 ratio. All the trend shows R value above 0.9.

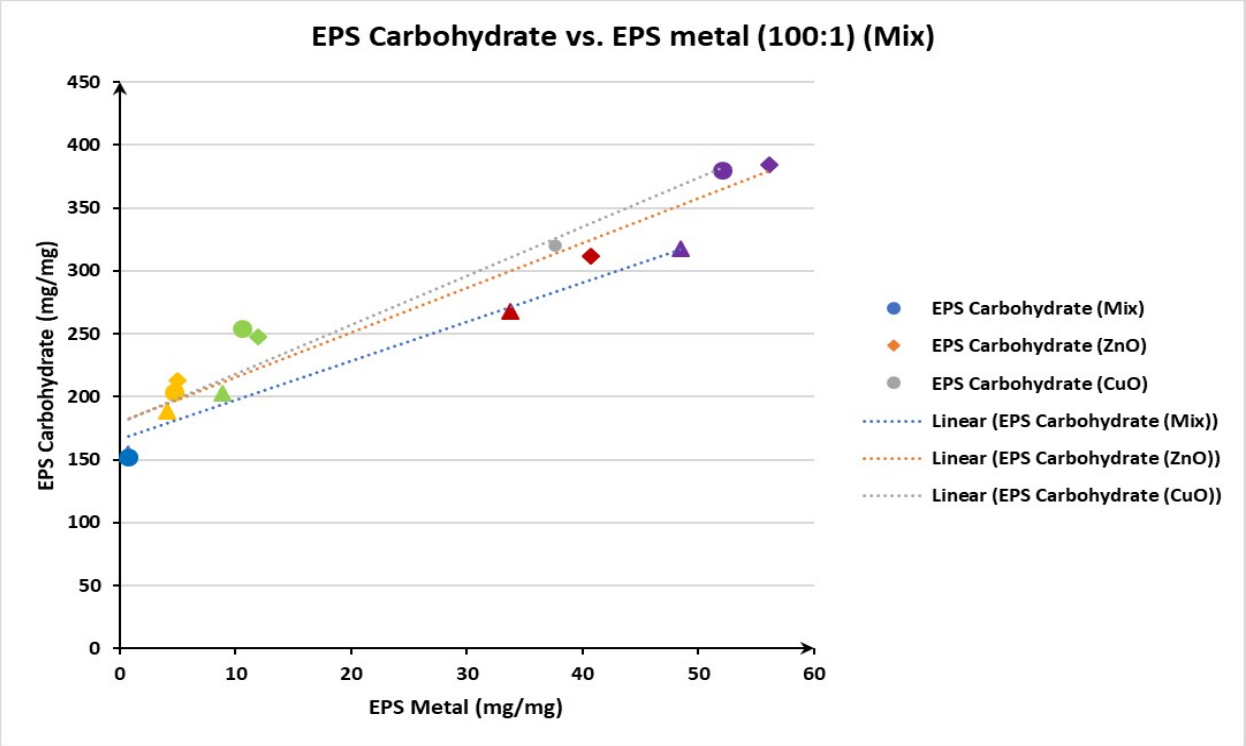

Figure 3.6.2: EPS Carbohydrate (normalised with biomass) (mg/mg) vs. EPS Metal (mg/mg) after t=96 hours at different concentrations (0, 0.1 mg/L, 1 mg/L, 10 mg/L, and 100 mg/L) at 100:1 ratio. Blue colour shows 0 mg/L, yellow colour shows 0.1 mg/L, green colour shows 1 mg/L, red colour shows 10 mg/L, and purple colour shows 100 mg/L. All the trend shows R value above 0.9.

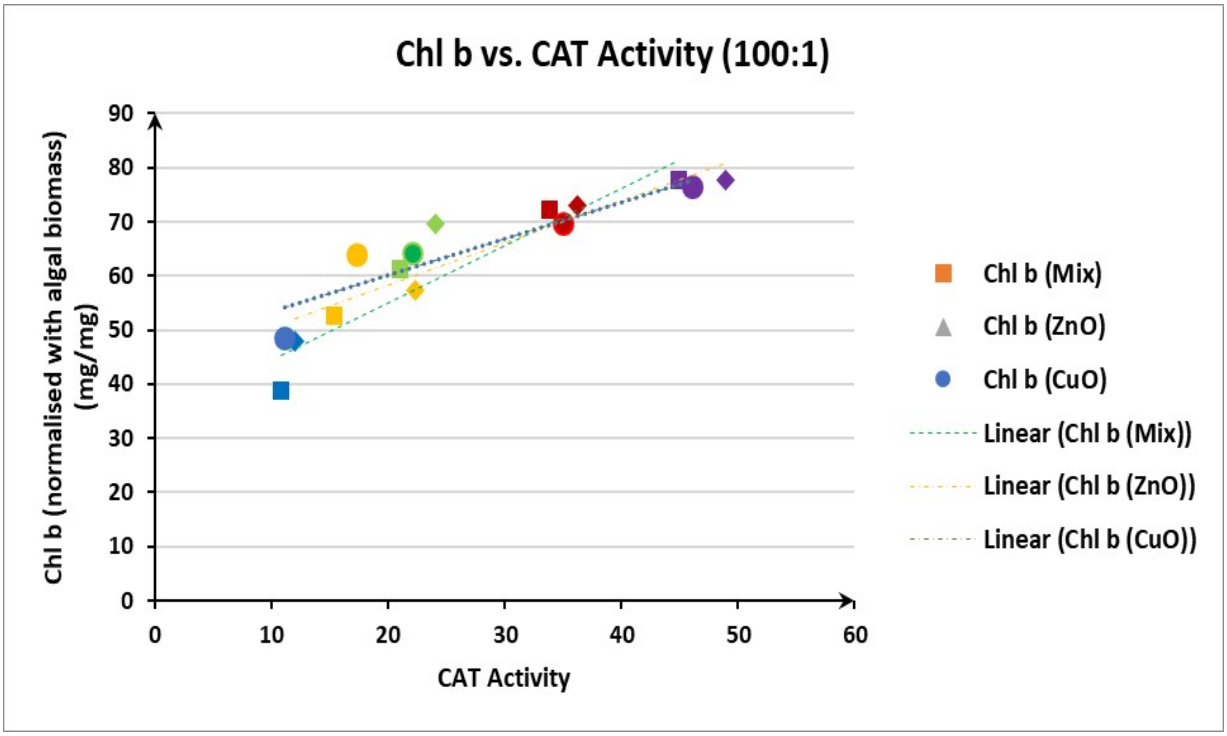

Figure C.6.3: Chl b (normalised with algal biomass) (mg/mg) vs. CAT Activity after t=96 hours at different concentrations (0, 0.1 mg/L, 1 mg/L, 10 mg/L, and 100 mg/L) at 100:1 ratio. R value was found to be above 0.9 for all the samples.

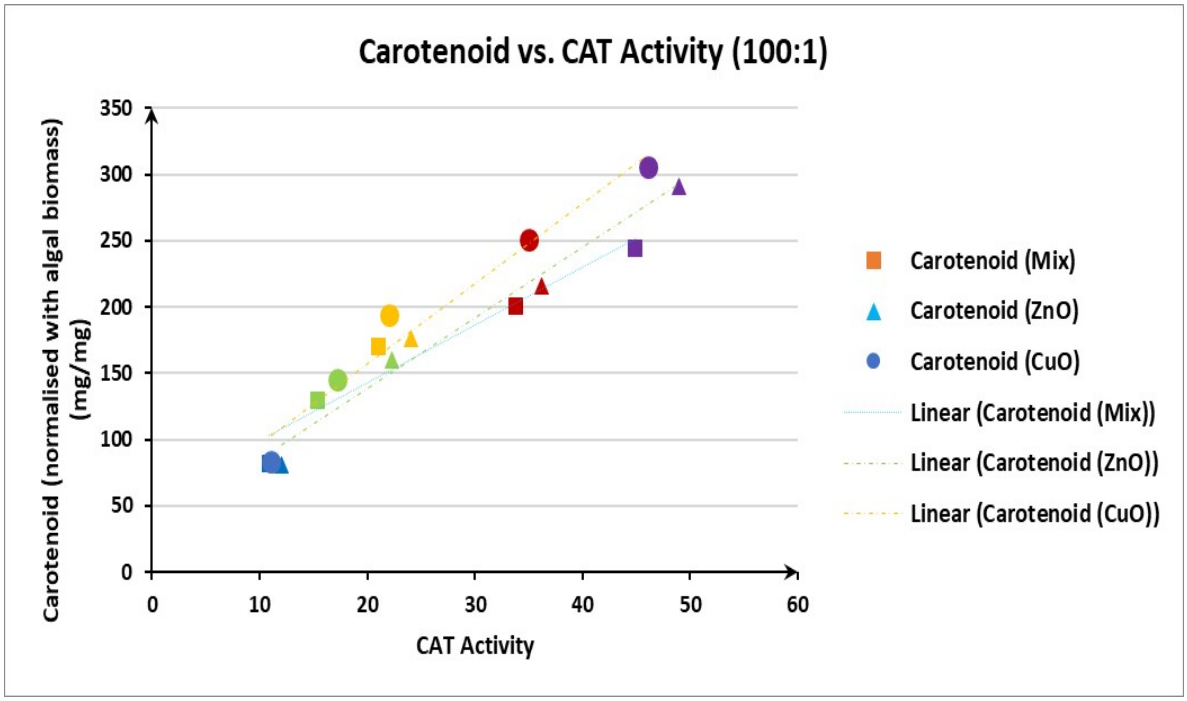

Figure C.6.4: Carotenoid (normalised with algal biomass) (mg/mg) vs. CAT Activity after t=96 hours at different concentrations (0, 0.1 mg/L, 1 mg/L, 10 mg/L, and 100 mg/L) at 100:1 ratio. Blue colour shows 0 mg/L, green colour shows 0.1 mg/L, yellow colour shows 1 mg/L, red colour shows 10 mg/L and purple colour shows 100mg/L concentration respectively. All the trend shows R value above 0.9.

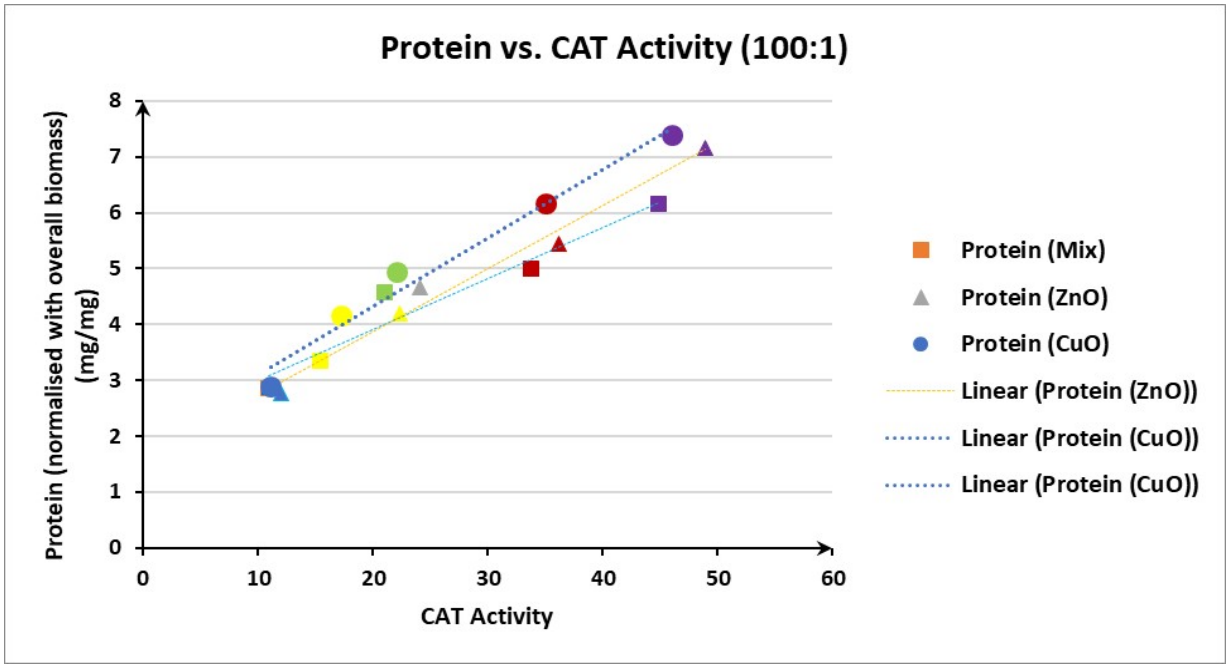

Figure C.6.5: Protein content (normalised with algal biomass) (mg/mg) vs. CAT Activity after t=96 hours at different concentrations (0, 0.1 mg/L, 1 mg/L, 10 mg/L, and 100 mg/L) at 100:1 ratio. Blue colour shows 0 mg/L, yellow colour shows 0.1 mg/L, green colour shows 1 mg/L, red colour shows 10 mg/L, and purple colour shows 100 mg/L. All the trend shows R value above 0.9.

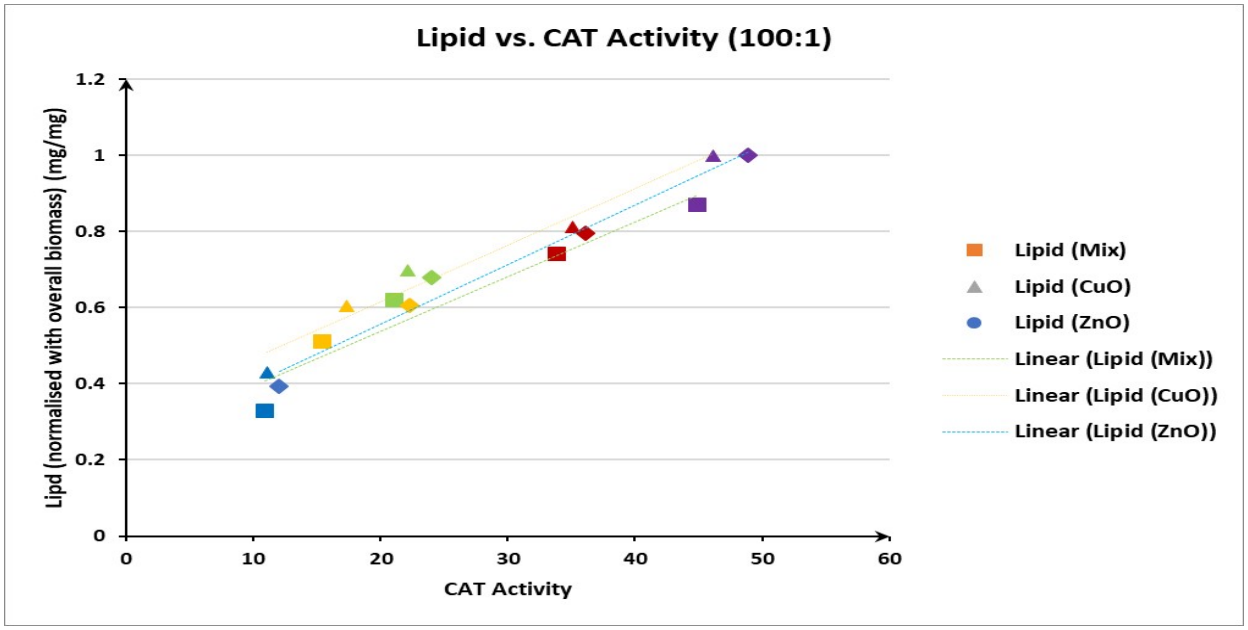

Figure C.6.6: Lipid (normalised with algal biomass) (mg/mg) vs. CAT Activity after t=96 hours at different concentrations (0, 0.1 mg/L, 1 mg/L, 10 mg/L, and 100 mg/L) at 100:1 ratio. Blue colour shows 0 mg/L, yellow colour shows 0.1 mg/L, green colour shows 1 mg/L, red colour shows 10 mg/L, and purple colour shows 100 mg/L. All the trend shows R value above 0.9.

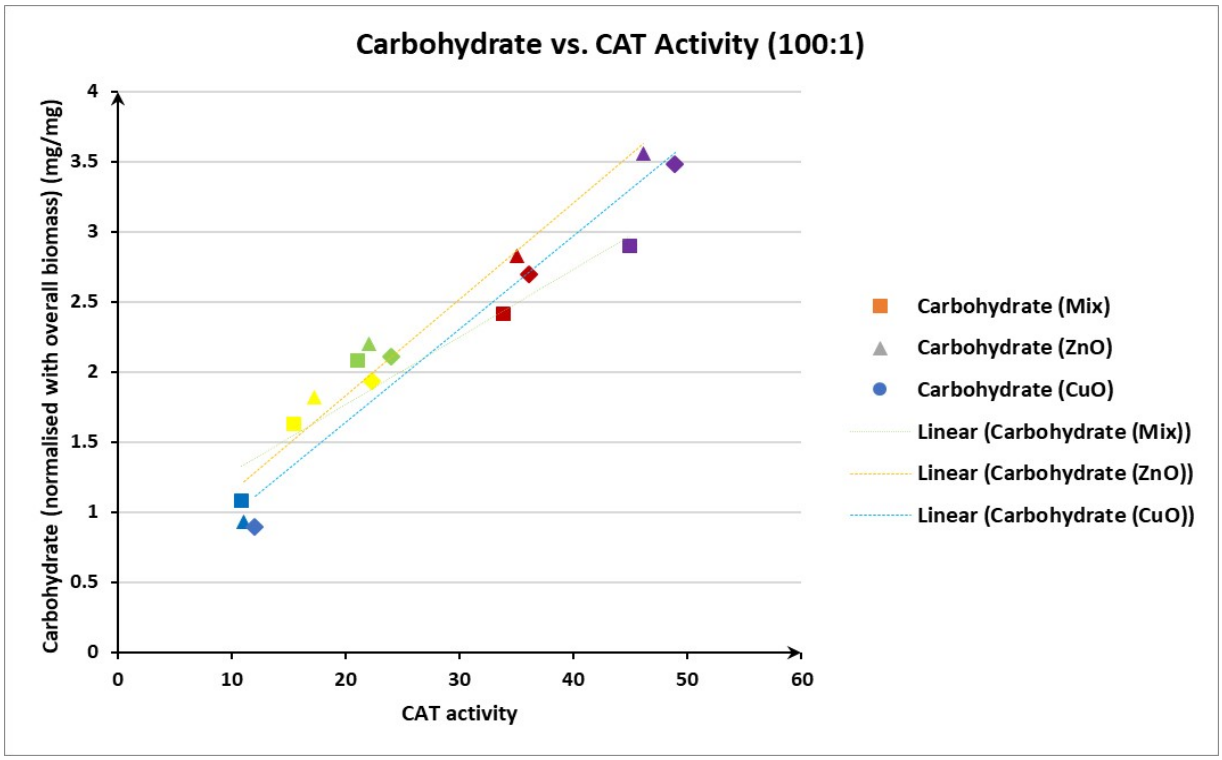

Figure C.6.7: Carbohydrate (normalised with algal biomass) (mg/mg) vs. CAT Activity after t=96 hours at different concentrations (0, 0.1 mg/L, 1 mg/L, 10 mg/L, and 100 mg/L) at 100:1 ratio. Blue colour shows 0 mg/L, yellow colour shows 0.1 mg/L, green colour shows 1 mg/L, red colour shows 10 mg/L, and purple colour shows 100 mg/L. All the trend shows R value above 0.9.

Table C.7: Showing Equation and R values of different parameters with NPs concentration.

Parameters	NPs Conc.	Equation of linear model	Coefficient of determination (R^2)
EPS-related protein vs metal on algal-bacteria consortium	CuO only	Y=0.0054x+0.1609	0.8749
	ZnO only	Y=0.0062x+0.1467	0.88
	Mixture of NPs	Y=0.005x+0.1564	0.916
EPS-related lipid vs metal on algal-bacteria consortium	CuO only	Y=0.2823x+28.543	0.901
	ZnO only	Y=0.7723x+32.039	0.9086
	Mixture of NPs	Y=0.8856x+27.088	0.986
EPS-related carbohydrate vs metal on algal-bacteria consortium	CuO only	Y=3.8919x+179.22	0.9368
	ZnO only	Y=3.5522x+179.91	0.9395
	Mixture of NPs	Y=3.1038x+166.37	0.9779
CAT activity vs metal on algal-bacteria consortium	CuO only	Y=2.674x+22.348	0.9761
	ZnO only	Y=2.6719x+26.639	0.99
	Mixture of NPs	Y=0.9723x+14.102	0.9655
Chl a vs. CAT activity	CuO only	Y=5.9368x+656.19	0.9749
	ZnO only	Y=5.3524x+684.56	0.9528
	Mixture of NPs	Y=6.824x+742.56	0.9974
Chl b vs. CAT activity	CuO only	Y=0.6682x+46.847	0.8312
	ZnO only	Y=0.7776x+42.808	0.8205
	Mixture of NPs	Y=1.0598x+33.804	0.901
Carotenoid vs. CAT activity	CuO only	Y=6.0438x+36.059	0.9628
	ZnO only	Y=5.331x+31.717	0.9726
	Mixture of NPs	Y=4.352x+55.518	0.9322
lipid vs. CAT activity	CuO only	Y=0.0149x+0.3174	0.9613
	ZnO only	Y=0.0157x+0.2432	0.9733
	Mixture of NPs	Y=0.0143x+0.2523	0.9278
Carbohydrate vs. CAT activity	CuO only	Y=0.0665x+0.309	0.9688
	ZnO only	Y=0.0689x+0.4527	0.9569

	Mixture of NPs	Y=0.00483x+0.8031	0.9287
Protein vs. CAT activity	CuO only	Y=0.1221x+1.8812	0.9763
	ZnO only	Y=0.113x+1.5996	0.9784
	Mixture of NPs	Y=0.0915x+2.0778	0.9405
Internal metal vs. CAT activity	CuO only	Y=2.674x+22.348	0.9761
	ZnO only	Y=2.6179x+26.639	0.99
	Mixture of NPs	Y=1.0446x+6.4977	0.9686

3.7. TEM

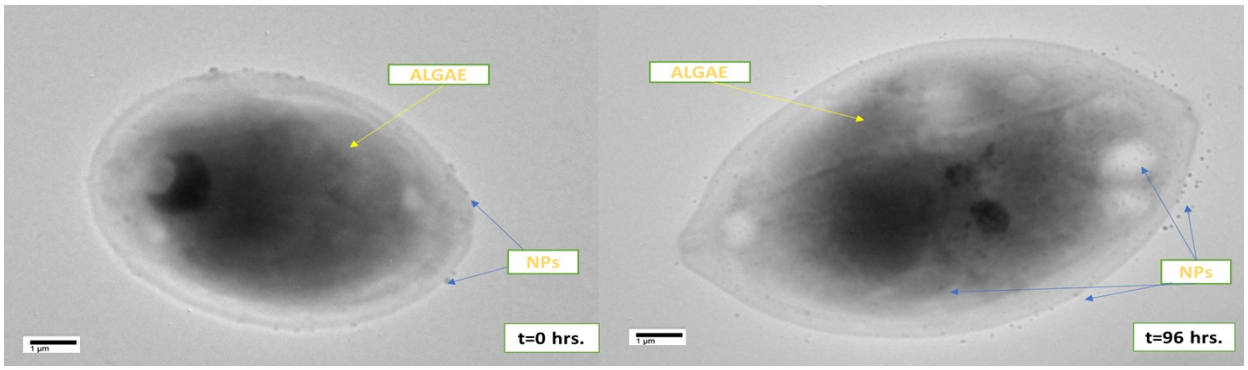

Figure C.7.1: TEM image showing algae in OECD media at t=0 hrs and t=96 hrs. in the presence of mixture of nanoparticles at 100:1 ratio for 100 mg/L nanoparticle concentration.

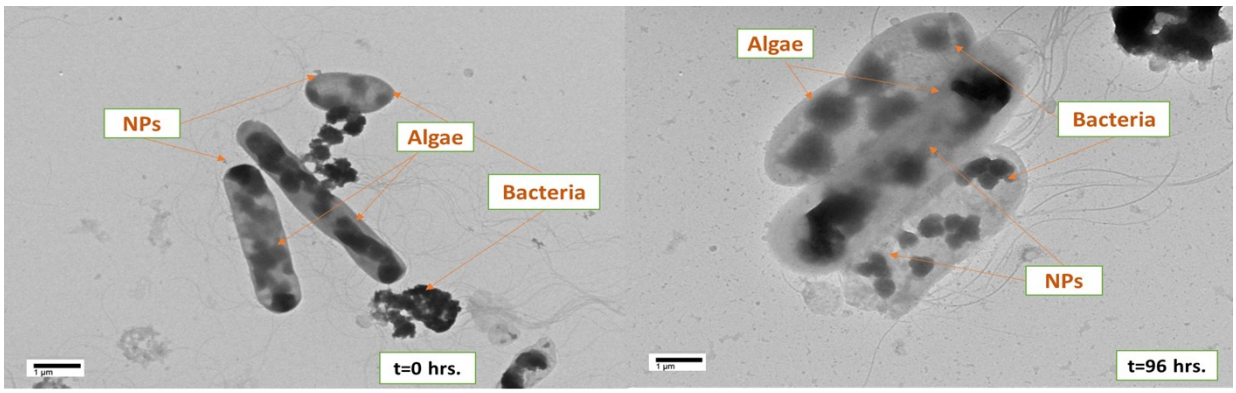

Figure C.7.2: TEM image showing algae-bacteria consortia in OECD media at t=0 hrs and t=96 hrs. in the presence of ZnO nanoparticles at 100:1 ratio for 100 mg/L nanoparticle concentration.

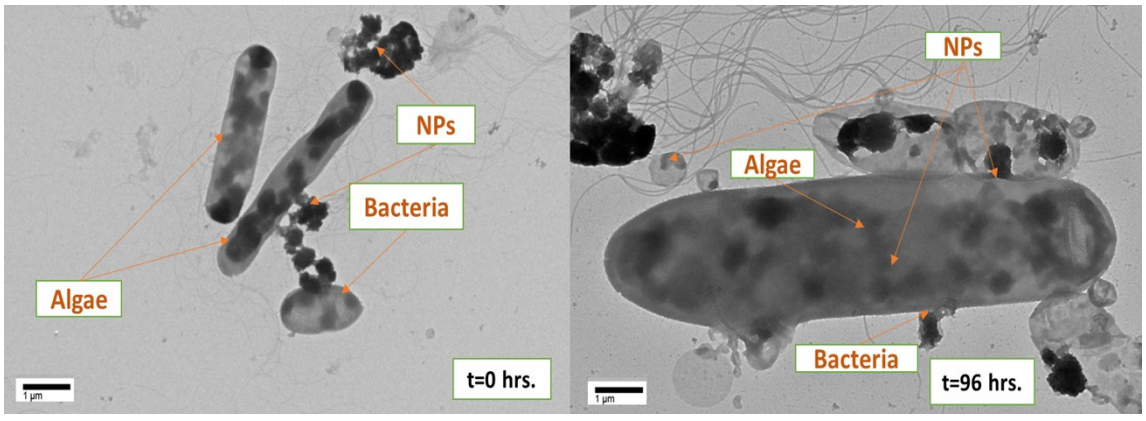

Figure C.7.3: TEM image showing algae-bacteria consortia in OECD media at t=0 hrs and t=96 hrs. in the presence of ZnO nanoparticles at 100:1 ratio for 100 mg/L nanoparticle concentration.

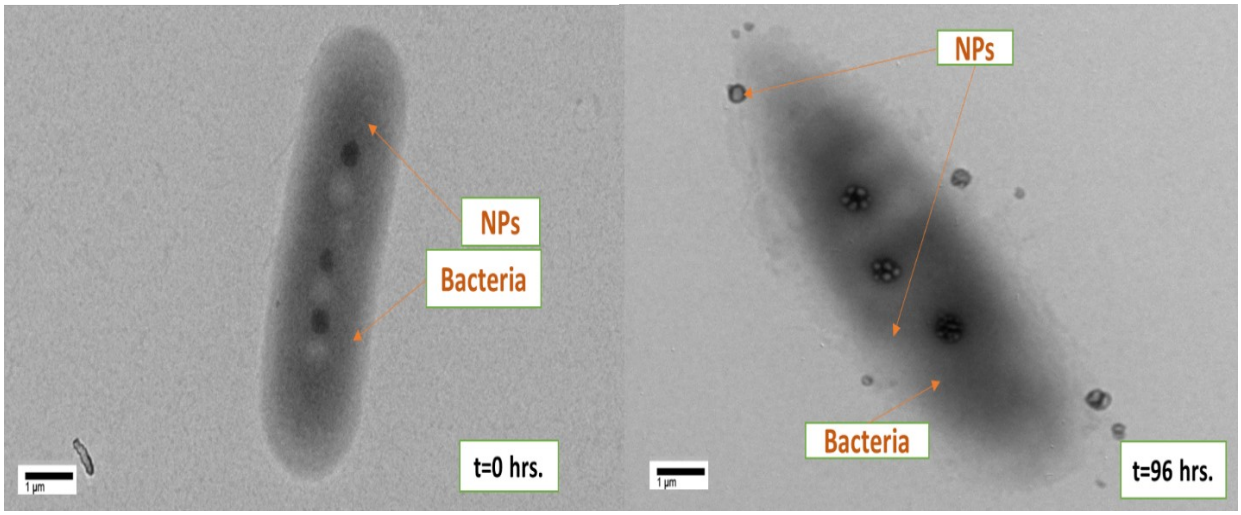

Figure C.7.4: ***TEM image showing bacteria at=0 hrs and t=96 hrs. in the presence of ZnO+CuO nanoparticles at 100:1 ratio for 100 mg/L nanoparticle concentration.***

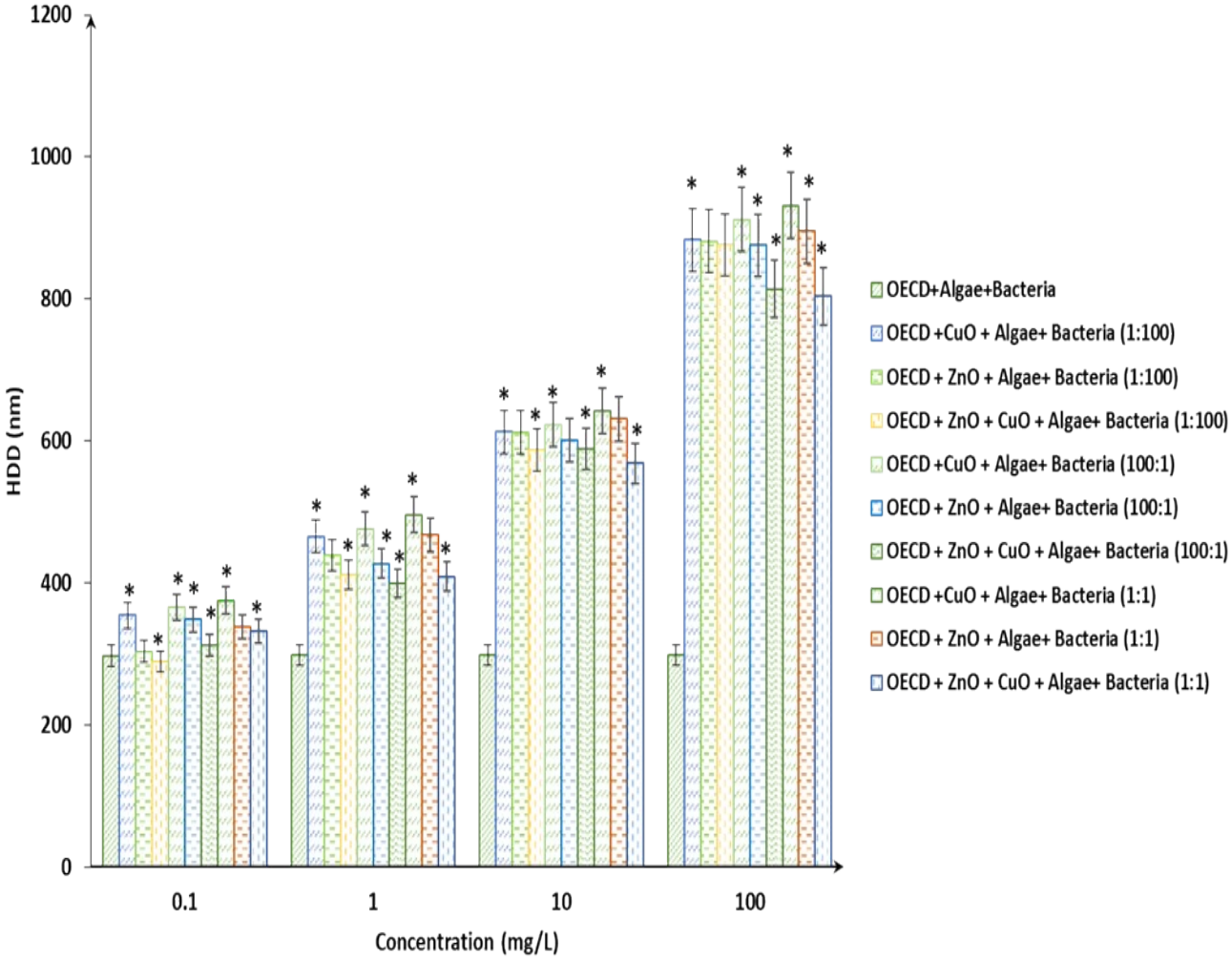

Figure C.7.5: HDD in the various samples containing algae+bacteria at different concentration (0, 0.1, 1, 10 and 100 mg/L) of ZnO, CuO and ZnO+CuO NPs in the OECD media at after 96 hours at different ratios. *Error bars indicate one standard deviation value of three replicates around average values;* ****:*** *significance ($p < 0.05$)*

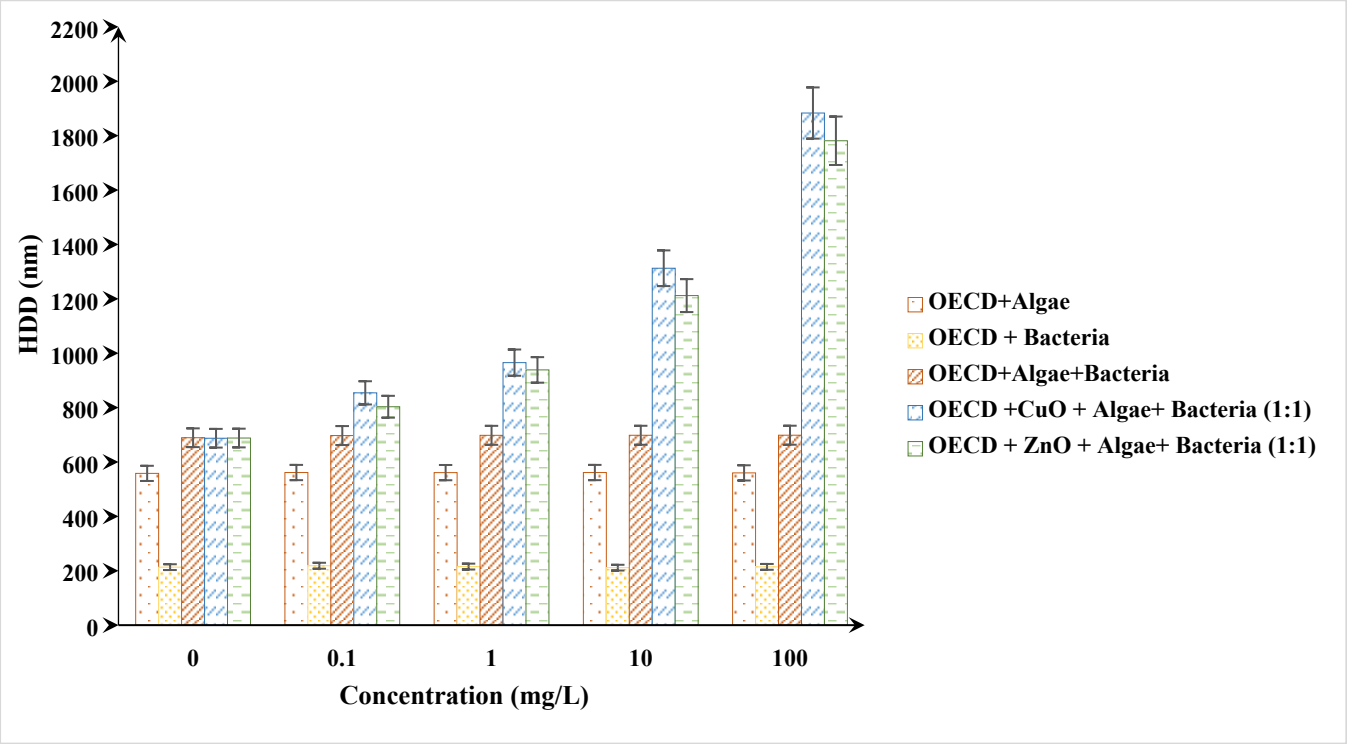

Figure C.7.6: HDD in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:1).

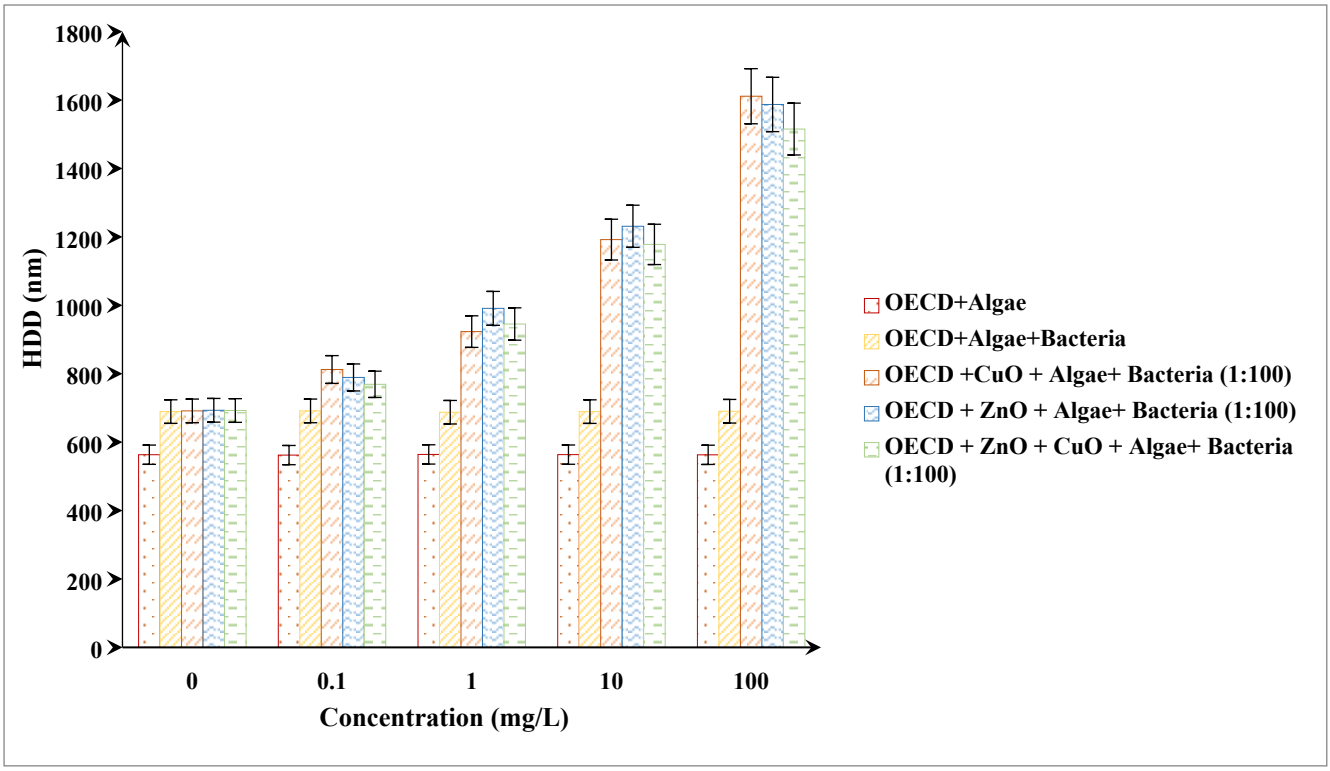

Figure C.7.7: HDD in the sample containing different concentrations of nanoparticles at algae-bacteria ratio (1:100).

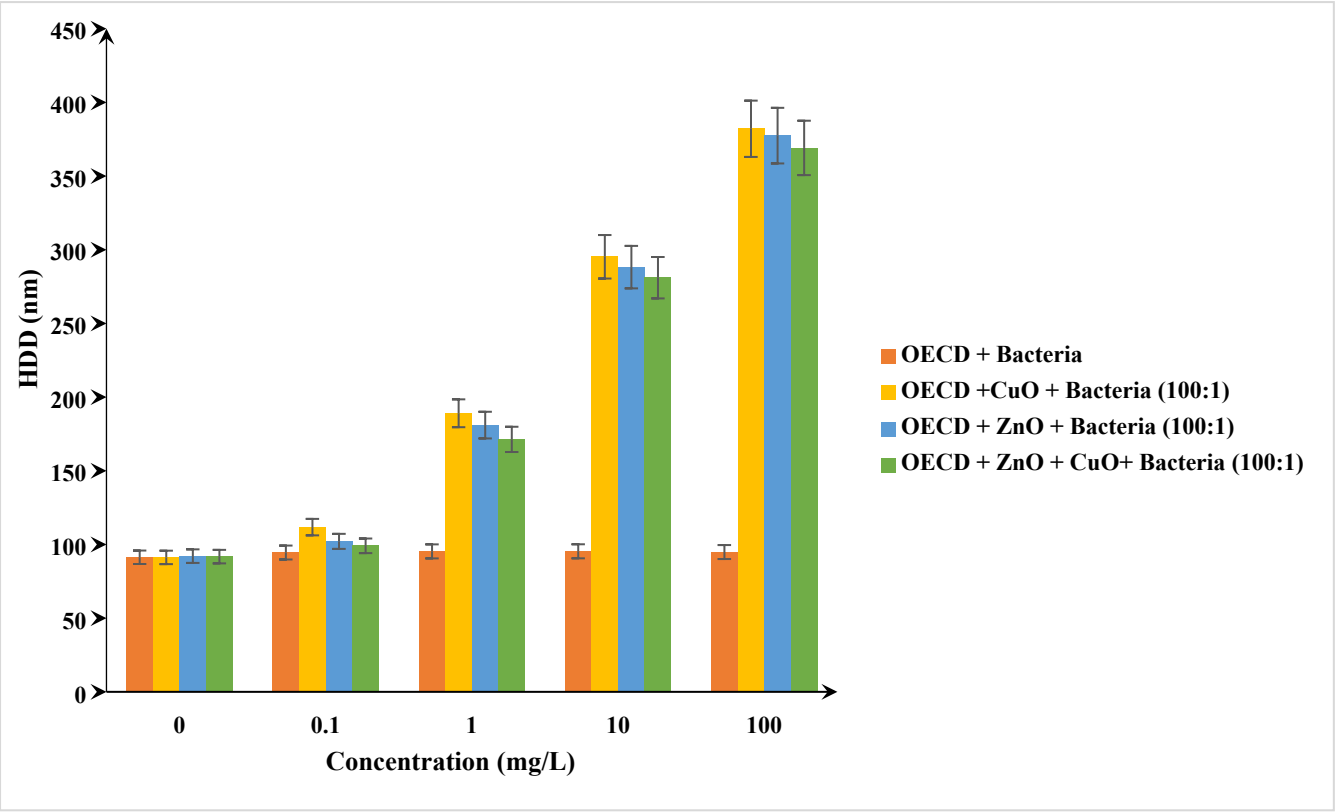

Figure C.7.8: HDD in the sample containing different concentrations of nanoparticles in bacteria alone.

Table C.8: Calculated values of rate constants of the kinetics of metal accumulation of algae-bacteria consortium after t=96 hours at 1 mg/L concentration (average and standard deviation values are shown here).

Nanoparticles presence	Total adsorption (k_{total})	Coefficient of determination of model fitting (R^2_{total})
ZnO only	0.587±2.76	0.932
CuO only	0.619±2.86	0.956
Both types of NPs	0.449±1.93	0.939

FTIR

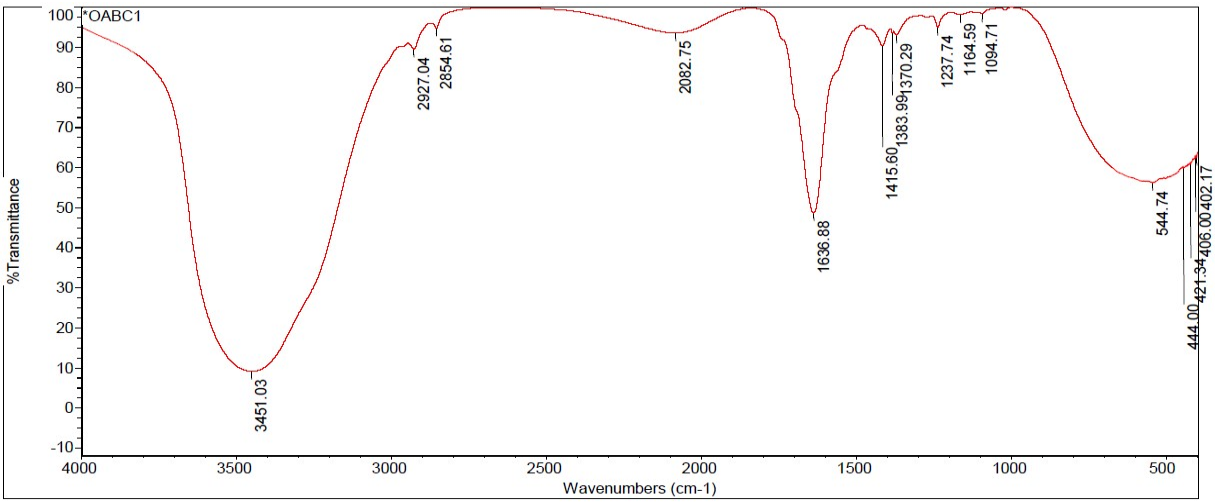

Figure C.8.1: FTIR spectra in the sample containing no nanoparticles at algae-bacteria ratio (100:1).

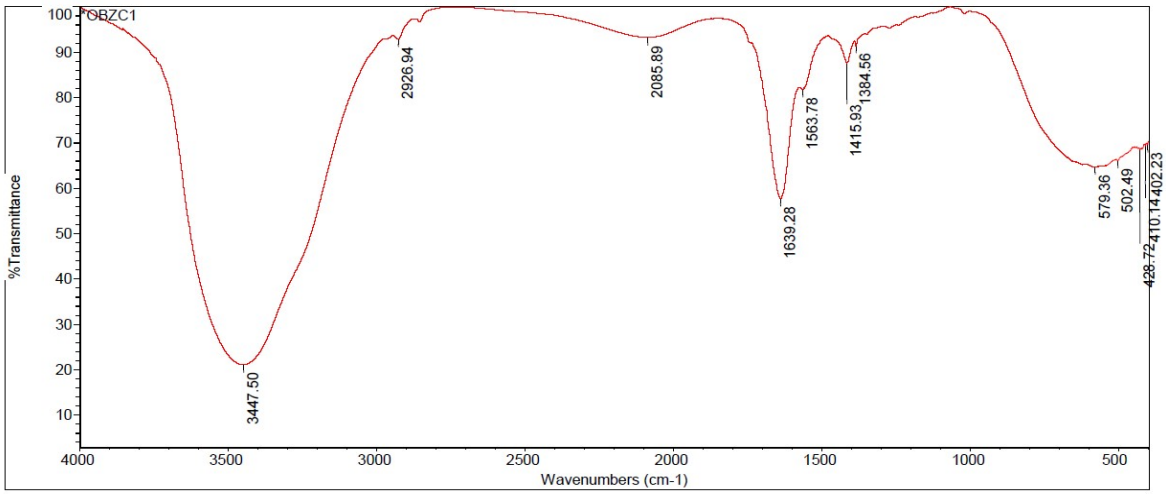

Figure C.8.2: FTIR spectra in the sample containing 100 mg/L ZnO nanoparticles at algae-bacteria ratio (100:1).

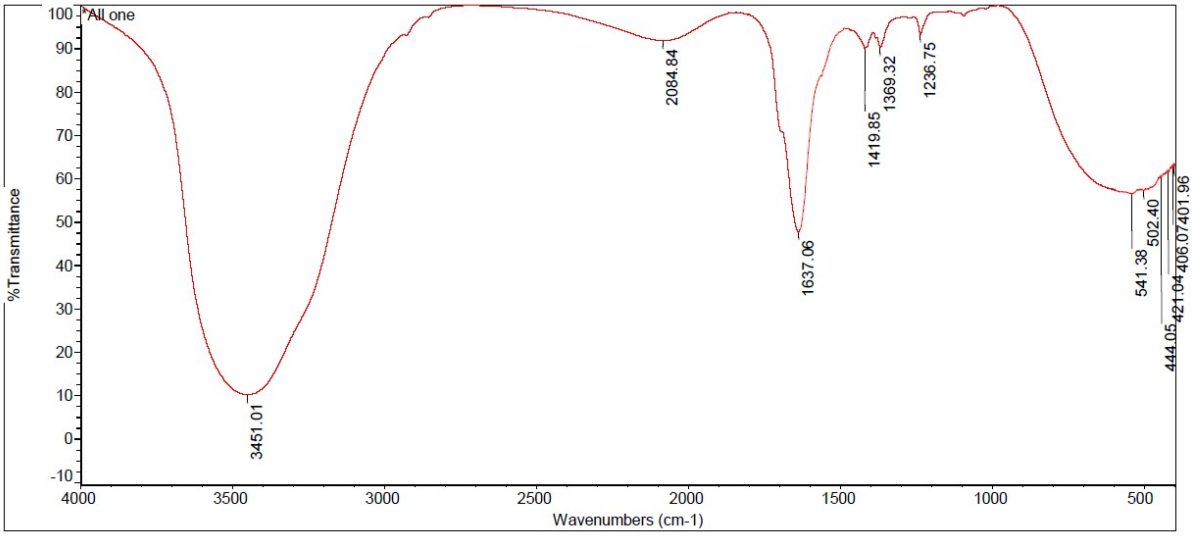

Figure C.8.3: FTIR spectra in the sample containing 100 mg/L CuO nanoparticles at algae-bacteria ratio (100:1).

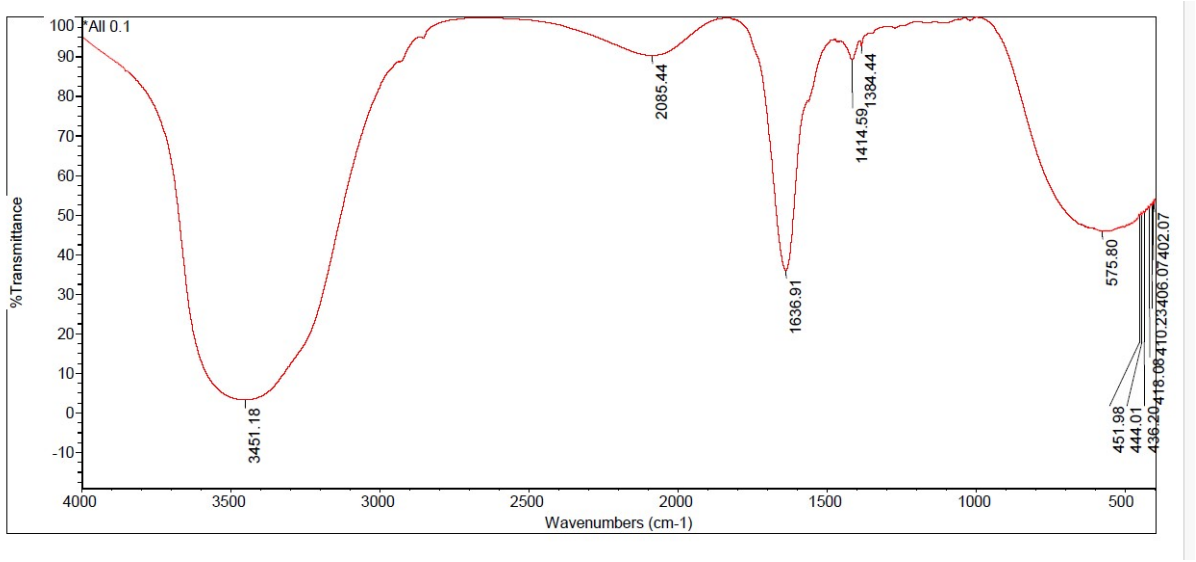

Figure C.8.4: FTIR spectra in the sample containing 100 mg/L CuO+ ZnO mixture of nanoparticles at algae-bacteria ratio (100:1).

Table D.1: Shows the comparison table between the parameters analysed in the present study vs. various toxicity studies done in the past with mixture of NPs and algae alone. Shows the parameters which are analysed in the past.

MIXTURE OF NANOPARTICLES+ALGAE

Algal alone/Algae-Bacterial Species used	NPs	Chl a	Chl b	Carotenoid	Cell Growth/ Cell Count	Biomass	Lipid	Protein	Chemical Bonding	Metal	Microscopic	Mechanism	References
Chlorella vulgaris	ZnO, CuO, TiO_2, NiO, Fe_2O_3		No	No		No	No	No	No	No	No	No	Ko et al., 2018
Scenedesmus obliquus, Chlorella vulgaris	TiO_2 NPs; TiO_2 NT	No	No	No		No	No	No	No	No		No	Wang et al., 2020
Scenedesmus obliquus	ZnO, GO	No	No	No		No	No	No	No			No	Ye et al., 2018

Chlorella sp.	TiO_2 (rutile and anatase phases)	No	No	No	No	No	No	No	No			No	Iswarya et al., 2015
Chlamydomonas reinhardtii and *Ochromonas danica*	AgNPs, HemNPs, PsNPs	No	No	No	No	No	No	No	No			No	Huang et al., 2019
Scenedesmus obliquus	TiO_2, ZrO_2, SiO_2				No	No	No	No	No	No		No	Liu et al., 2018
Chlorella pyrenoidosa	ENPs (nCeO	No	No	No		No	No	No	No	No	No	No	Wang et al., 2016

	$_2$ and $nTiO_2$), one antibiotic (florfenicol, FLO)												
Picochlorum sp.	ZnO and TiO_2 NPs		No	No		No	No	No	No			No	Hazeem et al., 2015

BACTERIA+SINGLE/MIXTURE OF NANOPARTICLES

Bacteria alone	NPs	Cell Growth/ Cell Count	Biomass	Lipid	Protein	Chemical Bonding	Metal	Toxicity test	Enzyme Assay	Microscopic	Mechanism	References

Pseudomonas aeruginosa Staphylococcus aureus	AgNP		No	No	No	No	No				No	Dorobantu et al., 2015
Escherichia coli, Staphylococcus aureus	AgNP		No	No	No	No	No			No	No	Greulich et al., 2012
Shewanella oneidensis; Bacillus subtilis	AuNP		No	No	No	No	No				No	Feng et al., 2015
Escherichia coli, Staphylococcus aureus; Pseudomonas aeruginosa	ZnO		No	No	No	No	No				No	Premanathan et al., 2010
E. coli	CuO	No	No	No	No	No				No	No	Bondarenko et al.,

												2012
Pseudomonas fluorescens OS8; E. coli; *Saccharomyces cerevisiae BY4741*	AgNP	No	No	No	No	No	No				No	Ivask et al., 2014
Streptomyces	CuO		No	No	No	No					No	Liu et al., 2018
Staphylococcus aureus; Escherichia coli; Pseudomonas aeruginosa	CuO and ZnO alone		No	No	No	No	No		No	No	No	Dadi et al., 2019
Staphylococcus aureus; Pseudomonas aeruginosa	CuO		No	No	No	No					No	Janani et al., 2020

Escherichia coli	CuO	No	No	No	No	No					No	Meghana et al., 2015
Streptococcus mutans; Lactobacillus casei; and L acidophilus	CuO	No	No	No	No	No			No	No	No	Amiri et al., 2017
Escherichia coli	ZnO		No	No	No	No				No	No	Li et al., 2011
Sinorhizobium meliloti	CeO_2 and ZnO NPs alone		No	No	No	No					No	Bandyopadhyay et al., 2012
Vibrio fischeri	ZnO and CuO NPs	No	No	No	No	No			No	No	No	Zhang et al., 2020

	mix											
Escherichia coli	ZnO and CuO NPs mix	No	No	No	No	No			No	No	No	Tong et al., 2015
Nitrosomonas europaea	n-TiO2, n-CeO2, and n-ZnO		No	No	No	No					No	Yu et al., 2016

ALGAE-BACTERIA CONSORTIA+SINGLE/MIXTURE OF NANOPARTICLES

Bacteria alone	NPs	Contaminant	Cell Growth/ Cell Count	Biomass	Lipid	Protein	Chemical Bonding	Metal	Toxicity test	Enzyme Assay	Microscopic	Mechanism	References
AgNP and HemNP tagged bacteria with Chlorella pyrenoidosa	No	Antibiotic	No	No	No	No	No	No	No		No	No	Cao et al, 2023
Bacillus sp. and Micrococcus sp (Bacteria) and. Scenedesmus acutus and Chlorella pyrenoidosa	No	Heavy metal Pb^{2+}+Cd^{2+s}		No	No					No		No	Chandrashekharaiah et al., 2022
Chlorella vulgaris and	No	Antibiotics		No	No	No	No	No	No		No	No	Zhou et al., 2023

Bacillus subtilis													
Chlorella vulgaris and Bacillus subtilis	No	Tetracycline	No	No	No	No	No	No	No			No	Zhou et al., 2023

Table 4: Various studies showing algae-bacteria consortia at different water matrix.

Reference	Algae/bacteria Information	Matrix	Findings	Parameters observed	Remarks/ Limitations
Han et al. (2016)	***Bacteria: Muricauda sp.*** **Axenic microalga:** ***Tetraselmis chuii, Cylindrotheca fusiformis & Nannochloropsis gaditana***	Wastewater	Microalgae-bacteria co-cultures effective strategy for microalgal cultivation under mixotrophic conditions. Algal cell density increases with bacteria.	Growth curve, algae-bacteria ratio, plating	No data on the Toxicity of NPs; Only growth studies done.
Berthold et al. (2019)	**Algae:** ***Characium sp.*** **Bacteria:** ***Pseudomonas composti***	BG-11 media	bacteria release of unidentified extracellular compounds which might affect the growth rate and lipid metabolism of algae.	Biomass, Lipid, FAME, Molecular phylogenetic analyses	No data on toxicity of NPs; Mechanism unexplored.
Ferro et al. (2019)	**algal strain:** ***Chlorella vulgaris*** **Bacteria*****: Rhizobium sp.***	Synthetic Municipal Wastewater	Culture stability along with high nutrient removal capacity even at HRTs of 5 and 3 days	Biomass, Specific growth rate, O_2, CO_2, N_2, Lipid, Protein, carbohydrate	No data of Toxicity of NPs.
Thøgersen et al. (2018)	**Alga** ***Emiliania huxleyi*** **bacterium** ***Phaeobacter inhibens*** **DSM17395**	Growth Media	The presence of the alga facilitated the attachment of the bacterium to a surface	DNA isolation, PCR, Fluorescence tagging	No data of Toxicity of NPs; Only growth study
Ashok et al. (2014)	**Algal-bacterial consortia.** ***Chlorella vulgaris. Chlamydomonas reinhardtii***	Synthetic wastewater	Almost 90 % removal of Nitrogen and Phosphorus and 80% of COD (2-day HRT)	Temp., pH, chl a, biomass, nitrogen, phosphorus, polysaccharides, alkalinity.	condition optimum for OECD condition or mixture of NPs
Holmes et al. (2019)	**Bacteria-** ***Escherichia coli*** **Algae-** ***Auxenochlorella protothecoides***	Simulated wastewater	In cocultures with algae, minimal or no acetate was observed; COD removal up to 66% faster than *E. coli* in co-culture.	Culture growth, qPCR, organic acid analysis	No data on toxicity of NPs; no consideration for mixture of NPs
Cao et al. (2019)	**Algae:** ***Isochrysis galbana*** **Bacteria*****: Pseudomonas stutzeri***	NMB3 media	Decrease in the chlorophyll contents by 23–74% in co-culture as compared with the axenic culture in the period of 6 days.	Growth, chl via fluorescence, DNA extraction, PCR,	No NPs toxicity; no information at cellular levels (EC_{50}, etc).
Segev et al (2016)	**Algae:** ***Emiliania huxleyi*** **Bacteria*****: Phaeobacter Inhibens***	Growth media	Naked algal cells covered by bacteria attached via their poles; Over time more attachment of algae with bacteria in co-culture conditions	Growth profile, flow cytometry, fluorescence, SEM, chl a, cell analysis.	No toxicological studies on co-culture; no toxicity study due to NPs
Fie et al (2019)	**Bacteria:** ***R. radiobacter*** **Algae:** ***C. variabilis.***	*Growth media*	*R. radiobacter*-derived nitrogen stimulates fatty-acid oxidation in *C. variabilis* and promotes its growth	Growth profile, nitrogen, carbon, FAMEs.	No toxicity studies; no consideration for OECD conditions

Lepine et al. (2018)	***Chlorella spp.***	Industrial wastewater	A microalgae-bacteria consortium was grown in a mixture of industrial wastewater.	Cell count, pH, growth, Lipid, FAME,	No NPs-related toxicity study
Grover et al. (2020)	***C. vulgaris with Nitrobacter***	Growth media	Co-culturing enhanced growth (w/ increased cellular composition and biomass content)	Growth profile, cell count. biomass	No NPs-related toxicity study
Contreras-Angulo et al. (2019)	***Co-culture of Azospirillum brasilense and Scenedesmus sp.***	Growth media	Symbiotic co-culturing of microalgae-bacteria on nitrogen-deficient media enhancing microalgae size and biomass biofuels.	Biomass, cell size, protein carbohydrate, fatty acids, nitrogen	No NPs-related toxicity study
Zhou et al, (2020)	***Chlorella pyrenoidosa; Bacteria: High-efficient ammonia-oxidizing strain FN5***	Antibiotic containing Wastewater	FN5 enhanced biomass concentration and lipid content of microalga *Chlorella pyrenoidosa; Chlorella pyrenoidosa-FN5* culture removed NH_3–N and accumulated lipid	Enzyme activity (SOD, MDA, CAT), SEM, EPS, IAA, Nitrogen removal, Phosphate, COD removal	No NPs-related toxicity study
Verma et al, (2020)	***Algae: Chlorella sp. Activated sludge***	Lakewater	Removals of 93% nitrates, 99% phosphates, and 73% COD; maximum biomass content =7.8g/L	Microalgal growth, biomass, SEM, COD, FTIR, nutrient removal	No NP-related toxicity studies
Xie et al. (2020)	***Microalgal strain C. sacchrarophila bacterium C. pealriver***	*Growth media*	*Chlorella sacchrarophila* was grown in bioreactor while a xylanolytic bacterium *Cellvibrio pealriver;* During the CTS strategy, the co-cultivation using xylan as feedstock	Microbial growth, SEM, total nitrogen concentration, lipid.	No NPs-related toxicity study
Xu et al. (2021)	***C. vulgaris, S. obliquus, Spirulina platensis Aerobic activated sludge***	Raw municipal wastewater	Nutrient removal was increased; season- dependent nutrient removal; Aeration helps in the removal efficiency.	Wastewater characteristics, biomass, pH, DO, N, P, plate count, TSS, Growth kinetics.	No NPs-related toxicity study
Loria et al. (2021)	***C. vulgaris, C. sorokiniana, S. dimorphus, Neochloris oleoabundans; Activated sludge (AS)***	Growth media, sludge	Several microalgal taxa bio flocculated with AS within 2 h; P removal was inconsistent in *C. vulgaris* and *N. oleoabundans* reactors, but stable and high in *S. dimorphus* in SBR reactors, though S. dimorphus reactors also exhibited the poorest settleability	Biomass, lipid, TSS, growth profile, nitrogen, phosphorous, DO	No NPs-related toxicity study
Huo et al. (2020)	***Algae Chlorella sp. Bacteria: Bacillus firmus and Beijerinckia fluminensis***	Vinegar production Wastewater	Nutrient removal rates were significantly increased after adding bacteria cultures; *B. fluminensis* enhanced the pigment content of *Chlorella sp.;* Co-culturing had more notable effect on fatty acid composition rather than oil content.	TN, TFA, Cell count, TN, TP, COD, Lipid, Fatty acid	No NPs-related toxicity study

Mujtaba et al. (2017)	***microalga Chlorella vulgaris; bacterium Pseudomonas putida.***	Municipal wastewater	higher removal of both nutrients and COD in coculture than each axenic culture; the best removal performance with suspended *P. putida* and immobilized *C. vulgaris*	TP, TN, COD, TOC, TSS, Cell growth, wastewater characterization	No NPs-related toxicity study
Xu et al. (2021 b)	***Chlorella vulgaris, Scenedesmus obliquus, Spirulina platensis***	Municipal wastewater	In the summer & autumn seasons high removal rates and biomass production percentages, the highest specific growth rate was 0.46 d−1; the highest TN removal rate was 2.34 d^{-1}; and the highest TSS removal efficiency was 96.3 ± 2.1%.	Biomass, plate count, COD, pH, DO, TP, TN, nutrient removal kinetics, growth kinetics	No NPs-related study
Xu et al, (2021 a)	***Algae: Chlorella vulgaris Bacteria: Bacillus.***	Growth media	Two bacterial strains of different genera were isolated from *Chlorella vulgaris;* Bacillus strain improved algae growth, photosynthesis, and nutrient removal; 7-day optimal co-culturing conditions with 10:1 bacteria-to-microalgae ratio	Biomass, cell count. Nutrient removal, growth kinetics, chl a	No NPs-related toxicity study
Tao et al. (2020)	***Chlorella sp. and Bacillus simplex***	Growth media	Consortium improved phenol degradation efficiency and *Chlorella sp.* Growth	Cell count, growth kinetics, phenol degradation	No NPs-related toxicity study
Li et al. (2021)	***Scenedesmus obliquus and Bacillus megaterium***		Co-culture was found more efficient in treating high-concentration biogas slurry compared with the pure microalgae culture, reducing various nutrients in biogas slurry and simultaneously accumulating biomass with higher biofuel characteristics.	Biomass, cell growth, chl a, chl b, lipid, TP, TN, COD, etc.	No NPs-related toxicity studies
Wang et al. (2021)	***Monoculture and co-culture of algae (Chlorella vulgaris) and bacteria (activated sludge)***	Swine manure	When co-cultivated, the algal growth and precipitation (harvest) were promoted, while aerobic bacteria growth was initially promoted, and then inhibited.	Biomass, pH, TN, COD, DO, cell count, SEM, DNA, Biomass settling efficiency	NPs toxicity was not studied.
Wang et al., 2022	***Algae-bacteria consortia (ABC) in activated sludge***	Cooking wastewater	ABC reactors show satisfactory removal ability. ABC can secrete large EPS to protect themselves and form flocs with good sedimentation performance under toxic and refractory organic wastewater stress.	COD, TN, pH, SS, biomass, EPS, BOD, chl a	NPs toxicity not discussed
Rossi et al., 2022	***Chlorellaceae (1·10⁶ cell·mL⁻¹), Scenedesmaceae (0.2·10⁶ cell·mL⁻¹), and Chlamydomonadaceae (0.2·10⁶ cell·mL⁻¹); bacterial culture: heterotrophs and nitrifiers.***	Piggery wastewater	Removal of NH_4^+, PO_4^{3-} (90%), and COD (59%), with 10.7 g/m^2/d biomass productivity. The process allowed to reduce the nitrogen spread to arable land by 77%, by increasing the nitrogen valorised as biofertilizers/ bio stimulants and the nitrogen released to the atmosphere.	TSS, TAN, COD, Biomass, chl a, FDA, SEM.	NPs were not present in this study.
Xu et al., 2023	***Cladophora, activated sludge***	Wastewater	The addition of microorganisms increases the	DO, biomass, TN, TP,	NPs not studied.

		Toxicant: atrazine	removal efficiency of TN in atrazine-containing wastewater by 43.70%, and the addition of Cladophora further increased by 3.82%. The protein signal produced by the microbial release of EPS triggered the algal resistance mechanism and approximately 3% more in the algae–bacteria consortia.	COD, chl a, chl b, SOD, POD, MDA, EPS.	
Cheng et al., 2022	***Laboratory-grown algae and bacteria from activated sludge***	Municipal wastewater	A new type of algae-bacteria biofilm reactor (ABBR) was designed. ABBR allowed a marked improvement on the removals of IMI, TDN, TDP, and cod during the 16-day operation. Meanwhile, more IMI degradation products were found in PBR while lower biological toxicity was detected in ABBR.	pH, TDS, TN, ICP-MS, biomass,	NPs presence was absent.
Wang et al., 2023	***Trebouxiophyceae, Saccharimonadales*, *Propion ibacteriaceae*, *Propioniciclav a*, and *Micropruina***	Municipal wastewater **Toxicant:** NPs, abiotic stress	The addition of algae led to an increase in sedimentation performance, biomass, and EPS. The AEBC had a maximum 77.15 % removal rate of C, 63.22 % of N, and 82.54 % of P, respectively. The effluent of algae-enhanced reactors suggested that algae had significant effects on pollutant removal.	Total DNA, PCR, SEM, biomass, chl a, chl b, carotenoid, PCA.	No NPs were used in this study.