Journal of Education for Pure Science
Vol.16, No.02 (2026)
DOI: https://doi.org/ 10.32792/jeps.v16i2.478
Website: jceps.utq.edu.iq Email: jceps@eps.utq.edu.iq

Impact of Nano-Polystyrene on Growth Rate and Physiological Metabolism of Microalgae *Chlorella vulgaris*

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Received 7/ 7 /2024, Accepted 20 / 09 /2024, Published 01 / 06 /2026

Abstract:

The world considers nano-plastic pollution to be one of the major emerging health threats due to human beings' poor consumption and recycling practices of the increasing waste products. To find out how microplastics affect primary productivity in the environment, we tested how harmful polystyrene microplastics (PS-MPs) are to the microalgae *Chlorella vulgaris* (*C. vulgaris*). In particular, the growth rate and antioxidant enzyme activity of the microalgae in the presence of different PS-MP concentrations (0, 30 µg/l, 60 µg/l, 120 µg/l, 0.5 mg/l, 5 mg/l, and 10 mg/l) were monitored under three spectra (red, blue, and white). These new data revealed a decrease in microalgae growth rates at the 10th day of exposure in the red spectrum in response to different-dose PS-MP exposure. In contrast, a blue spectrum shows an early increase in microalgae growth rate in response to low-dose PS-MP exposure. In contrast, a higher concentration of PS-MP solution leads to significant inhibition within 15th days. Meanwhile, the superoxide dismutase (SOD) level increases in the red and blue spectra and decreases in the white spectra. All spectra show an increase in CAT levels. Overall, our study comprehensively explores the toxicity of microplastics on microalgae, providing a thorough understanding of the potential effects of plastic pollution on the entire food web in the aquatic environment. We observed a reduction in *C. vulgaris* antioxidant enzyme activity in the red and blue spectra after exposure, along with an increase in antioxidant activities.

Keyword: CAT, Growth rate, Nanopolystyrene, SOD, spectra

1- Introduction:

Plastic products are a wide range of organic polymeric materials, most of which originate from coal, mineral oils, and natural gas, such as polyamide, polyethylene, polyvinyl chloride, polystyrene, etc. Moreover, its cost-effective and versatile design makes it suitable for use in industries such as medical, agricultural, electronic, electrical, and industrial [1]. Poor management of plastic waste has led to the proliferation of microplastics. [2,3].

Most of the plastic materials produced in the world are used in the packaging sector, where they are disposed of daily in marine and terrestrial ecosystems. Every year, we discard about 8 megatons of plastic waste into the oceans, where it swiftly breaks down into tiny fragments, and the biological decomposition

of organisms further reduces their size. UV radiation, mechanical corrosion, and microorganisms all play a role [4]. Microplastics reach the water body through industrial and domestic drainage, sewage plants, and surface runoff [5]. These microplastics distribute themselves between sediments and the water column, affecting living organisms based on their shape and size [6].

A large number of studies have reported on the ingestion of microplastics by various aquatic organisms, which may cause inflammatory diseases, and the ways in which some other pollutants enter the food web of the aquatic system [7]. Reports have indicated high risks to human health and food safety. Threats to aquatic organisms such as phytoplankton, zooplankton, molluscs, and fish species, potentially entering the human food chain [8, 9], have led to reports of microplastic pollution negatively affecting the growth of chlorella algae. Under biological, physical, and chemical conditions such as weathering, ultraviolet radiation, and high temperatures, small plastic particles can decompose into fine nanoplastics [10]. Aquatic environments are abundant with microplastics, and we can briefly explain their toxicity mechanism as an oxidative stress process in the organism's cell due to exposure to toxic pollutants [11]. Microorganisms, birds, and animals in the aquatic environment absorb particles of plastic materials due to their small size, light weight, durability, and stability, and they end up in the human body either through the food chain or through food materials that increase risks to human health [12].

It was found that microplastics have a high tendency to carry many toxic substances, such as endocrine-disrupting compounds, heavy metals, polybrominated diphenyl ethers, and polycyclic aromatic hydrocarbons, through the adsorption process, doubling the effect of these pollutants [13].

Microplastic-induced plastic pollution has resulted in environmental degradation, a topic that has garnered global attention from numerous scientists, governmental and non-governmental organizations, and the media. Few studies have explored the chemical behavior and toxicity of microplastics in the natural environment [14]. The point of this study is to look into how nanopolysteryne hurts the growth rate and oxidant enzymes of Chlorella vulgaris microalgae.

Methodology:

2-1 PS-NPs preparation and characterization:

To prepare nano-polystyrene, 0.1 g of PS powder and 4 ml of ethyl acetate were combined, weighed, and then added to a conical flask fitted with a magnetic stir bar to create the polystyrene particles [15]. We used scanning electron microscopy (FESEM) and Fourier transform infrared spectroscopy (FTIR) to confirm the size, shape, and surface chemical composition of the particles.

2-2 Algal Culture and Growth rate test:

A pure culture of C. vulgaris alga was obtained from the advanced ecology laboratory of the ecology and pollution department , Faculty of Sciences, Kufa University, and maintained in BG-11 medium. The algae were cultivated at 25 ± 1◦C in an incubator with a 14:10 (light:dark) photoperiod under three distinct light spectra (red, blue, and white). We cultivated exponential growth phase algal cells for 29 days in 400 mL culture medium, either with or without PS-NPs at dilutions of 0, 0.5, 5, and mg/L in 500 mL Erlenmeyer flasks. At the start of its cell density, each flask contained 25 mL of algae. There were three replicates made for every concentration. We shook the cultures three times a day during incubation to ensure optimal development. We measured the algal cell density at 680 nm using a UV spectrophotometer (USA). We used the following formula [16] to determine the growth constant (K):

$$K = \frac{(\mathrm{LnOD1} - \mathrm{LnOD0})}{\mathrm{T}}$$

2-3 Oxidative Enzymes Measurement:

2-3-1 SOD Enzyme :

We determined the measured SOD activity using a simple and rapid method based on the enzyme's ability to prevent pyrrogallol oxidation at pH 8.2.

Preparation of reagents

1. Tris-EDTA, pH 8.2
   2.85 g of Tris and 1.11 g of EDTA-Na_2 were dissolved in 1 L of DW.
2. Pyrogallol solution (0.2 mM)
   A weight of 0.252 g of Pyrogallol was dissolved in a solution of 0.06 ml of concentrated HCl diluted in 1 L of DW.

Procedure: as described in [17].
The optical spectrum was adjusted to read zero using Tris-EDTA buffer. We prepared the control and sample test tubes and subsequently pumped the contents into them.

Indicators	Test solution (µl)	Efficient solution (µl)
Sample	50	-
Buffer Tris	1000	1000
DW	-	50
Pyrogallol	1000	1000

At time zero and one minute after the addition of pyrrogallol, we measured the absorbance at a wavelength of 420 nm against the buffer solution.
Calculating the superoxide dismutase's effectiveness

$$\%\ inhibition\ of\ pyrogallol\ autoxidation\ = \frac{\Delta \mathrm{A}\ control\ -\ \Delta \mathrm{A}\ test}{\Delta \mathrm{A}\ control} \times 100$$

$$(Cu - Zn)\ SOD\ activity(\frac{U}{ml})\ = \frac{\%\ inhibition\ of\ pyrogallol\ autoxidation}{50\%}$$

2-3-2 CAT Enzyme:

We measured CAT using the Hadwan & Kadhum [18] method.

Reagents

1. We prepared a sulfuric acid solution (0.5 M) by appropriately diluting concentrated sulfuric acid in 200 ml of distilled water.

2. Prepare a solution of ammonium metaphenadate (0.01 M) by dissolving 0.2925 g of ammonium metaphenadate in 200 ml of sulfuric acid prepared in step 1.
3. Prepare phosphate buffer (50 mM; pH 7.0) by mixing solutions A and B in a ratio of 1:1. 5. We prepared solution (a) by dissolving 6.81 g of KH_2PO_4 in one liter of distilled water, and solution (b) by dissolving 8.90 g of $Na_2HPO_4.2H_2O$ in one liter of distilled water.
4. We prepared the standard hydrogen peroxide solution (10 mM) by mixing 0.1134 ml of 30% H_2O_2 with 100 ml of phosphate buffer solution.

Table (1) Steps of the working method.

Indicators	Sample Test tube (µl)	Standard Test tube (µl)	Efficient solution (µl)
Standard sample extract	100	-	-
Phosphate buffer	900	1000	3000
H2O2	2000	2000	2000
Mix of test tubes well, then incubate in a water bath at 37 C̊ at two minutes and the following regent added thereafter.			
Ammonium *Vanadate*	2000	2000	2000
Next, keep the tubes at room temperature for ten minutes, then read the absorbance at a wavelength of 452 nm.			

Calculator:

We calculated the catalase enzyme's activity using the following equation:

$$\text{Catalase Activity of test kU} = \frac{2.303}{t} * log \frac{S^o}{S} \quad ---(1)$$

Where S^o is the absorbance of the standard tube

S represents the absorbance of the model test tube.

We divide the resulting potency by the number of homogenized grams of the plant per liter of extract.

2- Statistical analyses:

Using IBM SPSS Statistics v26.0, univariate analysis of variance (ANOVA) was used to statistically examine all the data. The Tukey test revealed a significant difference between the means at $P < 0.05$.

3- Results and Discussion:

3-1 PS-NPs Description:

The sizes, shapes, and chemical compositions of PS-NPs were nanosized, as verified by FESEM (Figure 1) , FTIR (Figure 4), and XRD (Fig. 5). Prior to laboratory exposure, greater characterization of PS-NPs' physicochemical properties in the exposure medium is required to better understand their behavior and biological impact, as recommended by other investigations [19, 20].

The FESEM technique was used to characterise PS and NPS particles' morphology and particle size. Figures 1 and 2 show monodispersed polystyrene nanoparticles with a particle size distribution of around 34–47 nm. Surfactants are essential to the nanoprecipitation technique. Research has shown that in the absence of surfactant, polystyrene tends to aggregate instead of forming stable nanoparticles. The morphology of the PS nanoparticles prepared in the lab is not spherical, and their average size is about 40 nm.

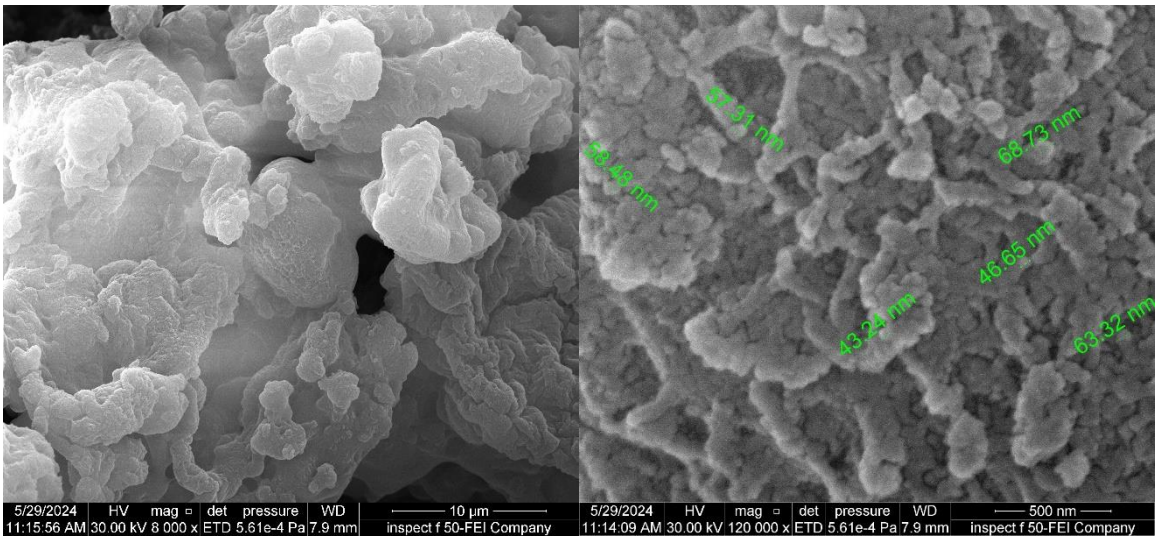

Fig1: FESEM Image of Nano polystyrene (500nm and 10µm)

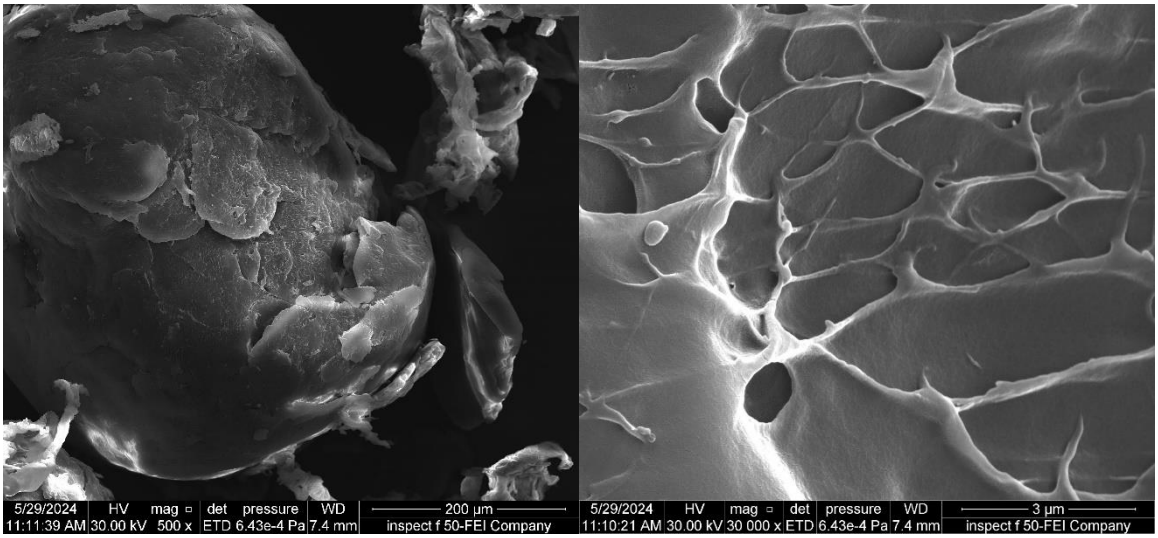

Fig 2: FESEM Image of polystyrene (3 µm and 200µm)

Figure 4 illustrates polystyrene's infrared absorption spectra, whose average particle size is about 40 nm. There are several absorption peaks with the corresponding wavenumber range. There are absorption peaks at wave numbers 3479 and 3417, which represent alcohol (O-H medium), and peaks at wave numbers 3049,

2970, and 2884, which represent (O-H stretching) carboxylic acid; wave number 1776 represents (C=O strong) stretching anhydride; peaks at wave number 1600 represent (medium C=C) expanding conjugated alkene; and peaks at wave number 1504 represent aromatic C=C stretching vibration absorption, which are following previously published data (Babaei et al., 2020). These absorption peaks indicate the existence of benzene rings. The absorption peaks at 765 and 711 wavenumbers are caused by C-H out-of-plane bending vibration absorption, and they show that there is only one substituent in the benzene ring [22]. These FTIR results have confirmed that styrene reacts to produce polystyrene through a polymerization reaction. In addition, the absorption peaks at wave numbers 3479 and 3471 correspond to the stretching vibration absorption of O-H, indicating the existence of hydroxyl. The hydroxyl may come from water or from the hydrolysis of a strong acid or weak alkali salt, such as sodium p-styrene sulfonate or potassium hydrogen carbonate.

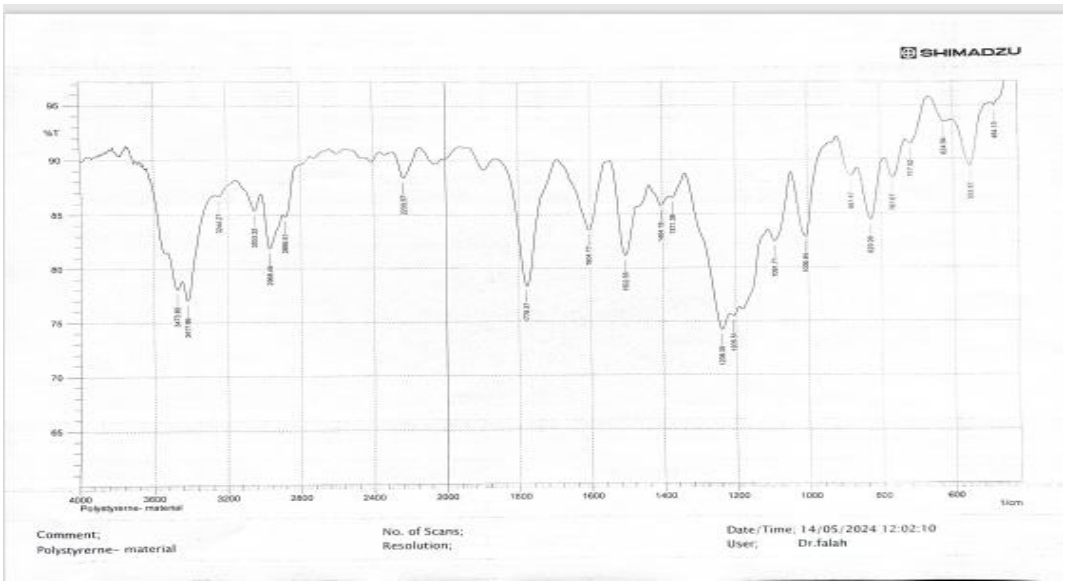

Fig.3: FTIR of polystyrene material

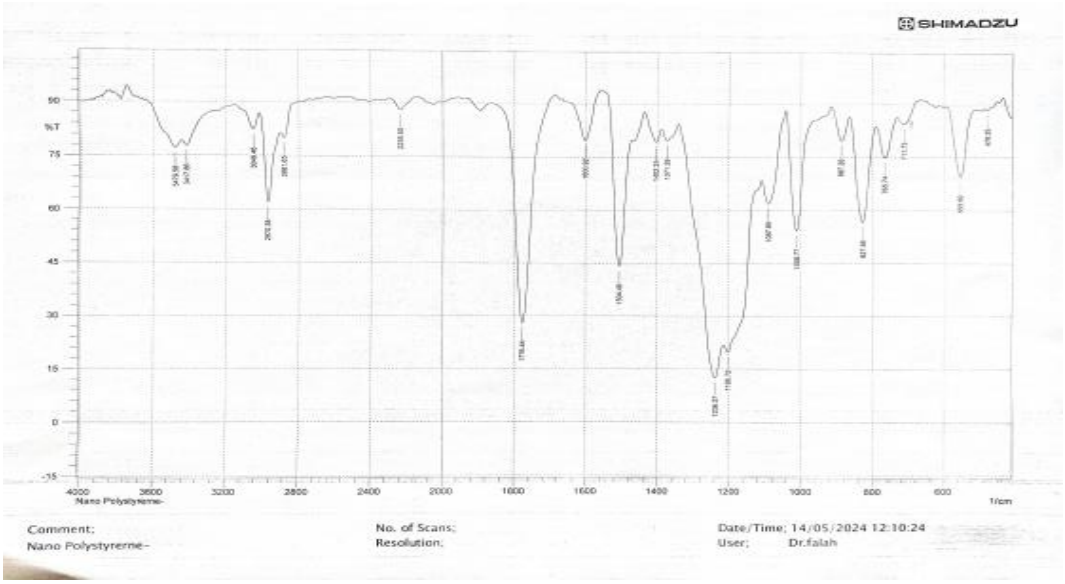

Fig.4: FTIR of Nano polystyrene material

For the purpose of characterizing a solid-state sample, X-ray powder diffraction is an effective method for characterizing a solid-state sample. Every crystalline species has a distinct pattern of X-ray diffraction. An investigator can use a diffraction pattern to identify an unknown species or to describe the atomic-scale structure of a recognized substance. Fig. 5 displays the polystyrene materials' XRD diffractogram. For polystyrene, the most noticeable peak in the nanopolystyrene is seen at 2θ = 18.0°. Crystalline polymer material is indicated by the presence of peaks at 2θ = 18.0 and 25.

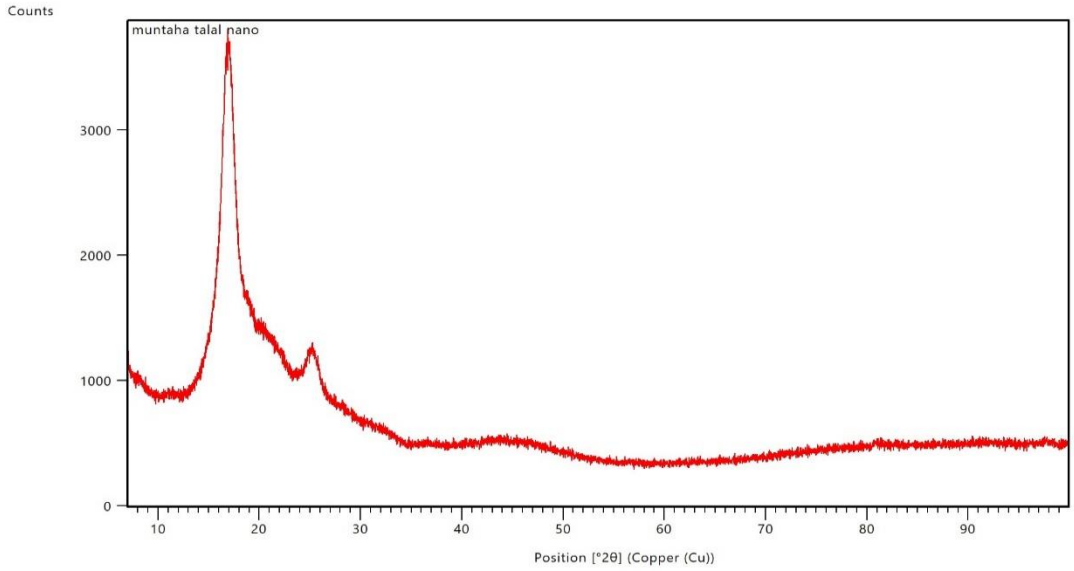

Fig 5 :XRD of the Nano-Polystyrene.

4-2 Growth rate:

We investigated the effects of PS-NPs on the growth rate of C. vulgaris at different doses ranging from 0 to 10 mg/L, as shown in Figure 6. After 29 days of exposure, PS-NPs suppressed the growth of C. vulgaris. All treatments showed a decrease in growth when compared to the control under red light. However, under blue light, the low concentrations (30 and 60 µg/l) began to increase in the early days. All treatments, except the control, decreased on the 16th day and then increased on the 19th day. The increase continued until the 28th day (Fig. 7). As for the white color, low concentrations (30 and 60) µg/l recorded in the first few days to the 10th day started to go down compared to the control. Then, all treatments started to go up, reaching 25th day higher than the control, then going down again in the 28th day (Fig. 8). Mao et al. [23, 24] also found that PS-NPs, at concentrations ranging from 10 to 100 mg/L, have a similar dose-responsive negative effect on the growth of freshwater microalgae in the logarithmic phase [24]. In terms of varying PS-NP concentrations, *C. vulgaris* appeared to be more susceptible to 0.5 mg/l of PS-NPs, exhibiting a 16-day half-life (Fig. 6A). In contrast, there was a clear ($p < 0.05$) reduction in another batch of algae treated with PS-NPs at concentrations of 5 and 10 mg/L, respectively. According to certain research, PS-NPs' impact on algal development rose as their size shrank [25].

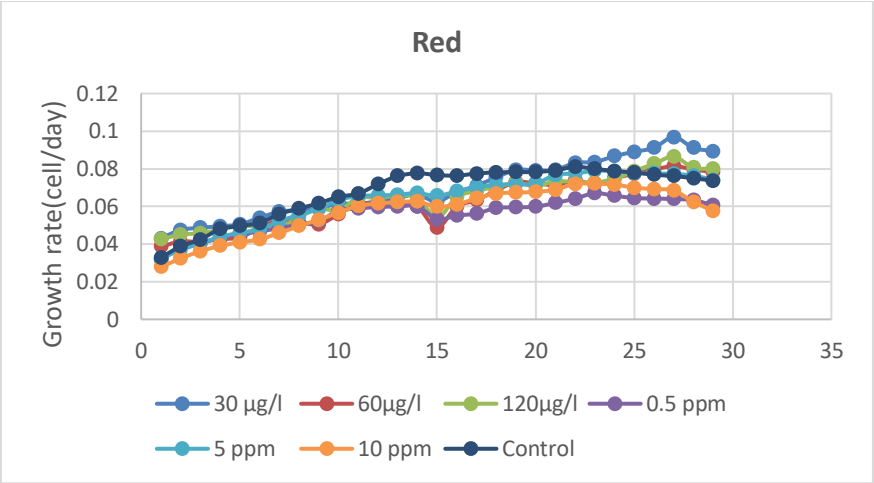

Fig.6: Toxicity of Nano polystyrene on growth rate of Chlorella vulgaris under red spectrum

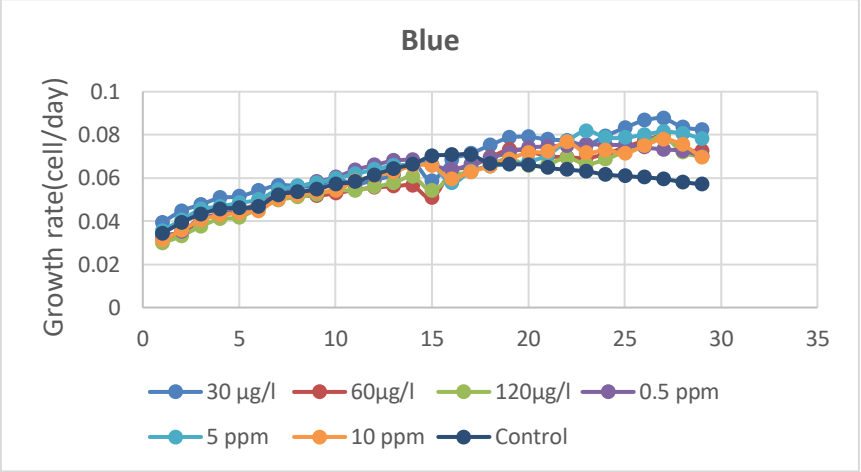

Fig.7: Toxicity of Nano polystyrene on growth rate of Chlorella vulgaris under blue spectrum

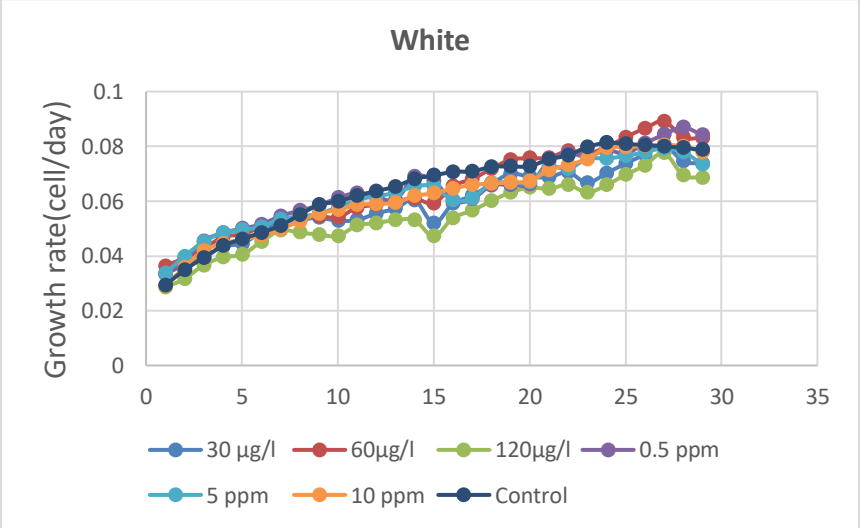

Fig.8: Toxicity of Nano polystyrene on growth rate of *Chlorella vulgaris* under white spectrum

4-3 Antioxidant enzymes:

Researchers have observed that PS-NPs' disruption of photosynthesis increases intracellular ROS buildup [26, 27]. This investigation discovered a similar occurrence. After being in PS-NPs for 29 days, the SOD levels of C. vulgaris showed higher responses to higher PS-NPs concentrations, especially in the 5 and 10 mg/l of PS-NPs treatment groups when viewed in the red spectrum (Figures 9 and 10). The SOD levels gradually rose over the course of the experiment, peaking on the 10th day (Fig. 11). In the blue spectrum, the SOD levels of *C. vulgaris* showed increased positive responses to PS-NPs concentrations, especially in the 5 and 10 mg/l treatment (figs. 12 and 13). During the experimentation days, the 4th day recorded a higher level than the 10th day (fig. 14). In the white spectrum, the SOD levels of C. vulgaris showed an increase in positive responses to PS-NPs concentrations, especially at the high concentration of 10 mg/l of PS-NPs treatment. However, all treatments showed a decrease compared to the control group (figs. 15 and 16). During the experimentation period, the 4th day recorded a higher level than the 10th day (fig. 17). All treatments, particularly the 120 µg/l and 10 mg/l PS-NPs treatments, showed an increase in CAT values across all spectra (Figs. 18, 19, 21, 22, 24, 25). On days where the 4th day was lower, compare the values of the 1st and 10th days under the red and blue spectra (Fig. 20 and 23). Under the white spectrum, the 10th day had a higher value than the 1st and 4th day. The elevated levels of SOD and CAT suggested that PS-NPs might cause C. vulgaris oxidative damage. Antioxidant enzymes may contribute to antioxidant protection processes [28, 29], but when ROS overproduction surpasses algal cells' natural defense mechanisms, their activity may be diminished or even blocked [30].

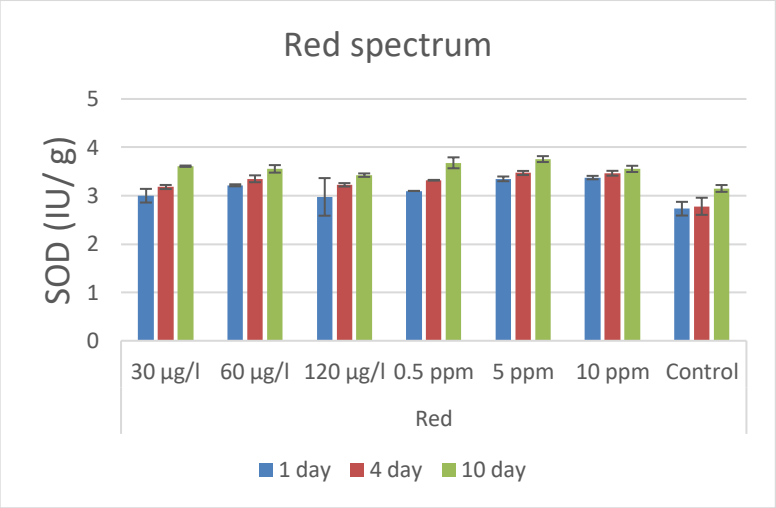

Fig.9: Toxicity of Nano polystyrene on SOD enzyme of *Chlorella vulgaris* under red spectrum

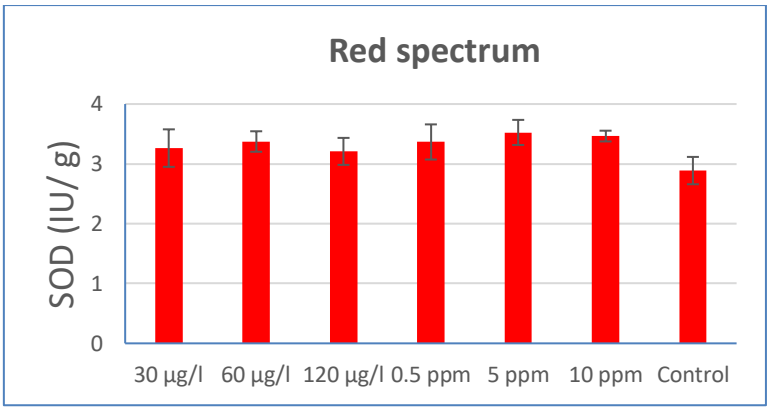

Fig.10: Mean of effects different concentrations of nanopolystyrene on the SOD enzyme of *Chlorella vulgaris* under red spectrum

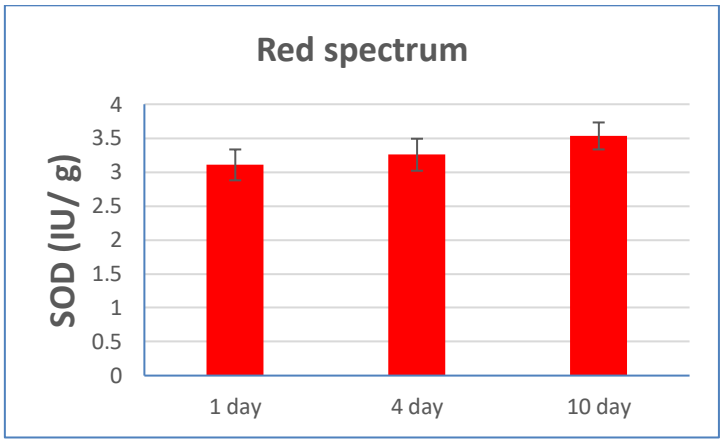

Fig. 11: Mean of effects of nanopolystyrene at different times on the SOD enzyme of *Chlorella vulgaris* under the red spectrum

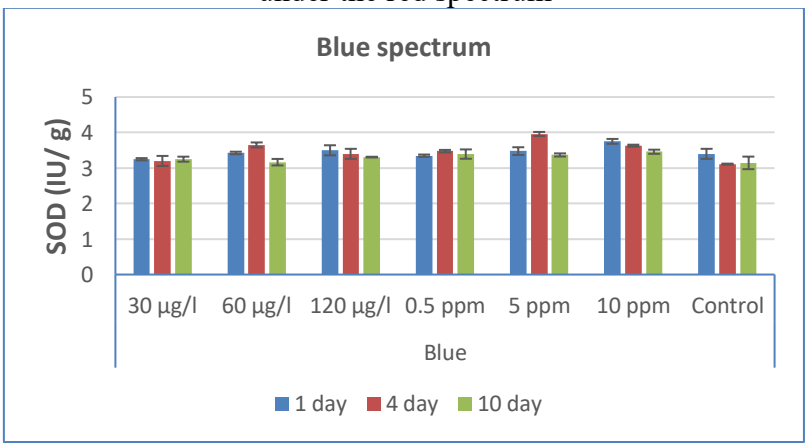

Fig.12: Toxicity of nanopolystyrene on the SOD enzyme of *Chlorella vulgaris* under blue spectrum

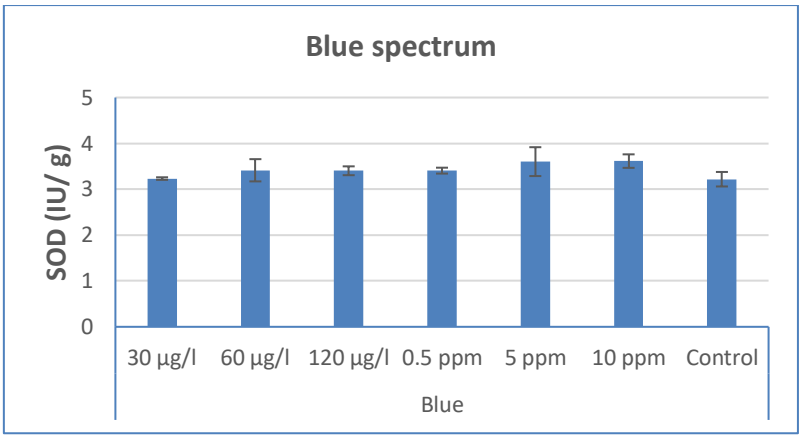

Fig.13: Mean of effects different concentrations of nanopolystyrene on the SOD enzyme of *Chlorella vulgaris* under blue spectrum

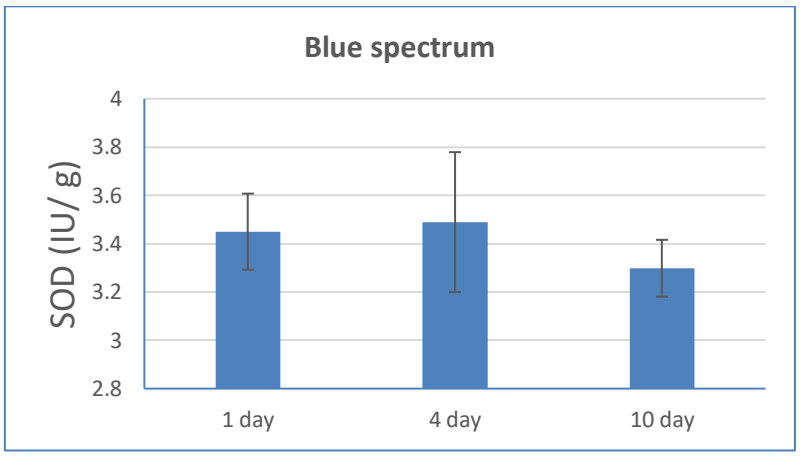

Fig. 14: Mean of effects of nanopolystyrene at different times on the SOD enzyme of *Chlorella vulgaris* under the blue spectrum

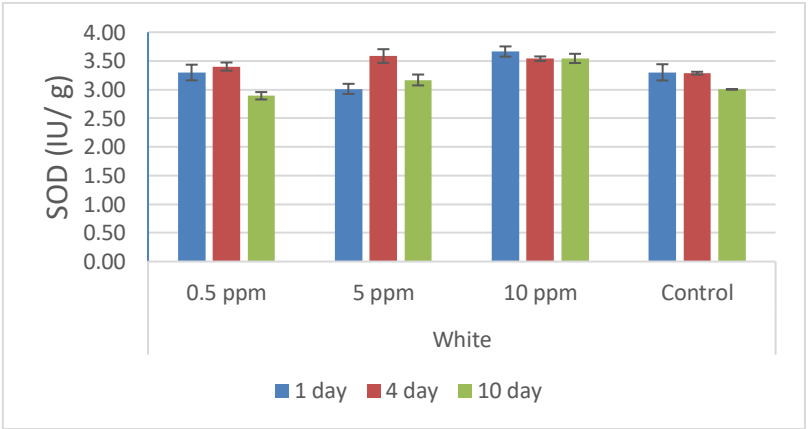

Fig.15: Toxicity of nanopolystyrene on the SOD enzyme of *Chlorella vulgaris* under white spectrum

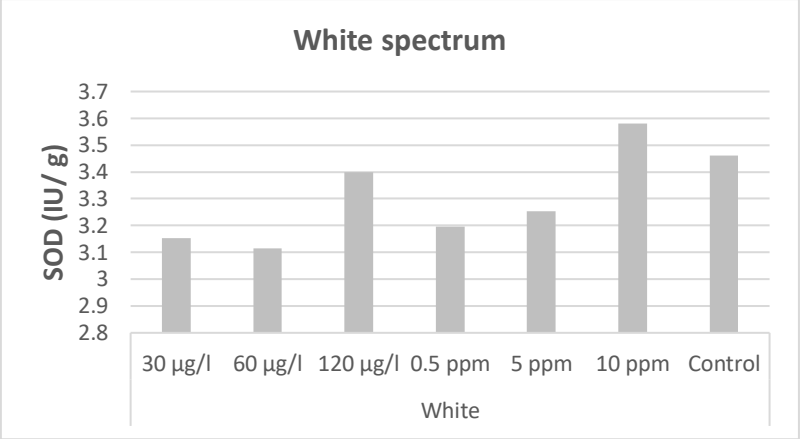

Fig.16: Mean of effects different concentrations of nanopolystyrene on the SOD enzyme of *Chlorella vulgaris* under white spectrum

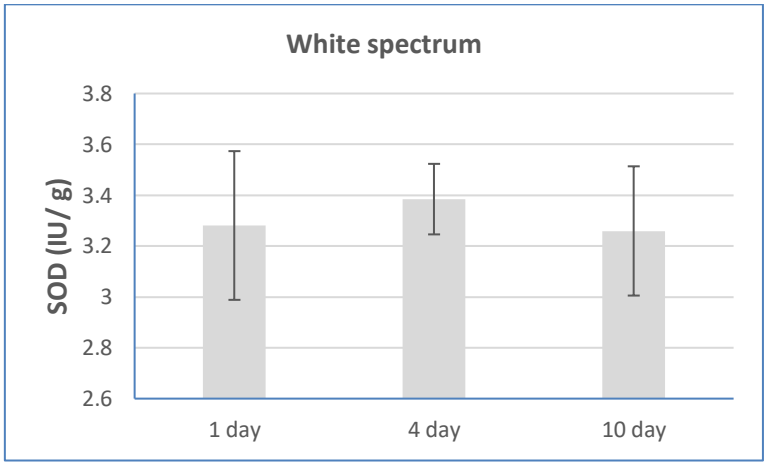

Fig. 17: Mean of effects of nanopolystyrene at different times on the SOD enzyme of *Chlorella vulgaris* under the white spectrum

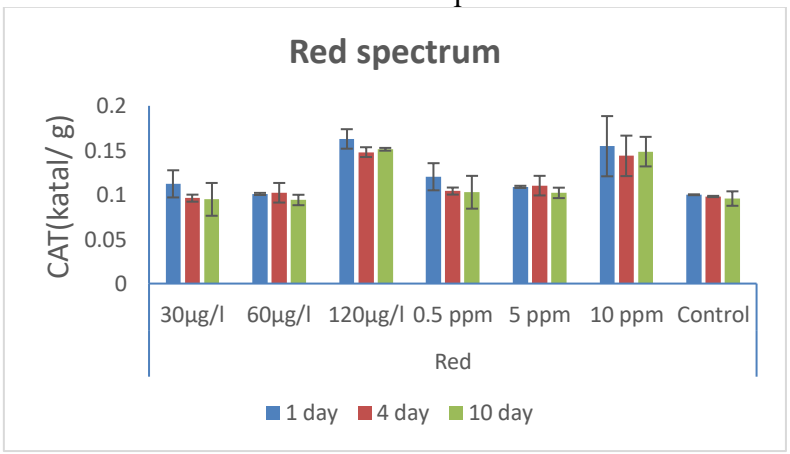

Fig.18: Toxicity of nanopolystyrene on the CAT enzyme of *Chlorella vulgaris* under red spectrum

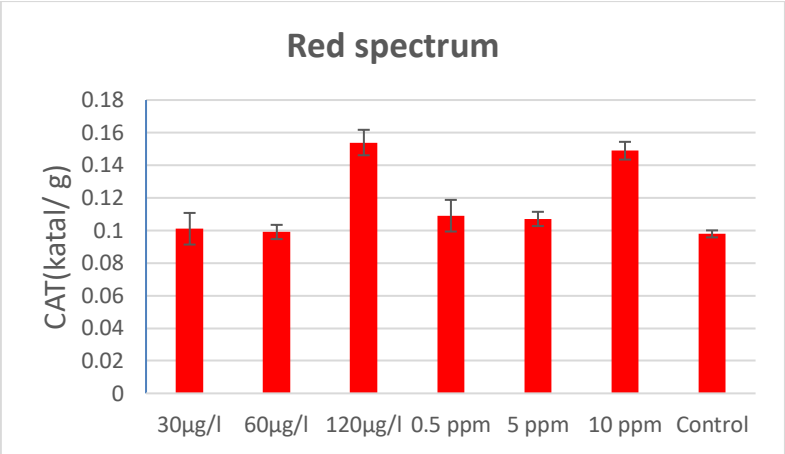

Fig.19: Mean of effects different concentrations of nanopolystyrene on the CAT enzyme of *Chlorella vulgaris* under red spectrum

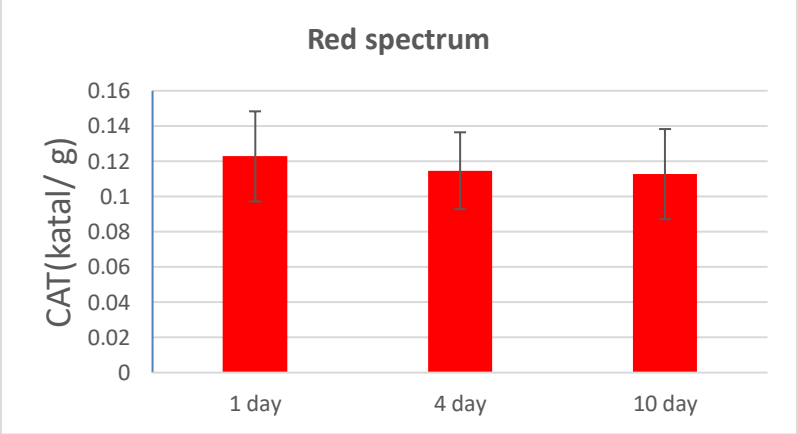

Fig. 20. The average effects of nanopolystyrene at various times on the CAT enzyme of Chlorella vulgaris under the red spectrum.

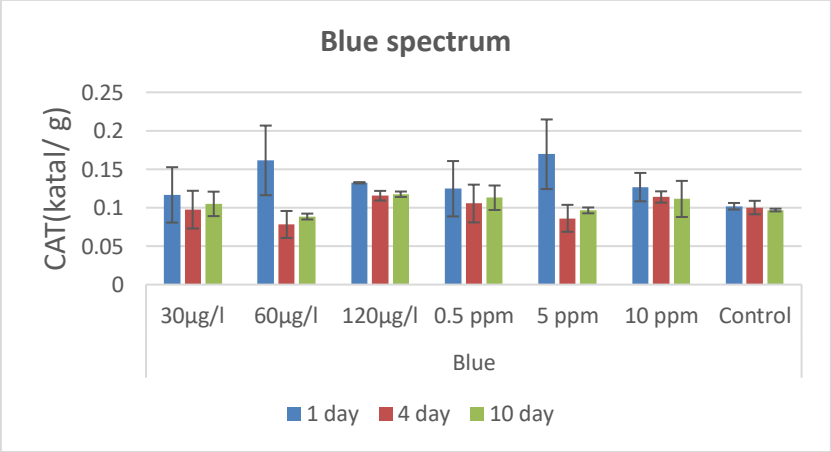

Fig.21: Toxicity of nanopolystyrene on the CAT enzyme of *Chlorella vulgaris* under blue spectrum

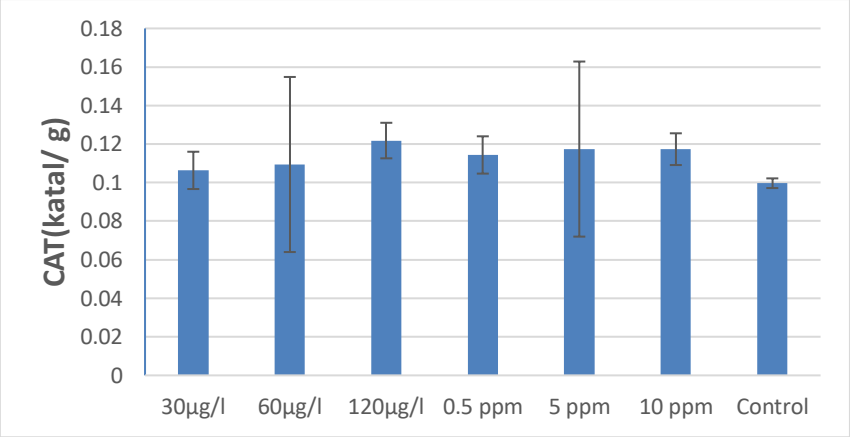

Fig.22: Mean of effects of different concentrations of nanopolystyrene on the CAT enzyme of *Chlorella vulgaris* under blue spectrum

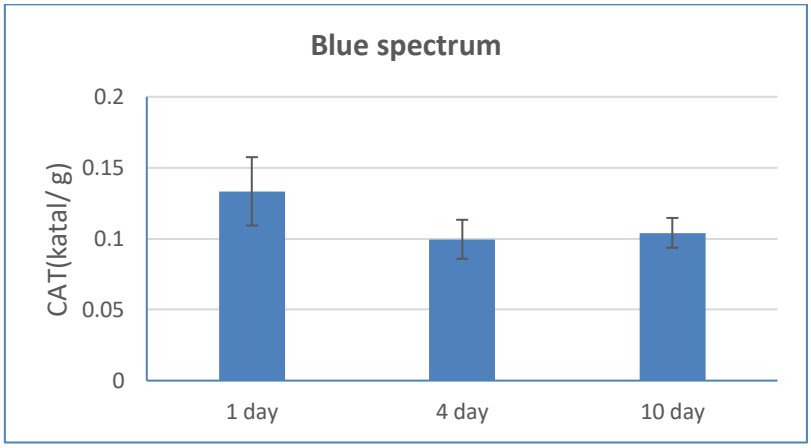

Fig. 23: Mean of effects of nanopolystyrene at different times on the CAT enzyme of *Chlorella vulgaris* under the blue spectrum

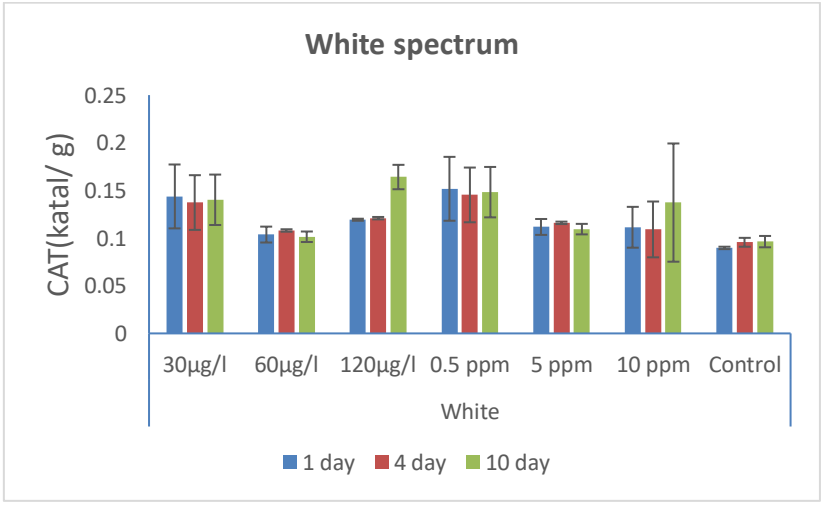

Fig.24: Toxicity of nanopolystyrene on the CAT enzyme of *Chlorella vulgaris* under white spectrum

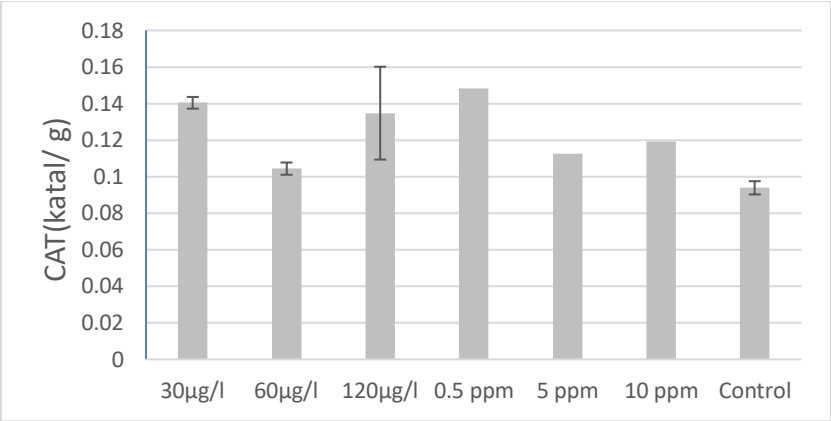

Fig.25: Mean of effects different concentrations of nanopolystyrene on the CAT enzyme of *Chlorella vulgaris* under white spectrum

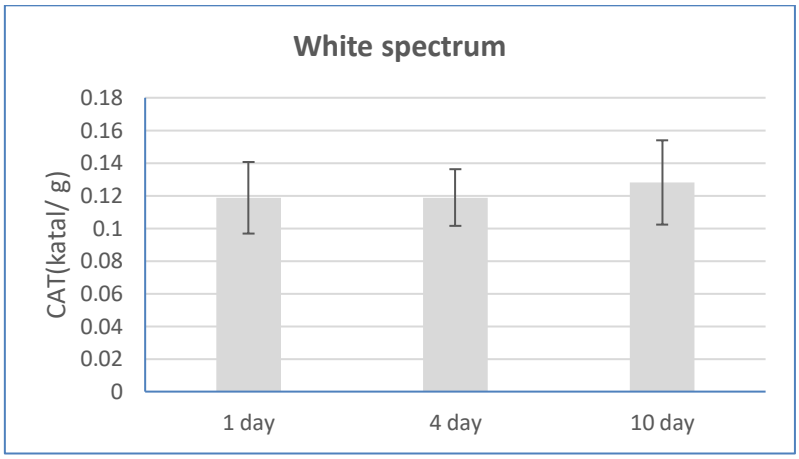

Fig. 26: Mean of effects of nanopolystyrene at different times on the CAT enzyme of *Chlorella vulgaris* under the white spectrum

Conclusion:

This work investigated the effects of PS-NPs on *C. vulgaris* at different concentrations (0, 30, 60, and 120 µg/l) (0.5, 5, and 10 mg/l) The results showed that *C. vulgaris* is acutely toxic to high concentrations of PS-NPs, which are the principal cause of adverse effects on algal growth rate. When compared to other PS-NP concentrations, 10 mg/l showed the most damaging effects on microalgae growth and antioxidant enzyme activity. These findings illustrate how important it is to consider how the characteristics of commercial PS-NPs may influence toxicity evaluation.

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