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Distinctive toxic repercussions of polystyrene nano plastic towards aquatic non target species *Nitrobacter vulgaris, Scenedesmus sp* and *Daphnia magna*

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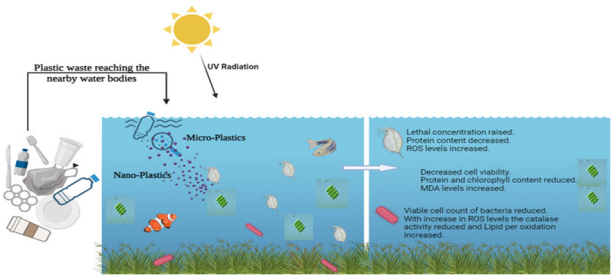
Abstract

The widespread application of plastics and its eventual degradation to micro-sized or nano-sized plastics has led to several environmental concerns. Moreover, nanoplastics can easily cascade through the food chain accumulating in the aquatic organisms. Thus, our study focussed on investigating the hazardous impact of nano-sized plastics on aquatic species including *Nitrobacter vulgaris*, *Scenedesmus sp*, and *Daphnia magna*. Various concentrations of polystyrene nanoplastics ranging from 0.01 mg/L to 100 mg/L were tested against *Nitrobacter vulgaris*, *Scenedesmus sp*, and *Daphnia magna*. The minimum inhibitory concentration of polystyrene nanoplastics in *Nitrobacter vulgaris* was found to be 25 mg/L, and in *Daphnia magna*, the median lethal concentration 50 was observed to be 64.02 mg/L. Exposure of *Scenedesmus sp* with increasing nanoplastic concentrations showed a significant decrease in total protein ($p < 0.001$), and chlorophyll content ($p < 0.01$), whereas the lipid peroxidation increased ($p < 0.001$) significantly. Similarly, *Nitrobacter vulgaris* and *Daphnia magna* showed a significant decrease in catalase activity ($p < 0.001$) and an increase in lipid peroxidation levels ($p < 0.01$). Concomitant with lipid peroxidation results, decreased superoxide dismutase levels ($p < 0.01$) and protein concentrations ($p < 0.01$) were observed in *Daphnia magna*. Besides, the increasing concentration of polystyrene nanoplastics displayed an elevated mortality rate in *Scenedesmus sp* ($p < 0.001$) and *Nitrobacter vulgaris* ($p < 0.01$). Further, scanning electron microscopy analysis substantiated the morphological alterations in *Nitrobacter vulgaris* and *Scenedesmus sp* on exposure to polystyrene nanoplastics.

Graphical Abstract



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Introduction

Plastics have become ubiquitous in modern society, finding extensive use across various industries such as food production, pharmaceuticals, packaging, textiles, paints, adhesives, biomedical applications, toys, electronics, and personal care products (Gigault et al., 2018; Hernandez et al., 2017; Koelmans et al., 2015; Kik et al., 2020). However, their widespread application has led to escalating environmental concerns due to pollution, impacting ecosystems globally (Geyer et al., 2017; Kik et al., 2020). Plastics enter the aquatic environment through anthropogenic activities, resulting in ecological disbalance. These plastics eventually degrade via physical, chemical, biological forces, UV radiation, etc. and result in the formation macro-plastics (> 20 mm size), meso-plastic (2–20 mm size), microplastics (< 5 μm) and nanoplastics (1 nm – 100 nm) (Dawson et al., 2018; Rodriguez-Hernandez et al., 2020; He et al., 2021). They are tiny plastic particles and are ubiquitous in nature. As the size of these nanoplastics are very small, they are being taken up by organisms in the environment and affect non-target species in the eco-system (Guzzetti et al., 2018; Hengstmann et al., 2021; Huerta Lwanga et al., 2016; Kalcikova et al., 2017; Lusher et al., 2015; Machado et al., 2020). Reports reveal that nanoplastics get accumulated in the body of the aquatic organisms and can be transferred along the food chain (Maocai et al. 2021; Bhagat et al., 2021; Cole et al., 2013; Cole et al., 2011).

Currently, freshwater contamination with plastic debris is one of the most intense issues worldwide. Aquatic species like the fishes, phytoplankton, zooplankton, oligochaetes, and mammals, etc. ingest these nano-sized particles, which may further get accumulated and cause harm (Edwin et al., 2014; Esperanza et al., 2016; Perumal, Muthuramalingam 2022). The hydrophobicity, large surface area, particle size, porosity, etc aid the penetration of nanoplastics into the cell membrane (Bhagat et al. 2022; Oliveri et al., 2020). A study by Manabe et al., 2011, demonstrated the penetration of nanoplastics through chorion in the embryos of *Oryzias latipes* (Manabe et al., 2011). Subjection of fishes to polystyrene nanoplastics (PS NPs) either directly through gills or indirectly via tropical transfer in the food chain can cause virulent effect on the same (Alimi et al., 2018; Lu et al., 2018; Mattsson et al., 2017). An experimental study by Cargo et al., 2018, showed that, 0.1 ppm, 1 ppm, 10 ppm fluorescently labelled PS NPs tested against zebrafish embryos and larvae lead to the penetration, uptake and distribution of PS NPs in yolk sac after 24 hpf. This later migrated to the gastrointestinal tract, gall bladder, liver, pancreas, heart, and brain at 120 hpf. Although the mortality rate or bioenergetics of mitochondria were not affected, the accumulation of these particles in various tissues of zebrafish, resulted in physiological and behavioural changes of the organism. Chronic exposure to PS NPs at 0.1 ml/L affected the reproduction and growth of the *Daphnia magna*, showing negative impact on the crustaceans. Long term exposure of *Nitrobacter* bacteria to PS NPs raised the extracellular polymeric substances (EPS) levels. Also, this study showed that reactive oxygen species produced damage to the membrane integrity of the bacteria (Valentina et al., 2019; Telouk et al., 2022; Vandermeersch et al., 2015; Das et al., 2023).

Reports have shown that nanoplastics cause adverse effects like genotoxicity, neurotoxicity, metabolic disorders etc, in various organisms including humans (Fan et al., 2023). Hence it is important to study the toxic effects of nanoplastics on aquatic species. While studies have shown the negative effect of PS NPs on aquatic species, the knowledge gap pertaining to the physiological and biochemical effects need to be addressed (Maocai et al., 2021). Thus, our present study focusses on exploring the toxic impact of Polystyrene nanoplastics on the biochemical properties of varying aquatic lifeforms such as, microorganism (*Nitrobacter vulgaris*), phytoplankton (*Scenedesmus sp*) and a zooplankton (*Daphnia magna*).

Materials and methods

All chemicals including Latex Beads-Polystyrene (100 nm), required for the study were purchased from Sigma-Aldrich, Bengaluru, Karnataka, India. *Daphnia magna* were procured from The Infanciya Oceans, Bengaluru, Karnataka, India. BG11 medium was purchased from Sisco Research Laboratories Pvt Ltd, Mumbai, India. All other chemicals purchased from Sisco Research Laboratories Pvt Ltd, Mumbai, India and Sigma-Aldrich, Bengaluru, India, and were of analytical grade.

Sample preparation

A standard stock solution of nanoplastics (1%) was prepared using Milli-Q water. From which 0.01 mg/L, 0.05 mg/L, 0.1 mg/L, 1 mg/L, 5 mg/L, 50 mg/L, and 100 mg/L working standards were prepared and tested against *Nitrobacter vulgaris*, *Scenedesmus sp*, and *Daphnia magna*. Here samples without any nanoplastics were used as control. All experiments were performed in triplicate and represented as mean ± SEM.

Characterisation of nanoplastics

Particle size analysis

The hydrodynamic mean particle size of the Polystyrene nanoparticles was analysed using a Nano Particle Analyser (SZ100, Horiba Scientific, Japan). Before the analysis, the samples were diluted using Milli-Q-Water and further loaded into 1 cm^3 cuvettes. The samples were analysed using the laser light scattering principle at a scattering angle of 90° (Ragavan et al., 2017).

Surface charge analysis

Zeta potential analysis was carried out to study the surface charge of the formulated emulsion droplets. Polystyrene nanoparticle samples before analysis were diluted using deionized water and introduced into a capillary cell for charge determination. Final recordings were used to analyse the repulsive forces among the particles present in the emulsion system using the Helmholtz-Smoluchowski equation. The zeta potential analysis was carried out using a Nano Particle Analyser (SZ100, Horiba Scientific, Japan) (Ragavan et al., 2017).

Nanoplastic toxicity studies on *Scenedesmus sp*

Fresh water collected from Kattigenahalli lake, Bengaluru, 13.1147^{o} N, 77.6286^{o} E, was used to isolate the algal species using a standard plating method. Here, 1 ml of water sample was used and serially diluted up to 10^{-7}. This mixture was plated onto BG11 agar plates and incubated for 15 days. The composition of BG11 purchased is per Mishra et al., 2019. Later pure colonies were streaked onto freshly prepared plates. This procedure was repeated until pure culture of *Scenedesmus* was obtained (Keesoo Lee et al., 2014). The isolated algal species were exposed to various concentrations of polystyrene ranging from 0.01 mg/L to 100 mg/L as mentioned earlier in sample preparation. This was incubated at suitable light: dark (10:14) and temperature for 4 days (96 h). To collate the results, *Scenedesmus sp* inoculated in 2 ml BG11 media was maintained as control throughout the experiment.

Cell viability test

A cell viability test was carried out using the cell counting method as per Cadena-Herrera et al. (2015). Each treated sample was vortexed for 30 s, from which 10 µl of sample was placed on the haemocytometer. All 4 squares on the haemocytometer were counted and average was taken to get total number of viable cells. Thus, the manual counting of viable cells after exposure to nanoplastics was performed. Further, the cell viability test was experimented through MTT assay as per Moshfegh et al., 2019 and Van Meerloo et al. (2011). Here, after 96 h of exposure to different concentrations of nanoplastics, 10 µl of each sample was taken, to which 100 µl of MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was included and incubated overnight. Later, to this mixture 100 µl DMSO (Dimethyl sulfoxide) was added to dissolve the tetrazolium formed and readings were taken at 650 nm.

Biochemical assays

Protein estimation

Bradford's method (1976) was used to estimate the protein content in algae after exposure to nanoplastics. 10 µl of each treated samples were added into different wells and volume was made up to 100 µl with distilled water, on a 96 well plate. To this, 200 µl of Bradford reagent was included into each well and incubated in dark for 15 to 20 min. Absorbance was taken spectrophotometrically at 650 nm. Bovine serum albumin of 2 mg/ml was used as the working standard.

Lipid peroxidation Assay/MDA assay

After 96 h of interaction with nanoplastics the lipid peroxidation level in *Scenedesmus sp* was measured via the MDA (Malondialdehyde) assay. This test was performed as per the process mentioned by Dubovskiy et al., 2008 and Gawel et al., 2004. Here, 1.5 ml of each sample were transferred into different centrifuge tubes along with 500 µl of 20% TCA (Trichloro Acetic Acid) and centrifuged at 14000 rpm for 10 min. Later, 1200 µl of supernatant was taken into a new centrifuge tube and to this 800 µl of 0.8% TBA (Thiobarbituric Acid) was added and kept in hot water bath for 60 min at 100°C. The aldehydic products generated reacts with the TBA and give a reactive substance called thiobarbituric acid reactive substance (TBARS), which was measured spectrophotometrically at 532 nm.

Pigmentation assay

The chlorophyll content present before and after treatment of algae with nanoplastics was investigated through the pigmentation assay. Chlorophyll a, chlorophyll b and total chlorophyll were extracted using 80% acetone and spectrophotometrically measured as defined by Mantoura and Llewellyn, 1983; Su et al., 2010; Simon, Helliwell (1998). Below formula 1A, 1B, 1C (Poonghuzhali 2015)

was used to calculate chlorophyll content.

Formula1A : $\text{Chlorophyll a}(\mu g/ml) = 12.7(A_{663}) - 2.69(A_{645})$

Formula1B : $\text{Chlorophyll b}(\mu g/ml) = 22.9(A_{645}) - 4.68(A_{663})$

Formula1C : $\text{Total Chlorophyll}(\mu g/ml) = 20.2(A_{645}) + 8.02(A_{663})$

where, A = Absorbance at respective wavelength.

SEM for *Scenedesmus sp*

SEM analyses was performed using the Sputter Coater – Quorum SC7620 and Carl Zeiss EVO-18 instrument, at the Central Instrumentation Facility (CIF), University of Agricultural Sciences, Bangalore (UASB), GKVK, Bengaluru. Here, *Scenedesmus sp*., after 96 h of exposure to the control, 0.01 mg/L and 100 mg/L of PS NPs, were selected for SEM imaging. The algae from each of these concentrations was applied onto Whatman filter paper no 1, using a paint brush, then air dried. Further, the images were analysed (Mishra et al., 2016).

Nanoplastics toxicity study on *Nitrobacter vulgaris*

A fresh water sample collected from Kattigenahalli lake, Bengaluru, 13.1147^{o} N, 77.6286^{o} E, was used to isolate the bacteria. Here, 1 gram of soil was mixed in 9 ml of sterile distilled water and serially diluted up to 10^{-3}. Aliquots from each dilution were plated on to winogradsky agar and incubated for 2–3 days at room temperature. Greyish, mucoid, flat colonies of gram-negative bacteria were morphologically identified, and further nutrient broth was used for culturing and maintaining the bacterium throughout the study (Douglas, and Dilosi 2019).

Minimal inhibitory concentration (MIC)

Initially, minimal inhibitory concentration of the bacteria with polystyrene was determined using a process explained by Panacek et al., 2018. Here, each well contained 50 µl of nutrient broth. In the first well 50 µl of 100 mg/L nanoplastic was added and further this was serially diluted. To each well 50 µl of bacterial culture was added and incubated at 27 °C overnight. A control was used without adding nanoplastics. Further absorbance was measured at 650 nm. The minimal inhibitory concentration determined was used for biochemical analysis and other assays.

Cell viability studies

MTT assay

10 mg/L, 25 mg/L and 50 mg/L of nanoplastics were tested the against the control. After 24 h of exposure to polystyrene, 100 µl of MTT dye was added to 10 µl of each sample and incubated overnight. To this mixture DMSO of 100 µl was added and readings were taken at 650 nm (Moshfegh et al., 2019; Van Meerloo et al. 2011).

Biochemical experiments

Lipid peroxidation assay

Following 24 h of exposure to nanoplastics, 1.5 ml of each treated sample were taken to which 500 µl of 20% TCA was added and centrifuged at 14,000 rpm for 10 min. Further, 1200 µl of supernatant was transferred into a fresh test tube to which 800 µl of 0.8% TBA was added and placed in a water bath for 60 min at 100 °C. The aldehydic product formed was spectrophotometrically measured at 532 nm (Dubovskiy et al., 2008; Gawel et al., 2004).

Catalase activity

400 µl of 10 Mm H_2O_2, 2 ml of phosphate buffer, with 40 µl of enzyme extract was added to each of the test samples and the breakdown of hydrogen peroxide by the catalase enzyme was allowed for 3 min. Initial and final absorbance was noted at 240 nm and catalase activity was calculated using Formula 2 (Rezayian, et al., 2019; Cheng, et al., 2016).

Formula2 : Catalase activity = Initial readings
$-$Final readings Time(3mins).

SEM for *Nitrobacter vulgaris*

Bacterial cells from the control and those following 24 h of exposure to the 100 mg/L nanoplastics, were fixed as per the process explained by Chao and Zhang (2011). Further, Sputter Coater – Quorum SC7620 and Carl Zeiss EVO-18 instrument, at the Central Instrumentation Facility (CIF), University of Agricultural Sciences, Bangalore (UASB), GKVK, Bengaluru was used for the images.

Nanoplastic toxicity study on *Daphnia magna*

Daphnia magna were maintained in laboratory conditions using infusoria culture. The water parameters included, 6.5–9.5 pH, with 16 – 20 °C. Here, *Scenedesmus* algae was

used to feed the *Daphnia magna* twice a day. A photoperiod of 16 h:8 h, dark-light cycle was followed, and the culture water was changed every 2 weeks. Young Daphnids were used throughout the process (OECD guideline 202, 2004).

Lethal indices studies

Acute toxicity studies were performed as per the OECD guidelines (202). Various concentrations of nanoplastics ranging from 0.01 mg/L to 100 mg/L, were tested against *Daphnia magna*. In each concentration, 10 young Daphnids were used. The mortality rate was recorded after 24 h of treatment with different concentrations of nanoplastics. The acquired results were fed into Medcalc tool to get LC_{25}, LC_{50} and LC_{90} values (Lin et al., 2019). Table 1 gives the lower, median and upper limit of the lethal concentrations. Following this, the median limit was chosen to test the possible biochemical changes the organism undergoes.

Biochemical analysis

Protein analysis

Total protein content was estimated for *Daphnia magna* using the process described by Bradford's (1976). Ten *Daphnia magna* were used from each tested concentrations and crushed using motor and pestle. From this, 10 µl of sample was taken and the volume was made up to 100 µl with distilled water. To the mixture, 200 µl of Bradford's reagent was added and incubated in the dark for 15 min and absorbance was noted at 650 nm. BSA was used as standard.

Lipid peroxidation Assay: After 24 h of incubation with the nanoplastics, 1.5 ml of finely crushed samples were transferred into centrifuge tubes along with 500 µl of 20% TCA and centrifuged at 14000 rpm for 10 min. Later, 1200 µl of supernatant was taken and to this 800 µl of 0.8% TBA was added and kept in a hot water bath for 60 min at 100 °C. The aldehydic product formed was measured at 532 nm (Dubovskiy et al., 2008; Gawel et al., 2004).

Table 1 Lethal Indices of Polystyrene Nano-plastic Tested against *Daphnia magna*

Lethal Concentration	Median Limit (mg/L)	Lower Limit (mg/L)	Upper Limit (mg/L)
LC_{25}	38.12	13.13	73.33
LC_{50}	64.02	40.3	129.5
LC_{90}	113.23	76.5	257.6

Lethal Indices concentration of *Daphnia magna* after 24 h of treatment with polystyrene nano-plastics.

Catalase activity

To the test samples, 2 ml of phosphate buffer, 400 µl of 10 Mm hydrogen per-oxide and 40 µl of enzyme extract were added and this mixture was analysed at 240 nm. Initial readings were recorded, and after 180 sec the final readings were noted. Formula 3 was used to calculate the catalase activity (Rezayian et al., 2019; Cheng et al., 2016).

$$\textbf{Formula}3: \text{Catalase activity} = \text{Initial readings} - \text{Final readings}/\text{Time}(3\text{mins}).$$

Super-oxide dismutase (SOD) assay

One of the first lines of defence against oxidative stress is SOD, which catalyses the dismutation of superoxide into hydrogen peroxide and oxygen. 24 h post treatment with nanoplastics, ten *Daphnia magna* were homogenised to prepare the sample homogenate. Later, 100 µl of sample was taken from each concentration. To this, 20 µl of Hydrochloric acid + 20 µl of TEMED + 20 µl of EDTA and 20 µl of Quercetin, were included and incubated for 5 min. The radicals formed were measured at 406 nm (Lushchak et al., 2005).

Statistical analysis

All experimental results of the study were statistically evaluated using GraphPad Prism version 8.0. One-way ANOVA, followed by Dunnett's test was preformed to check the statistical significance of the results obtained. The lethal indices concentration for the *Daphnia magna* was calculated using MedCalc software. All the assays were performed in triplicate and results were represented in mean ± SEM.

Results

Characterisation of nanoplastic

Dynamic Light Scattering (DLS) determined the mean size of polystyrene nanoplastic to be 98.1 nm (Fig. 1a) and −23.4 mV surface charge of the particle was displayed through zeta potential (Fig. 1b). The zeta potential value of the polystyrene depicts a good stability for coalescence (Kadu, et al., 2011).

Toxicity analysis of polystyrene against *Scenedesmus sp*

Once pure culture was obtained, the algae was cultured and maintained using BG11 broth media throughout the study.

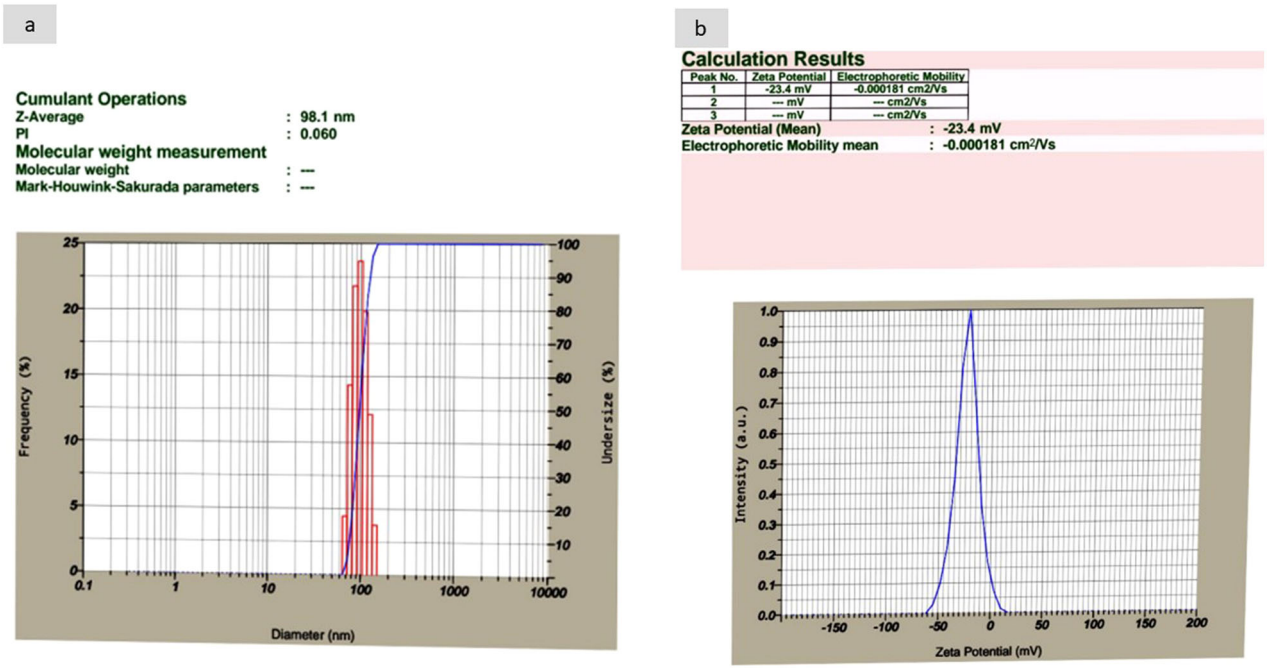

Fig. 1 **a** Characterization of Polystyrene Nanoplastic via Dynamis light scattering **b** Characterization of Polystyrene Nanoplastic via Zeta Potential

The algae were morphologically observed under microscope and recognised as *Scenedesmus* as per Gour et al. (2016).

Cell viability assays

Haemocytometer

After 96 h of exposure to different concentrations of nanoplastic, manual counting of the cells via haemocytometer exhibited the reduction in viable cell number with respect to control. Although the growth rate in all samples was constant, viable cell count reduced as concentration of polystyrene increased. On exposure to PS NPs, at 0.01 mg/L concentration 136.5 cells/10 µl ($p < 0.01$) were observed after 96 h, whereas 5 mg/L showed 109 cells/10 µl ($p < 0.001$). The least number of viable cells i.e., 80.2 cells/10 µl were observed on exposure to 100 mg/L of nanoplastics ($p < 0.001$) as compared to control, which had 168.5 cells/10 µl viable cells. The haemocytometer graph is depicted in Fig. 2a.

MTT Assay: At 0.05 mg/L, an absorbance of 0.1510 was observed ($p < 0.01$), while at 1 mg/L, an absorbance of 0.131 ($p < 0.01$) was observed at 650 nm. At 100 mg/L, an absorbance of 0.1050 was noticed ($p < 0.01$), which showed a significant reduction as compared to control absorbance of 0.181 (Fig. 2c).

Biochemical assays

Bradford's method of protein estimation showed that the highest concentration of protein, i.e., 0.6914 mg/L was found in control compared to test samples. In 0.05 mg/L of nanoplastic concentration, 0.517 mg/L protein content ($p < 0.001$) was observed, whereas 50 mg/L and 100 mg/L showed 0.300 mg/L ($p < 0.001$) and 0.235 mg/L protein content ($p < 0.001$), respectively at 650 nm. Figure 2b, represents the protein content reduction with an increase in polystyrene levels.

In the lipid peroxidation assay, an elevated MDA level was observed in the samples treated with higher concentrations of nanoplastics. 0.1 mg/L and 1 mg/L exhibited 0.013 MDA/mg of protein and 0.0180 MDA/mg of protein respectively after 4 days of exposure, nevertheless, a significant increase in lipid peroxidation level was observed with increasing nanoplastics concentrations of 5 mg/L ($p < 0.001$), 50 mg/L ($p < 0.001$) and 100 mg/L ($p < 0.001$). At 100 mg/L, a significant increase to 0.0350 MDA/mg of protein ($p < 0.001$) was noticed. This shows that as concentration of nanoplastics increased, the ROS levels also elevated, which caused the destruction of lipid membrane of the algae leading to cell damage (Dubovskiy et al., 2008). This has been depicted in Fig. 2d.

The Pigmentation assay was performed in *Scenedesmus sp* and the chlorophyll content was found to decrease with in the highest concentration of nanoplastics treatment as

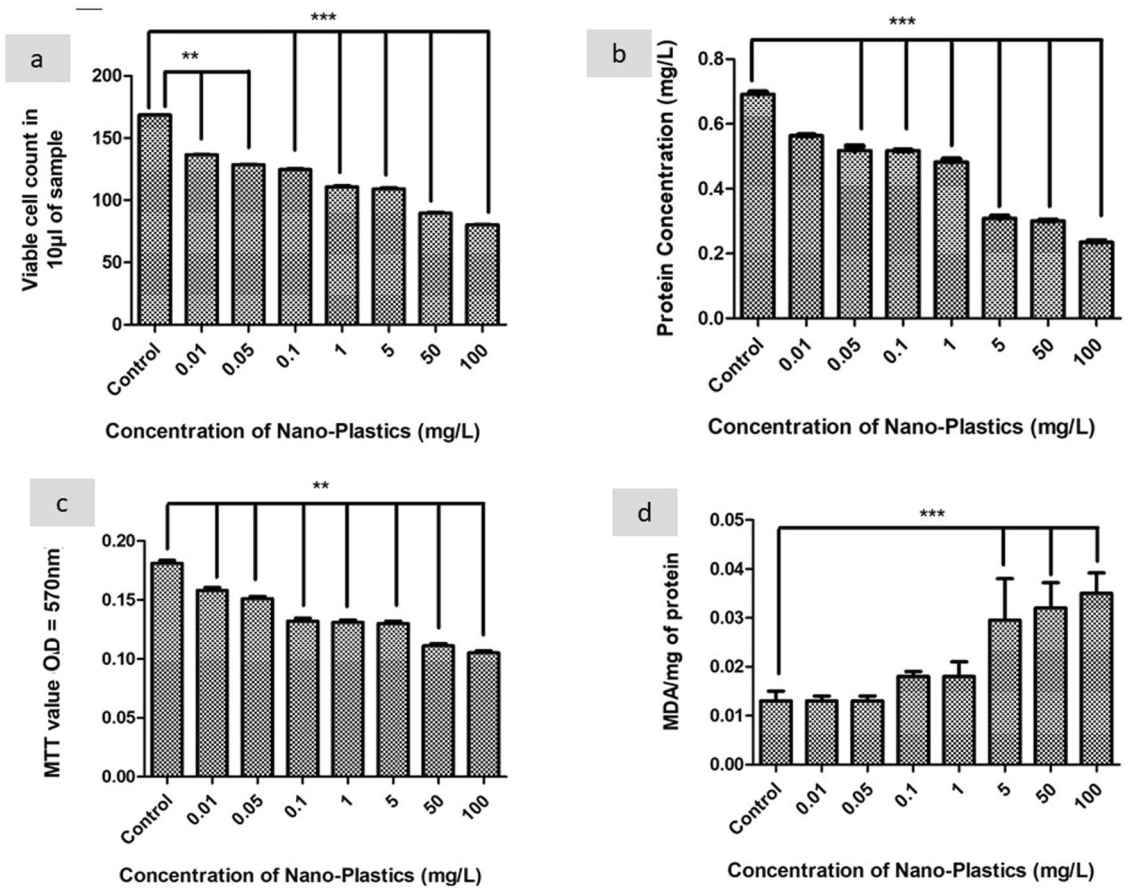

Fig. 2 **a** Haemocytometer assay: Cell viability of *Scenedesmus sp* through cell counting method upon exposure of polystyrene nanoparticles (0.01–100 mg/L) in comparison with control counterpart. **b** Protein Estimation: Total protein concentration of *Scenedesmus sp* upon exposure of polystyrene nanoparticles (0.01–100 mg/L) in comparison with control counterpart. **c** MTT Assay: Cell viability of *Scenedesmus sp* through MTT assay method upon exposure of polystyrene nanoparticles (0.01–100 mg/L) in comparison with control counterpart. **d** Lipid peroxidation: MDA concentration raised in tested algae with increase in nanoplastic levels. Highest TBARS, formation was observed in 100 mg/L. Corresponding error bar represents standard deviation of three replicates (SD, $n = 3$)

compared to the control. The mean value of control was observed to be 8.92 mg/L for chlorophyll a, 16.15 mg/L of chlorophyll b and total chlorophyll was 25.07 mg/L. Treatment at 100 mg/L depicted 1.56 mg/L of chlorophyll a ($p < 0.001$), 2.97 mg/L of chlorophyll b ($p < 0.01$), and 4.53 mg/L ($p < 0.01$) of total chlorophyll. Figure 3a–c depicted a decrease in chlorophyll a, chlorophyll b and total chlorophyll contents, respectively, with relation to various concentrations of nanoplastic tested on the algae.

SEM analysis

After 96 h of exposure to polystyrene, SEM analyses was performed to check the morphological changes. Figure 4 depicts the SEM images of *Scenedesmus sp* tested with nanoplastics. In the control, *Scenedesmus sp* displayed intact morphology, whereas, algal cell count decreased significantly in highest concentration (100 mg/L) as compared to control. Also, at this concentration the algal cells were distorted and displayed disturbed morphology in comparison with the control. While at 0.01 mg/L, morphology and algal cell counts were not disturbed as compared to the highest concentration (100 mg/L).

Toxicity of polystyrene nanoplastic on *Nitrobacter vulgaris*

The bacterium was morphologically identified as rod-shaped, gram-negative after isolation. Further, sequencing and purity check was performed at Medauxin, Bangalore, Karnataka, India, where it was recognized as *Nitrobacter vulgaris* with similarity to accession number AM286393-1.

Minimal inhibitory concentration (MIC)

As per the process explained by Panacek et al. (2018), MIC was determined to be 25 mg/L. Later, three different concentrations, i.e., 10 mg/L, 25 mg/L and 50 mg/L, were used for testing nanoplastic toxicity against *Nitrobacter vulgaris*.

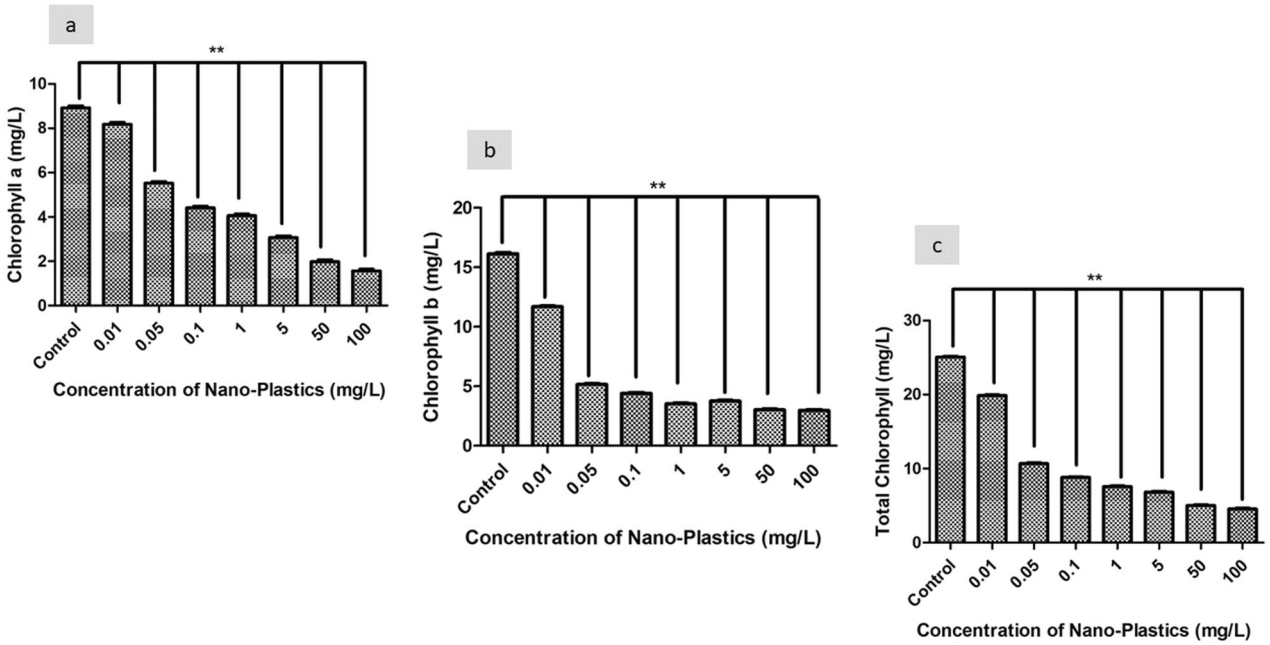

Fig. 3 **a** Pigmentation Assay: Chlorophyll a content in *Scenedesmus sp* upon exposure of polystyrene nanoparticles (0.01–100 mg/L) in comparison with control counterpart. **b** Pigmentation Assay: Chlorophyll b content in *Scenedesmus sp* upon exposure of polystyrene nanoparticles (0.01–100 mg/L) in comparison with control counterpart. **c** Pigmentation Assay: Total Chlorophyll content in *Scenedesmus sp* upon exposure of polystyrene nanoparticles (0.01–100 mg/L) in comparison with control counterpart. Corresponding error bar represents standard deviation of three replicates (SD, $n = 3$)

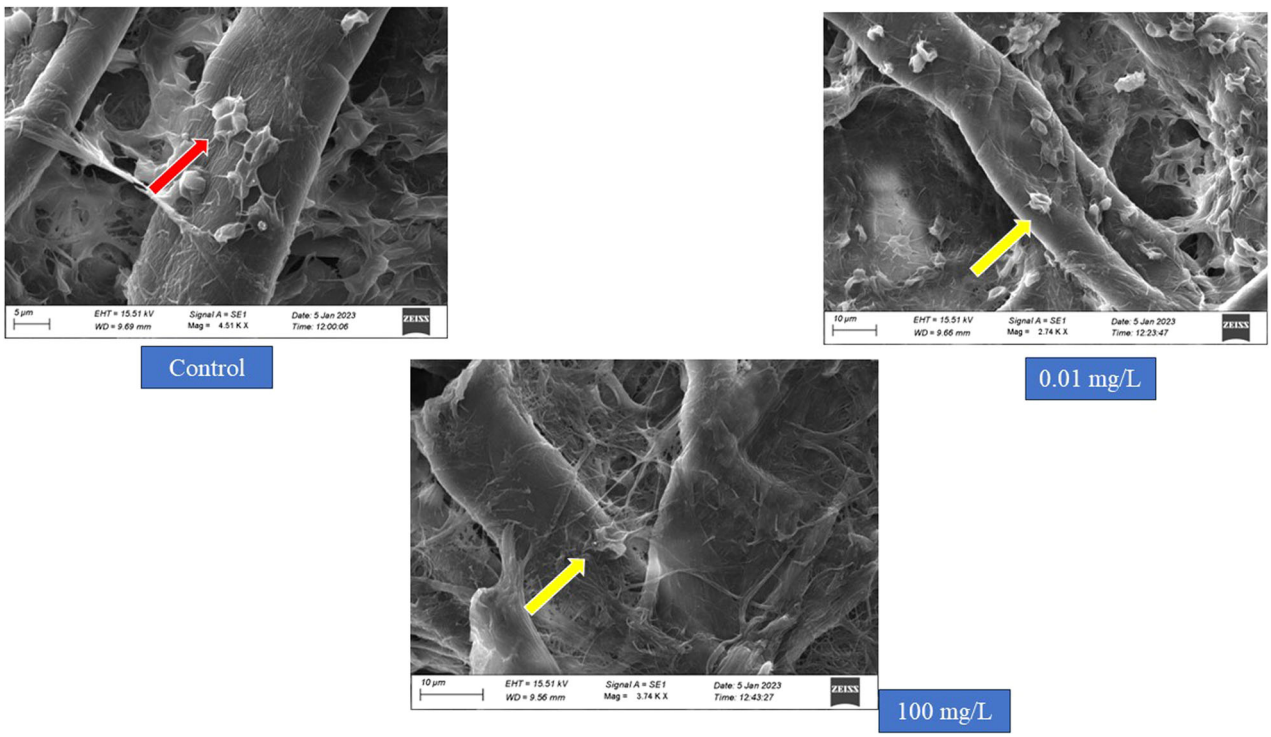

Fig. 4 SEM images of *Scenedesmus sp:* SEM images of *Scenedesmus sp* exposed to nanoplastics. Control, 0.01 mg/L, 100 mg/L for 96 h have been depicted here. The red arrow in control indicates the *Scenedesmus* cell without deformities. Yellow arrows in 0.01 mg/L and 100 mg/L indicate the deformed *Scenedesmus*. Also, the number of cells in 100 mg/L was less compared to control

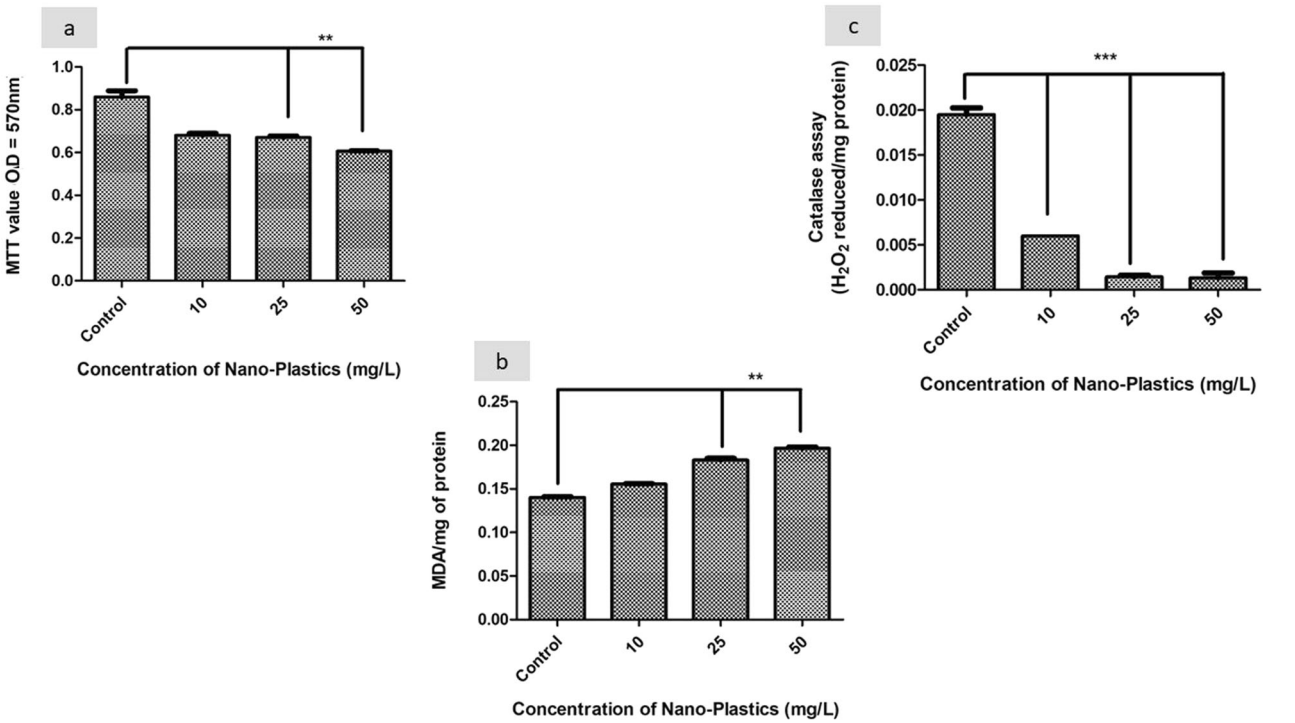

Fig. 5 **a** MTT assay: Cell viability of *Nitrobacter vulgaris* through MTT assay method upon exposure of polystyrene nanoparticles (10–50 mg/L) in comparison with control counterpart. **b** Lipid peroxidation: Oxidative stress analysis in *Nitrobacter vulgaris* through Lipid peroxidation assay method upon exposure of polystyrene nanoparticles (10–50 mg/L) in comparison with control counterpart. **c** Catalase activity: Oxidative stress analysis in *Nitrobacter vulgaris* through Catalase assay method upon exposure of polystyrene nanoparticles (10–50 mg/L) in comparison with control counterpart. Corresponding error bar represents standard deviation of three replicates (SD, $n = 3$)

Cell viability assay

Following 24 h of exposure to 10 mg/L, 25 mg/L and 50 mg/L of nanoplastics, readings were taken at 650 nm. The control showed an absorbance of 0.8595, whereas, 25 mg/L and 50 mg/L of nanoplastics showed a decrease in absorbance to 0.67 ($p < 0.01$) and 0.606 ($p < 0.01$) respectively (Fig. 5a).

Biochemical assays

The lipid peroxidation level did not show any change as compared to the control at 10 mg/L nanoplastic concentration. However, at 25 mg/L and 50 mg/L, a significant increase in lipid peroxidation level of 0.183 MDA/mg of protein ($p < 0.01$) and 0.196 MDA/mg of protein ($p < 0.01$), respectively was observed as compared to the control group. In Nitrobacter, a gram-negative bacterium, the disruption of the lipid membrane is seen to increase with the augmented nanoplastic levels. A graph showing the relationship between the control, the nanoplastic concentrations tested and, lipid peroxidation in Nitrobacter is depicted in Fig. 5b.

Catalase activity was measured at an absorbance of 240 nm. The catalase enzyme breaks protein present in bacteria with help of H_2O_2 releasing water and oxygen. In the treated samples, reduced catalase activity is observed due to nanoplastic activity as compared to control. An average of 0.0195 U/mg of protein was observed in the control, whereas 10 mg/L showed a significant decrease to 0.006 U/mg of protein ($p < 0.001$) at 240 nm. Also, at 25 mg/L and 50 mg/L, a significant decrease in catalase activity to 0.001425 U/mg of protein ($p < 0.001$) and 0.001327 U/mg of protein ($p < 0.001$) was observed. Figure 5c shows the catalase graph.

SEM analysis

SEM images of bacteria revealed the morphological changes in *Nitrobacter vulgaris*. The control showed clear rod-shaped cells with smooth surfaces. At the highest concentration (50 mg/L), the biofilm formed by extracellular polymeric substance (EPS) was observed. EPS are usually produced by bacterial cells to cope up with the toxicity produced in the surrounding environment (Chaali et al., 2021). Here the bacterial cells had fewer cells with smooth surfaces compared to control. Figure 6 represents the SEM images of *Nitrobacter vulgaris* treated with control and highest concentration (50 mg/L).

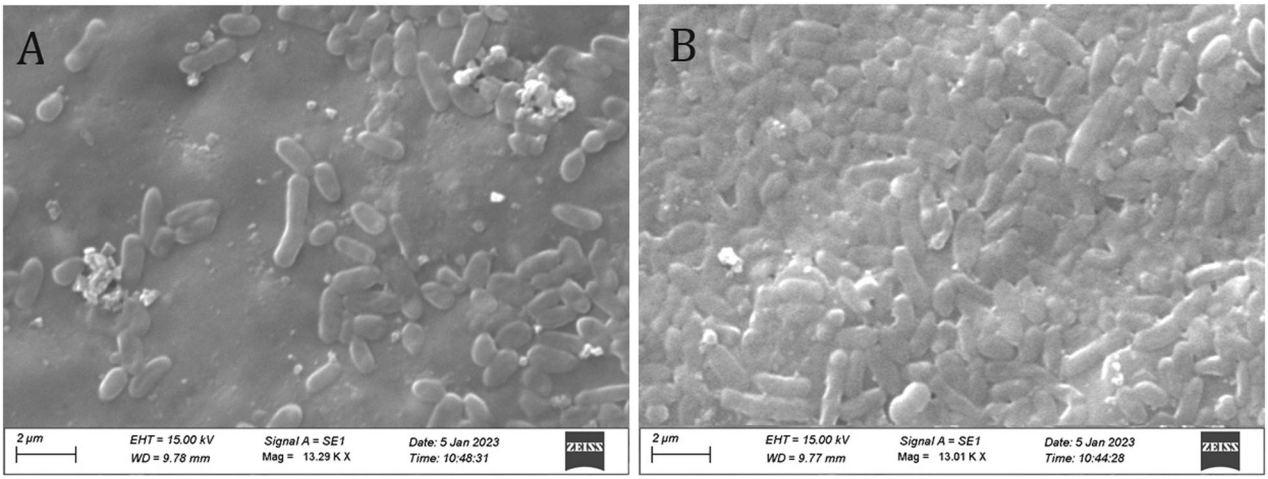

Fig. 6 SEM images of *Nitrobacter vulgaris:* SEM images of *Nitrobacter vulgaris* exposed to nanoplastics. Control and 50 mg/L, for 24 h have been displayed here. In control **A** cell morphology is distinct and individual cells are observed. While in the treatment i.e., 50 mg/L **B** the entire cells are substantially adhered together due to toxic response

Toxicity analysis of polystyrene on *Daphnia magna*

The mortality in *Daphnia magna* due to the effect of various concentrations of nanoplastics was noted and the values were fed into MedCalc tool to obtain LC_{25} (38.12 mg/L), LC_{50} (64.02 mg/L) and LC_{90} (113.23 mg/L) values. Table 1 gives the lethal indices of Polystyrene nanoplastic tested against *Daphnia magna*.

Biochemical assays

The protein content in highest concentration of nanoplastics, i.e., LC_{90} was found to be 0.51 mg/L ($p < 0.01$), also LC_{50} and LC_{25} depicted 0.53 mg/L ($p < 0.01$) and 0.62 mg/L ($p < 0.01$) at 650 nm after 24 h of exposure to nanoplastic, whereas control showed 0.65 mg/L of protein. The protein content significantly reduced with respect to control. This is represented in Fig. 7a.

Catalase activity was determined to check the oxidative stress of *Daphnia magna* against nanoplastics. In the control, 0.085 U/mg of protein ($p < 0.001$) was observed, which was found to be reduced in LC_{25}, LC_{50} and LC_{90} respectively. The catalase activity was found to be 0.05 U/mg of protein ($p < 0.001$) in LC_{25}, 0.032 U/mg of protein ($p < 0.001$) in LC_{50} and 0.0280 U/mg of protein ($p < 0.001$) in LC_{90}. Here, due to the interference of polystyrene, the absorbance was significantly reduced in the test samples as compared to the control. Figure 7c shows this graph.

In the lipid peroxidation assay, MDA formed after 24 h of treatment with nanoplastics was measured at 532 nm. The control showed, 0.31 MDA/mg of protein. Whereas the MDA in LC_{25}, LC_{50} and LC_{90} increased significantly to 0.35 MDA/mg of protein ($p < 0.01$), 0.39 MDA/mg of protein ($p < 0.01$) and 0.44 MDA/mg of protein ($p < 0.01$) respectively. This shows that lipid peroxidation increased in test samples compared to the control. Increase in MDA levels with increase in nanoplastic concentrations is represented by Fig. 7b. To add discernment for the change in antioxidant enzymes, SOD was analysed for *Daphnia magna*. In this assay, the control displayed 0.27 U/mg of protein, LC_{25}, LC_{50} and LC_{90} depicted 0.25, 0.23 and 0.18 U/mg of protein at 406 nm. The activity of SOD decreased with increase in concentration of polystyrene here (Fig. 7d).

Discussion

Microplastics (0.1 µm − 5 mm in size) and nanoplastics (less than 1µm) formed due to uncontrolled plastic usage and their breakdown have become a major concern for health, as this can enter the nearby water system and affect aquatic organisms (Windsor et al., 2019; Karthikeyan et al., 2023; Bhagat et al. 2022; Wang et al., 2021; Wang et al., 2024). Recent reports reveal the neurotoxicity, genotoxicity and metabolic disorders caused due to interaction of nanoplastics with different organisms (Fan et al., 2023).

Our study aims at investigating the altered biochemical and physiological properties of different aquatic species on exposure to various concentrations of polystyrene nanoplastic. The size and surface charge of PS NPs was found to be 98.1 nm and −23.4 mV, which ensures the stability of PS NPs from coalescence (Kadu et al., 2011). Further, an increasing concentration of PS NPs was shown to raise the mortality rate of *Scenedesmus sp* and decreased chlorophyll pigment. Consistent with our findings, Yan et al., 2021, demonstrated the inhibition of photosynthetic pigments when *C.reinhardtii* was treated with 50–500 mg/L of

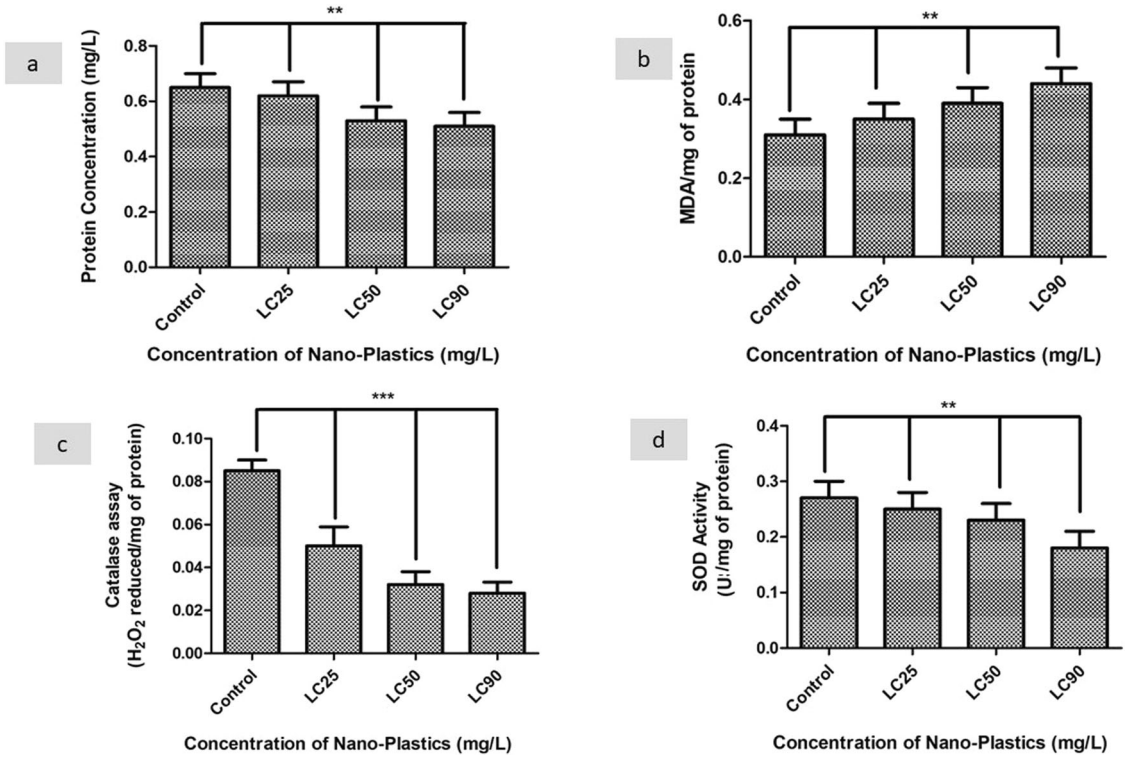

Fig. 7 **a** Protein assay: Total protein concentration of *Daphnia magna* upon exposure to LC_{25}, LC_{50}, LC_{90} concentration in comparison with control counterpart. **b** Catalase assay: Oxidative Stress analysis through catalase assay of *Daphnia magna* upon exposure to LC_{25}, LC_{50}, LC_{90} concentration in comparison with control counterpart. **c** Lipid peroxidation: Oxidative Stress analysis through Lipid peroxidation assay of *Daphnia magna* upon exposure to LC_{25}, LC_{50}, LC_{90} concentration in comparison with control counterpart. **d** Superoxide dismutase: Oxidative Stress analysis through Lipid peroxidation assay of *Daphnia magna* upon exposure to LC_{25}, LC_{50}, LC_{90} concentration in comparison with control counterpart. Corresponding error bar represents standard deviation of three replicates (SD, $n = 3$)

microplastics and nanoplastics. The same study also showed a significant increase in LPO levels in *Scenedesmus sp* with increase in PS NPs concentration. Modulation in SOD, CAT and POD content, and rise in intracellular ROS and LPO levels with an increase in micro and nanoplastic concentrations in *C.reinhardtii* for 96 h of exposure was noticed (Yan et al., 2021). An interesting investigation by Zhu et al., 2021, discussed that the PS NPs may act as barrier between microalgae and its surrounding environment, which may lead to release of reactive oxygen species or cause physical damage leading to cell mortality in the microalgae. The decrease in protein content observed could be due to the generation of ROS that damages protein, DNA and lipid, which may affect the viability of *Scenedesmus sp* (Yang et al., 2021; Schieber and Chandel, 2014). Further, this analysis was supported by the SEM imaging where, more distorted cells and decreased viable cells were observed in highest concentration (100 mg/L) as compared to the cells in control or in least concentration (0.01 mg/L). Similarly, the SEM images of *S. obliquus* depicted the damaged and deformed cells when treated with carbon nanotubes (CNT), nano Fe_2O_3 and nano MgO. The highest concentrations also revealed the aggregates of nanoparticles and cells formed (He et al., 2017).

Our study on polystyrene nanoplastic toxicity against *Nitrobacter vulgaris* also demonstrated decreased cell viability, catalase activity and increased LPO at higher PS NPs concentration. Destruction in the lipid membrane can lead to damage of the cell membrane and cause death of bacteria. Here the highest concentration tested (50 mg/L) had more effect on the bacterial cells compared to the lowest concentration and control. Previous investigation by Machado et al., 2020, demonstrated that exposure of marine *Synechococcus sp*. PCC 7002 bacteria to anthropogenic stressor like polyethylene nanoparticles and microparticles influences the cell morphology and viability (Douglas, and Dilosi, 2019). In another study the cell envelope of gram-positive *B.subtilis* was destroyed due to pore formation when treated with nanoplastics. A similar analysis showed that in a gram-negative bacterium the nanoplastics entered the cell membrane and caused more cell stress which led to higher level of reactive oxygen species (ROS) (Dai et al., 2022). A comparative study by Qian et al., 2021, depicted that there was non-negligible cellular oxidative stress induced by all three

tested nanoplastics, i.e., carboxyl-modified PS (100 nm, PS-COOH), amine-modified PS (100 nm, PS-NH_2), and unmodified PS (100 nm). PS-NH_2 showed cell membrane damage compared to other two nanoplastics. The SEM analysis clearly differentiated the smooth surfaced cells in control and formation of biofilm by EPS (extracellular polymeric substance) was seen in 50 mg/L. A study by Chaali et al., 2021 revealed a similar biofilm formation by the nitrifying bacteria, where these bacterial cells were adhered to the surface of extracellular polymeric substance formed. In the present study all tested concentrations of nanoplastics revealed sustainable inhibition of cell growth with maximum effect at 50 mg/L.

Similarly, different concentrations of polystyrene nanoplastic were tested against the zooplankton *Daphnia magna*. This organism also displayed toxic effects from the lower tested concentration, with observed elevation in SOD, catalase, LPO and decreased protein concentration. An analysis by Lin et al., 2019, manifested an increase in the MDA activity in response to polystyrene nanoplastics and depicted the modulation in antioxidant enzymes against *Daphnia magna*. In the study conducted by Liu et al., 2021, *Daphnia pulex* revealed a decrease in antioxidant enzymes such as catalase, total SOD and protein expression as dosage of polystyrene nanoplastics increased (Liu et al., 2021). Moreover, MPs are readily taken up by zooplanktons compared to algal or bacterial cells due to the resemblance to its prey size leading to toxicity at least concentration (Pikuda et al., 2022; He et al., 2022).

Thus, exposure of non-target species to polystyrene nanoplastics may induce adverse effects on the organisms (Loos et al., 2014). The tropical transfer of nanoplastics through the food web is also reported. For instance, research by Chae et al., 2018, demonstrated the transmission of nanoplastics from *Chlamydomonas reinhardtii* to *Daphnia magna*, which later passed on to *Oryzias sinensis*, a secondary consumer in the food chain, and further these nanoplastics moved on to the *Zacco temminckii* fish. Consistent with our results, a few other studies revealed that higher concentrations of nanoplastics may cause increased risks leading to negative impacts on cellular function and the metabolic secretions of algae (Nigam et al., 2022). A similar analysis by Mishra et al., 2019 revealed that increasing concentrations of PS NPs caused significant cytotoxic effects on human RBC and lymphocyte cells. Overall, the current experimental analysis reveals that various concentrations of polystyrene nanoplastics tested against *Scenedesmus sp*, *Nitrobacter vulgaris* and *Daphnia magna* showed toxic impacts, affecting antioxidant enzyme level, cellular growth and inducing morphological damage.

Conclusion

In conclusion, the study highlights the significant impact of polystyrene nanoplastics on various non-target aquatic species. The findings reveal that even minimal concentrations of nanoplastics can inhibit cell growth and disrupt biochemical processes, leading to oxidative stress and cell damage. However, a significant effect was noticed with increasing concentration of PS NPs in *Scenedesmus sp* and *Nitrobacter vulgaris*, substantiating the dose dependent adverse effects of PS NPs. The observed effects on lipid peroxidation, catalase, superoxide dismutase, protein content, and chlorophyll pigments underscore the widespread interference of nanoplastics in aquatic ecosystems. Moreover, SEM imaging illustrates the morphological damage inflicted on algal and bacterial cells by polystyrene nanoplastics. These results emphasise the urgent need for further research to fully understand the toxic effects of nanoplastics and implement measures to mitigate their impact on aquatic biodiversity and ecosystem health.

Data availability

All data supporting the findings of this study are available within the paper.

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Author contributions All authors contributed to the study conception and design. Materials preparation, data collection and analysis were performed by (Sowmya Sri N and Yerimma G). The first draft of the manuscript was written by (Sowmya Sri N and Yerimma G) and all authors commented on previous versions of the manuscript. The whole process of performing experiments and writing paper was guided by (Prabhakar Mishra, Yuvashree Muralidaran, Sikandar I. Mulla, Diana Miguez, Luiz Fernando Romanholo Ferreira and Juliana Heloisa Pine Americo-Pinheiro). All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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