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Toxicological impacts of PLA microplastics on freshwater microalgae (*Chlorella pyrenoidosa*)

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Abstract *- Tiny plastic particles known as microplastics are a major environmental concern due to their wide presence and harmful Impact on aquatic ecosystems. To tackle this issue, efforts have been made to improve waste management, adjust policies, and develop new technologies. One effective solution is to use biodegradable plastics instead of traditional ones, but the environmental impact of these biodegradable microplastics needs further investigation to address potential risks. The investigation focused on studying the toxicological impact of polylactic acid (PLA) microplastics on freshwater microalgae, specifically C. pyrenoidosa. During a 96-hour co-incubation experiment, the effects of various concentrations of PLA microplastics (ranging from 0 to 100 mg/L) on the growth of C. pyrenoidosa were examined. Different concentrations of PLA microplastics had varying effects on the growth of C. pyrenoidosa over the 96-hour period. For instance, a promotion rate of 11.68% and 5.4% was observed at a PLA concentration of 100 mg/L after 72 hours of incubation. Additionally, a growth promotion effect of 5.4% was noted at a concentration of 20 mg/L during the same incubation period. The study also found that Chl-b levels decreased significantly across all incubation periods, particularly at higher concentrations of PLA, with fluctuations observed at different time points. In PLA-treated samples, there were slight increases in Chl-a levels at specific concentrations. The fluorescence efficiency of C. pyrenoidosa samples treated with PLA microplastics was notably lower compared to the control treatment, especially at concentrations of 20 mg/L and 50 mg/L. As PLA concentrations increased, protein content decreased in C. pyrenoidosa. The research highlighted the varied effects of PLA microplastics on the fluorescence efficiency of different microalgae species, with C. pyrenoidosa showing greater susceptibility. The response of nitrate concentration varied, with MDA levels decreasing as PLA microplastic concentrations increased. The diverse reactions observed among different time and concentrations underscore the necessity for further investigation in order to fully comprehend the ecological consequences of biodegradable microplastics in water environments. This highlights the pressing requirement for additional research on the environmental risks associated with biodegradable microplastics in aquatic ecosystems.*

Key words: Microplastics, Biodegradable Plastics, Microalgae, Plastic pollution.

1. INTRODUCTION

Plastic items are often used in human production and daily activities because of their excellent mechanical properties, light weight, strong durability, and cost-effectiveness.[1]. The most commonly used plastics, such as polyethylene, polypropylene, and polystyrene, are primarily derived from petroleum. These plastics have high molecular weights and strong chemical bonds[2]. Plastic particles originating from petroleum can persist in the environment for many centuries, or even millennia, and are recognized as emerging pollutants that pose a threat to the aquatic ecosystem[3]. Biodegradable plastics derived from sustainable sources such as starch, cellulose, bioethanol, and lignin serve as a viable substitute for conventional petroleum-based plastics[4]. These biodegradable plastics possess the ability to completely decompose into carbon dioxide (CO2) and water (H2O) within specific environments like water, soil, or compost, thereby allowing them to reintegrate into the natural cycle [5]. Polylactic acid (PLA), polybutylene adipate terephthalate, polyhydroxyalkanoates, polycaprolactone, and polybutylene succinate are the most prevalent types of biodegradable plastics. Among these, PLA stands as the most extensively utilized variant, finding applications across diverse industries including waste bags, agricultural coverings, food packaging, medical sutures, and materials for 3D printing. As reported by Zhang et al. (2021), PLA accounts for approximately 45% of the global biodegradable plastics market[6].

This study investigates the impacts of PLA microplastics on the cultivation of *Chlorella pyrenoidosa*, an extensively studied microalgae species that plays a pivotal role in aquatic ecosystems. Microalgae play a critical role in the global carbon cycle by absorbing carbon dioxide through photosynthesis and converting it into biomass, which supports various trophic levels in

marine and freshwater environments. However, the growing presence of plastic pollution poses a significant threat to these vital microorganisms. Microplastics, in particular, can disrupt the growth, reproduction, and metabolic functions of microalgae. Research indicates that microplastics can diminish the photosynthetic efficiency of microalgae, resulting in reduced biomass production and altered nutrient cycling in aquatic systems [7]. This disturbance can have far-reaching consequences throughout the food chain, affecting higher trophic levels such as fish and other marine organisms that depend on microalgae as a primary food source.

The presence of PLA microplastics in aquatic environments has raised concerns regarding their effects on microalgae, which can affect their growth, metabolic functions, and ecological roles [8]. This portion of the study methodically analyzes the impact of PLA microplastics on the physiology and biochemistry of *C. pyrenoidosa* using specific experiments such as OD680, chlorophyll levels, chlorophyll fluorescence, and a range of biochemical assessments. The findings of this research provide significant understanding into the wider ecological consequences of microplastic contamination.

2. MATERIALS AND METHODS

2.1 Materials used in the study

The microalga *Chlorella pyrenoidosa* (strain no. FACHB-9) was obtained from the Institute of Hydrobiology, Chinese Academy of Sciences. Microalgae were grown in liquid SE medium which consisted of (KNO_3 1.25 g/L, KH_2PO_4 1.25 g/L, $MgSO_4$.$7H_2O$ 1 g/L, EDTA 0.5 g/L, H_3BO_3 0.1142 g/L, $CaCl_2$.$2H_2O$ 0.111 g/L, $FeSO_4$.$7H_2O$ 0.0498 g/L, $MnCl_2$.$4H_2O$ 0.0142 g/L, MoO_3 0.0071 g/L $CuSO_4$.$5H_2O$ 0.0157 g/L, Co (NO_3) .$6H_2O$ 0.0049 g/L and $ZnSO_4$.$7H_2O$ 0.0882 g/L) 5000ml deionized water added to a 5L beaker with a magnetic stirrer on the stirring machine, then the above reagents were added as we record actual mass measured. The beaker was covered with a foil while adding the reagents to minimize contamination. After all the reagents have been added we leave the medium for 30 minutes to stir up to the reagents fully dissolved. Then the medium pH adjusted to 6.1 and transferred it to a Vertical High Pressure Steam Pressure overnight to autoclave. The plastic we have used to study the effect on microalgae was polylactic acid microplastic (PLA MPs). We have used the following instruments for different purposes through our study time:

Table-1 Apparatus used in the experiment

Apparatus	Purpose
BluePard Incubator	Store the cultivated *C. pyrenoidosa*
Cimo GZX-300BS-III incubator	Store the *C. pyrenoidosa* growth assay
Cence H1850R Centrifuge machine	Centrifuge the samples
Vertical High Pressure Steam Sterilizer LDZF-50L-II	Sterilize the basal medium culture and the Erlenmeyer flasks
Ultrasonic Cell Crusher Noise Isolating Chamber	Carry out the cell lysate process of the algal samples
Double Beam UV-Visible Spectrophotometer	Measure samples absorbances
Mettler Toledo Electric Measuring Scale	Measure the reagents and microplastics to be used.

2.2 Experimental analysis methods

2.2.1 Determining the optical density (OD_{680}) test

The determination of microalgae growth using an OD680 test is crucial as it provides a quick and convenient means to quantify the biomass concentration of the algae culture. This method enables real-time monitoring of experimental progress, allowing for timely adjustments to culture conditions. Moreover, it offers a non-invasive approach to assess microalgae growth without the need for cell harvest or disruption. In essence, the OD680 test serves as a valuable tool for investigating microalgae culture dynamics and enhancing growth conditions for various applications. On this work to measure

the OD value of algae liquid, a 3ml sample of the liquid is collected and its optical density value is measured using a UV-visible spectrophotometer at a wavelength of 680 nm.

2.2.2 Chlorophyll content measurement

In this work we utilized absolute ethanol to quantify chlorophyll a, chlorophyll b, and carotenoids. The procedure involved taking 5 ml of algal liquid, centrifuging it at 10,000 rpm for 2 minutes, discarding the supernatant, and retaining the algal sludge. The sludge was then subjected to three freeze-thaw cycles. After freezing for 15 minutes and thawing three times, 5 mL of absolute ethanol was added to the algal slurry, mixed thoroughly, and stored in the dark in a refrigerator for 24 hours. The extract was then centrifuged at 10,000 rpm for 2 minutes to separate the supernatant, which was transferred to a new test tube. The absorbance of the extract at wavelengths of 663, 645, and 470 nm was measured using a UV spectrophotometer to determine the pigment content of *C. pyrenoidosa* and *M. aeruginosa*, calculated according to a specific formula [9].

$$Chl\ a\ \left(\frac{mg}{L}\right) = 12.21_{A663} - 2.81_{A645} \quad (1)$$

$$Chl\ b\ \left(\frac{mg}{L}\right) = 20.13_{A645} - 5.03_{A663} \quad (2)$$

$$Chl\ (a+b)\left(\frac{mg}{L}\right) = Chla + Chl\ b \quad (3)$$

$$Carotenoids\ \left(\frac{mg}{L}\right) = \frac{(1000_{A470} - 3.27_{chla} - 104_{chlb})}{229} \quad (4)$$

Where, A_{470}, A_{663}, A_{645} are the extract in absorbance at 470 nm, 663 nm and 645 nm respectively.

2.2.3 Chlorophyll fluorescence activity

A 1mL aliquot of the algae solution was introduced into a centrifuge tube with a volume of 10mL. Subsequently, the algae were diluted by a factor of 20 using deionized water. Following this, 1mL of deionized water was added to the same tube, while a separate tube received an addition of 4.5mL of deionized water. Subsequently, 0.5mL of the diluted algae solution was transferred from the initial tube and added to the new tube. The resulting mixture in the new tube was then stored in darkness for a duration of 20 minutes. To assess the chlorophyll fluorescence activity, the Aqua Pen PSI was employed. The aforementioned steps were repeated, with the only variation being that the tubes were stored in a light rather than dark adapted.

2.2.4 Protein test

Lipid peroxidation tests are useful in assessing the levels of oxidative stress caused by microplastics, which is a common indicator of cellular damage. In this experimental work, the test utilized the supernatant obtained from both the control and treatment samples during the Lysate step. To create a 'work solution', the Coomassie brilliant blue dye was diluted with deionized water at a ratio of 1:4. The samples and reagents were then added in a specific sequence as outlined in Table 2.4. After allowing the samples to incubate for 20 minutes, the protein content was determined using a Double Beam UV-visible spectrophotometer at a wavelength of 595nm.

2.2.5 Superoxide dismutase (SOD) test

The assessment of Superoxide Dismutase (SOD) in microalgae toxicity studies involving microplastics is crucial for evaluating the oxidative stress that may occur due to exposure to microplastics. SOD is an essential antioxidant enzyme that plays a vital role in safeguarding cells against damage caused by reactive oxygen species (ROS), which are produced during cellular metabolism and can accumulate under stressful conditions like exposure to microplastics. The instructions stated that for the SOD (Superoxide Dismutase) test in this work were, 10mL centrifuge tubes corresponding to the samples should be obtained in duplicates, with two additional tubes for a Blank and a Standard. It was advised to add reagents in the following sequence.

Table-2 Reagents for SOD test

Reagents	Blank	Standard	Test sample	Test blank
Absolute alcohol	0.2mL	---	---	---
Standard solution	---	0.2mL	---	---
Test sample	---	---	0.2mL	0.2mL
Reagent 1	0.2mL	0.2mL	0.2mL	0.2mL
Reagent 2	3mL	3mL	3mL	3mL
Reagent 3	1mL	1mL	1mL	---
50% Glacetic acetic acid (GAA)	---	---	---	1mL

2.2.6 Malondialdehyde (MDA) Test

The MDA Test kit was utilized to conduct this test, employing the supernatant obtained from both the control and treatment samples during the Lysate step. The addition of reagents and samples followed a specific sequence as outlined in Table 2.6, with the tubes being vortexed after each addition. Subsequently, the samples were transferred to a water bath set at 90oC for a duration of 30 minutes. After cooling down, the absorbance was measured at 532nm using a Double Beam UV-visible spectrophotometer. The MDA level was determined using the provided formulas.

Table- 3 MDA Test reagents and samples sequence

Reagents	Blank	Standard	Test sample
Absolute ethanol	0.2mL	------	------
Standard solution	-----	0.2mL	------
Test sample	------	------	0.2mL
Reagent 1	0.2mL	0.2mL	0.2mL
Reagent 2	3.0mL	3.0mL	3.0mL
Reagent 3	1.0mL	1.0mL	1.0mL
50% Glacetic acetic acid	------	-------	-----

2.2.7 Nitrate test

The algal control and treatment samples underwent filtration and were subsequently transferred into 10mL centrifuge tubes. To prepare the samples for analysis, 100µL of each sample was transferred into 10mL glass test tubes in duplicate, and then filled up to the 10mL mark with deionized water. Following this, 200µL of Hydrochloric acid (HCl) and 200µL of Sulfamic acid (H3NSO3) were added to the samples. It is important to thoroughly mix the samples after the addition of each reagent. The samples were then allowed to stand for a duration of 15 minutes at room temperature, after which their absorbance was measured using a UV spectrophotometer at wavelengths 220nm and 275nm. The nitrate levels were subsequently determined using the calibration curve equation.

$$y = 4.1244x - 0.0268 \quad (5)$$

Where;

y= absorbance

x= concentration

2.3 Data collection and analysis methods

The statistical analysis of the results was performed utilizing OriginPro 9 software, in addition to Microsoft Office Excel 2021 for further data manipulation and visualization. The significance of the findings derived from the completely randomized experimental design was evaluated through one-way analysis of variance (ANOVA), employing a predetermined significance level of $p < 0.05$. This approach facilitated the comparison of average values across replicated datasets. The variability was assessed by systematically calculating the standard deviation (SD) values, which were subsequently represented as error bars in graphical presentations. By employing advanced statistical tools in conjunction with Excel, a comprehensive and accurate analysis of the experimental data was conducted, thereby enhancing the reliability of the study's findings.

3. RESULTS AND DISCUSSIONS

3.1 Infrared and particle size of PLA Microplastics

3.1.1 Infrared structural analysis

The physical and chemical attributes of PLA microplastic particles significantly influence their interaction with freshwater microalgae during toxicity assessments, ultimately affecting the potential toxic effects on these vital aquatic organisms. The FTIR spectrum confirmed the chemical structure of PLA. In the IR spectrum of poly (lactic acid) (PLA) (Fig -1), the C = O bond stretching is represented by a strong band at 1757.33 cm−1. Additionally, the bands at 2996.37 cm−1 and 2945.82 cm−1 correspond to the C-H stretching of -CH 3. The most notable absorption for ester C-O stretching occurs at 1187.45 cm−1. The FTIR spectrum of PLA aligns with the IR spectra documented in the literature [10].

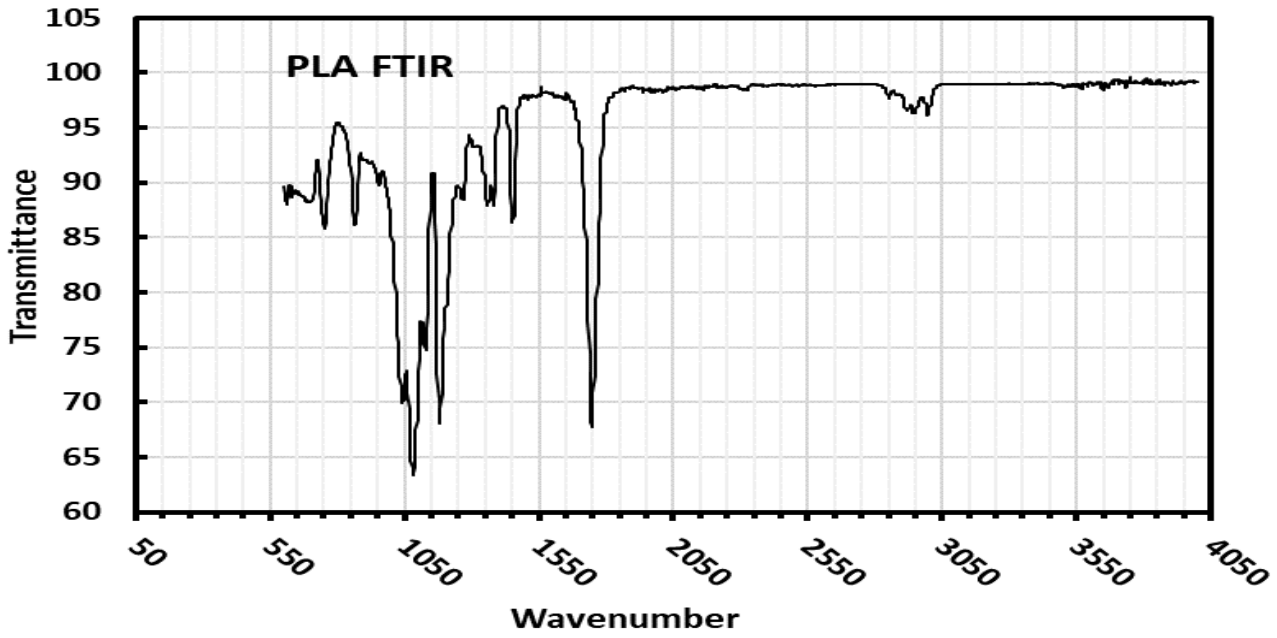

Fig -1 Polylactic acid microplastic FTIR.

3.1.2 PLA microplastic particle size

The size of particles plays a crucial role in ecology, as it is considered the primary factor influencing how microplastics interact with organisms and their impact on the environment [11]. The distribution of microplastics in natural environments, wastewater, and aquaculture systems varies significantly, often influenced by the sources and pathways through which these particles enter the ecosystems. In natural waters, microplastics primarily result from the degradation of larger plastic debris caused by physical and chemical weathering. The size distribution of these particles can vary greatly, with a substantial portion measuring less than 5 mm in diameter. Research has shown that particle sizes commonly range from 0.5 mm to 4 mm, with notable concentrations found in the 3–4 mm and 0.5–0.99 mm ranges [12, 13].

Wastewater treatment plants (WWTPs) play a major role in introducing microplastics into aquatic environments. Despite their high removal efficiencies, significant quantities of microplastics still find their way into water bodies through effluent. Microplastics in wastewater exhibit a diverse range of sizes, reflecting the various sources from which they originate, such as personal care products, synthetic fibers from laundry, and industrial waste. Typical sizes of microplastics in wastewater range from several micrometers to a few millimeters [12]. In aquaculture systems, microplastics often arise from the degradation of equipment like nets and ropes. The particle size distribution in these environments is similar to that in natural waters, with a predominance of particles measuring under 5 mm. However, due to specific sources like feed and equipment, there may be a higher occurrence of certain sizes and types of microplastics, such as fibers and fragments [13].

The distribution curve for the particle size of PLA microplastic is illustrated in Fig -2(a) and (b)**.** The analysis revealed that the particles measured fell within the size range of 2.4 - 272.4 µm, with 90% of them having an average size between 26 -163 µm and below 165 µm. Homogeneity was assessed by calculating the span values for each formulation, where the span value was determined using the formula: Span value = (D90 – D10)/ D50. Here, D90, D10, and D50 represent the particle distribution at 90%, 10%, and 50% respectively. A span value lower than 1 indicates a homogeneously dispersed size distribution [14]. The results suggest that the particle distribution of microspheres falls within an acceptable size range for parenteral injection.

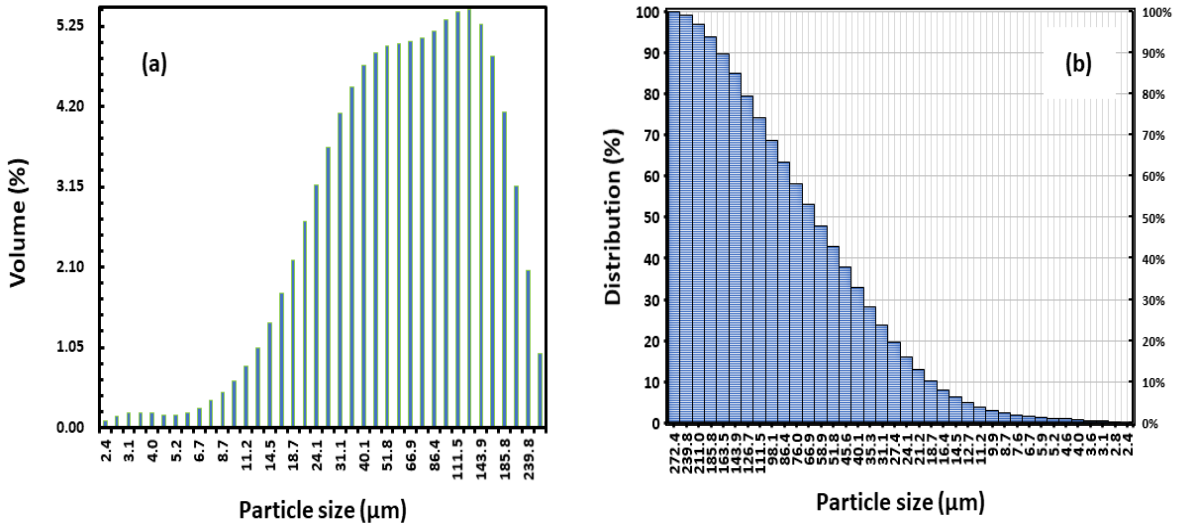

Fig -2 The particle size of PLA microplastic (a) volume % (b) distribution %.

3.2 Microalgae growth

The OD_{680} generally mirror the microalgae cell development and biomass focus [15]. The OD_{680} upsides of *Chlorella pyrenoidosa* under various growth of polylactic acid microplastic focuses are displayed in Fig -3. At the initial stage of the culture period, with the growth of time, the addition of microplastic could promote the growth of *Chlorella*, as shown the OD_{680}

curve in (Fig -3). PLA MPs with concentration of 100 mg/L presented the most promoting effect at the culture time of 96 hours on *C. pyrenoidosa* with rate of 11.68% than control treatment. Whereas, the concentration of 20 mg/L shows the lowest growth promoting effect on the microalgae at exposure time of 72 hours with rate of 4.8 but, 5 mg/L PLA microplastic concentration treatment was the lowest promoting effect of *C. pyrenoidosa* growth at same to growth time of 100 mg/L which is 96 hours. The graph analysis demonstrates the influence of Polylactic acid (PLA) microplastics on the growth of *C. pyrenoidosa* within a span of 96 hours. The optical density at 680 nm (OD680) is used as a measure of algal biomass, where higher OD values usually indicate greater algal growth[16].

Throughout the 24-hour period, the OD680 values for all concentrations of PLA microplastics, including the control group (0 mg/L), exhibit a similar trend. This observation suggests that the presence of microplastics does not have an immediate adverse impact on algae growth. This phenomenon could be attributed to the resilience of the algae or a delay in the manifestation of toxic effects induced by the microplastics. Moving forward to the 48-hour mark, the OD680 values remain consistent across the different concentrations of PLA, with a slight increase and decrease noted at 20 mg/L and 50 mg/L, respectively, in comparison to the control group. However, the significance of these fluctuations implies that the algae may still be able to thrive in the presence of PLA microplastics (Fig -3). Subsequent to 72 hours, a continuous decrease is observed at a PLA concentration of 50 mg/L, while other treatments show minor increases, except for the 100 mg/L concentration where the growth-promoting effect of PLA is most pronounced compared to the control. By the 96-hour mark, a notable decline in OD values is evident at a concentration of 50 mg/L of PLA microplastics, followed by 5 mg/L, indicating that PLA microplastics may have a multifaceted impact on algae growth, with the severity of negative effects potentially dependent on the duration of exposure. Studies have showed that, PE, PA, and PS concentrations ranging from 50 mg/L to 100 mg/L had no significant inhibition effects on *C. pyrenoidosa* growth until 96 h exposure in the microplastic toxicity experiment [17] the same thing is here the PLA microplastic insignificant positive effect ($P > 0.05$) on *C. pyrenoidosa.* Farther more the previous research indicated that MPs (PE, PET, PVC) stimulated growth in *C. pyrenoidosa*, while hindering the growth of Phaeodactylum tricornutum [18]. Conversely, it was discovered that mPA, mPLA, and mPBS exhibited the highest inhibition rate at 100 mg/L, with mPLA showing the most significant inhibition rate at 47.95% after 11 days of exposure for *C. vulgaris* microalgae [19].

PLA microplastics can have the potential to both stimulate growth and exhibit toxicity towards microalgae biomass for a variety of reasons. Firstly, in terms of growth promotion, PLA microplastics offer a surface area for biofilm development, thereby improving nutrient accessibility and fostering growth at lower concentrations[20]. Additionally, the biodegradability of PLA allows for the release of nutrients during degradation that can be beneficial for microalgae growth under specific circumstances[21]. On the other hand, toxicity can arise from high concentrations of PLA microplastics causing physical stress by obstructing light penetration necessary for photosynthesis. Furthermore, chemical leaching during degradation can result in the release of lactic acid and other harmful by-products that may negatively impact the metabolic processes of microalgae. Moreover, the production of reactive oxygen species (ROS) induced by PLA microplastics can lead to oxidative stress in microalgae, ultimately damaging cellular structures and impeding growth [20]. These contrasting effects underscore the intricate relationship between PLA microplastics and microalgae, which can be influenced by varying concentrations and environmental factors.

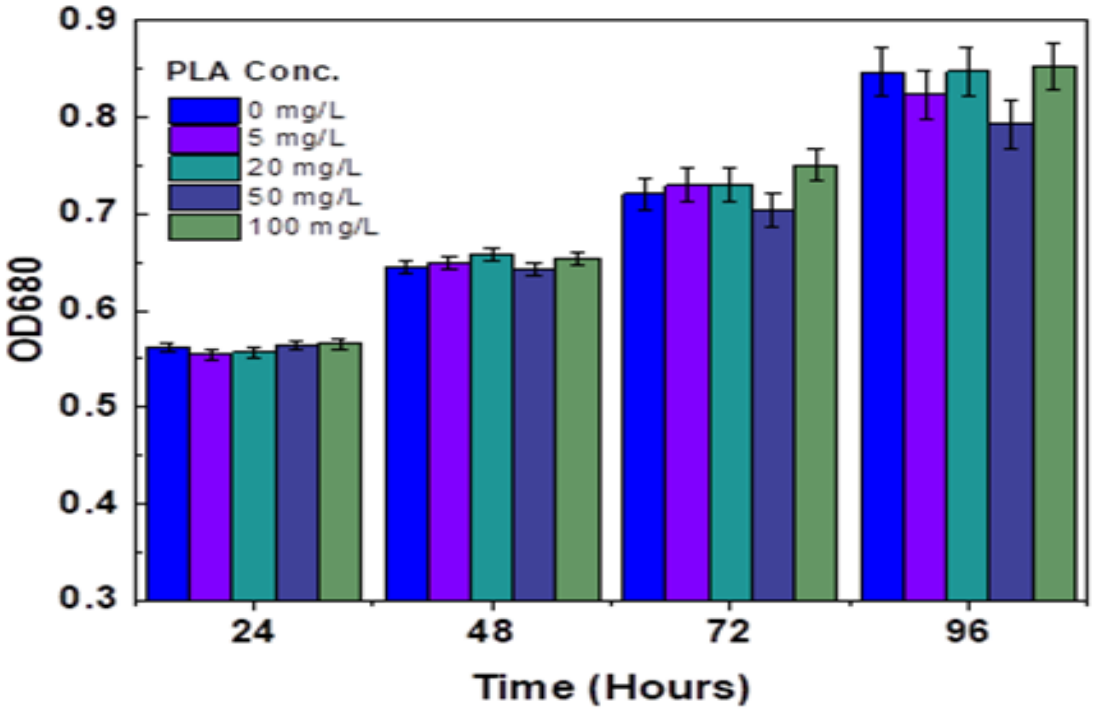

Fig -3 PLA MPs effect on OD680 values of Chlorella pyrenoidosa

3.3 Microalgae intracellular substances

Chlorophyll, an essential compound for photosynthesis in higher plants, serves as the primary pigment in green plants. Chl-a and Chl-b are recognized as the inherent pigments responsible for absorbing and transmitting light energy during the process of photosynthesis [22, 23]. Chl-a natural green pigment, holds significant commercial value as it plays a crucial role in plants and algae by absorbing light energy and converting it into chemical energy through the process of photosynthesis [24]. The interruption of chlorophyll results in a direct decrease in the capacity for photosynthesis, consequently impacting the rate at which plants grow [25]. The absorption and conversion of light energy heavily rely on photosynthetic pigments such as Chl-a, Chl-b, and carotenoids. In this study, during the *C. pyrenoidosa* microalgae exposure to PLA, the results were presented in (Fig -4 a-c). Chl-a exhibited a slight increase in PLA treated samples[26] compared to the control at concentrations of 50 mg/L after 96 hours, 100 mg/L after 24 hours, and 5 mg/L after 24 hours, with rates of 3.77%, 3.45%, and 2.87% respectively. Conversely, there was a negligible inhibition observed at 100 mg/L after 24 hours, 20 mg/L after 48 hours, and 100 mg/L after 72 hours, with rates of 5.78%, 4.4%, and 2.92% respectively (Fig. 3.4 a). On the other hand, Chl-b displayed inhibition rates[27] at 20 mg/L after 24 hours, 100 mg/L after 24 hours, 100 mg/L after 72 hours, and 5 mg/L after 24 hours, with inhibition rates of 10.94%, 10.49%, 6.88%, and 6.4% compared to the control, respectively. Furthermore, carotenoids in *C. pyrenoidosa* were depicted in (Fig -4 c), where inhibition of carotenoids was observed at PLA MPs concentrations of 100 mg/L after 24 hours, 20 mg/L after 48 hours, and 50 mg/L after 48 hours, with inhibition rates of 3.4%, 2.17%, and 1.42% respectively. Conversely, an insignificant positive impact of PLA on *C. pyrenoidosa* microalgae carotenoids content was noted at concentrations of 50 mg/L after 72 hours, 20 mg/L after 72 hours, and 5 mg/L after 72 hours of incubation, with rates of 3.5%, 2.33%, and 1.2% respectively.

Microplastics have been found to have a detrimental impact on microalgal pigmentation primarily by inducing oxidative stress and physically interfering with photosynthetic processes. This interference can lead to a reduction in chlorophyll content and photosynthetic efficiency, ultimately resulting in decreased pigmentation. Research has demonstrated that exposure to microplastics, such as polystyrene and PVC, can lead to lower chlorophyll concentrations and impaired photosynthetic activity in microalgae species like *Chlorella vulgaris* and *Skeletonema costatum*. Additionally, the physical

presence of microplastics can obstruct light penetration and cause damage to cellular structures, further exacerbating the negative effects on pigmentation and overall health of microalgae [28].

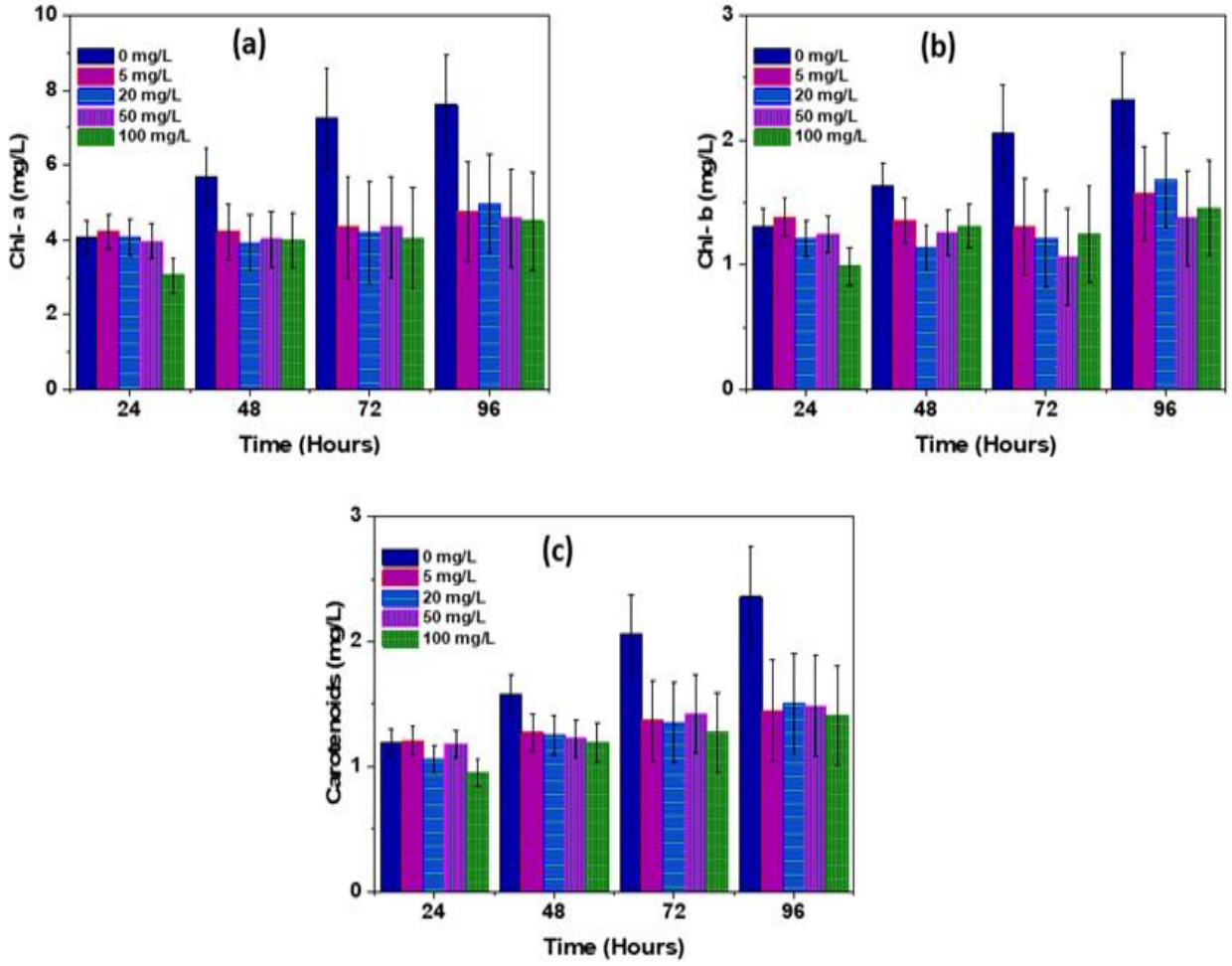

Fig -4 Effects of PLA-MP on the contents of Chlorophyll concentration for C. pyrenoidosa microalgae (a) chl-a, (b) chl-b, and (c) carotenoids.

The accumulation of metabolites in microalgal biomass is significantly affected by the conditions in which they are cultivated. Microalgae have the ability to adjust their metabolic pathways in response to various stresses, leading to the production of biomolecules triggered by different environmental factors [29]. The study consisted of a control group with no PLA concentration (0 mg/L) and experimental groups with varying concentrations of PLA, including 5 mg/L, 20 mg/L, 50 mg/L, and 100 mg/L. For *C. pyrenoidosa* microalgae, the protein content exhibited a decline (see Fig -5 a) as the concentration of PLA microplastics increased [30]. Specifically, at 100 mg/L, there was a decrease of 44.37% in protein content compared to the control treatment. Similarly, at 5 mg/L, the protein content decreased by 38.38%, while at 50 mg/L and 20 mg/L, the protein content decreased by 33.03% and 12.08% respectively, in comparison to the control treatment. These findings indicate that higher levels of PLA microplastics resulted in a more significant reduction in protein content for *C. pyrenoidosa* [31].

Additionally, a carbohydrate test on *C. pyrenoidosa* revealed a slight decrease at through the incubation period at all PLA MPs concentrations (see Fig -5 b). Overall, the effects of PLA microplastics on protein and carbohydrate content differed between different microalgae species [32]. The impact of polylactic microplastics on the biochemical composition of

microalgae can be better understood by examining the changes in proteins and carbohydrates. These changes may indicate alterations in metabolic processes, stress responses, or nutrient uptake mechanisms in microalgae following exposure to polylactic microplastics. An increase in proteins and carbohydrates could suggest a cellular response to stress caused by the presence of microplastics, potentially leading to changes in energy allocation, growth, or defense mechanisms. Conversely, a decrease in these biomolecules may indicate disruptions in normal cellular functions or nutrient availability due to the interaction with microplastics. Analyzing the variations in proteins and carbohydrates in microalgae exposed to polylactic microplastics is essential for comprehending the physiological and biochemical effects of microplastic pollution on these organisms.

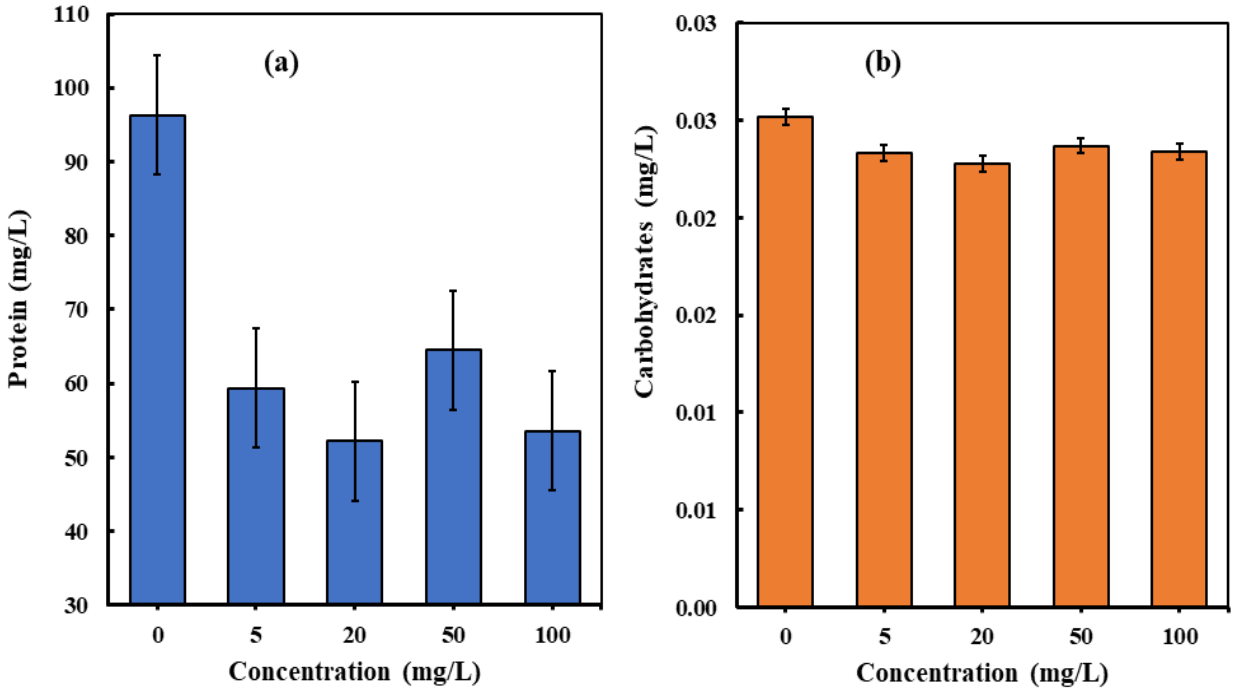

Fig - 5 Protein and carbohydrates contents of microalgae Chlorella pyrenoidosa (a) protein contents and (b) Carbohydrate contents after inoculated with PLA microplastic of different concentrations.

3.4 Microalgal photosynthesis

Photosynthetic parameters in microalgae encompass a range of quantifiable factors that shed light on the effectiveness and vitality of the photosynthetic process occurring within the cells. These parameters serve as indicators of the efficiency and overall health of photosynthesis. Among the commonly measured parameters is Fv/Fm, which stands for Maximum Quantum Yield of Photosystem II. This particular parameter gauges the utmost efficiency at which light absorbed by Photosystem II (PSII) is utilized for photochemistry while in a dark-adapted state [33]. A decrease in the Fv/Fm value signifies the presence of stress or damage to the photosynthetic apparatus. By monitoring changes in these parameters, it becomes possible to detect stress in microalgae. Environmental stressors such as variations in light intensity, nutrient availability, temperature, or the presence of pollutants like microplastics can be identified through alterations in these parameters. For instance, a decline in Fv/Fm suggests that the microalgae are under stress [34], potentially leading to harm to the photosystem II .

The fluorescence activity of *C. pyrenoidosa* samples treated with PLA MPs was lower compared to the control treatment [35] (see Fig -6). Specifically, at a concentration of 20 mg/L of microalgae under light-adapted conditions, there was a significant decrease in fluorescence parameters. The decrease rates for Fv/Fm were measured at 12.58% to the control treatment. Similarly, at a concentration of 50 mg/L of PLA MPs, the decrease rates for Fv/Fm were 12.42%, with other treatments showing similar decrease rates to the control. Additionally, dark-adapted treatments also exhibited decreased levels of

fluorescence parameters. At a concentration of 20 mg/L, the decrease rates for Fv/Fm were 16.64%. Furthermore, at concentrations of 5 mg/L and 100 mg/L, the decrease rates for Fv/Fm showed a consistent decrease rate of 16.14% to the control treatment.

The study findings recommend that the fluorescence efficiency of *C. pyrenoidosa* samples treated with PLA MPs was consistently lower than that of the control treatment [35]. The Fv/Fm ratio, which measures the maximum quantum yield of photosystem II, could potentially decrease in the presence of polylactic microplastics, indicating potential photoinhibition or stress in the photosynthetic apparatus [36]. Monitoring these fluorescence parameters can enhance our comprehension of the impacts of microplastic exposure on microalgae, providing insights into the ecological implications of microplastic pollution in aquatic environments.

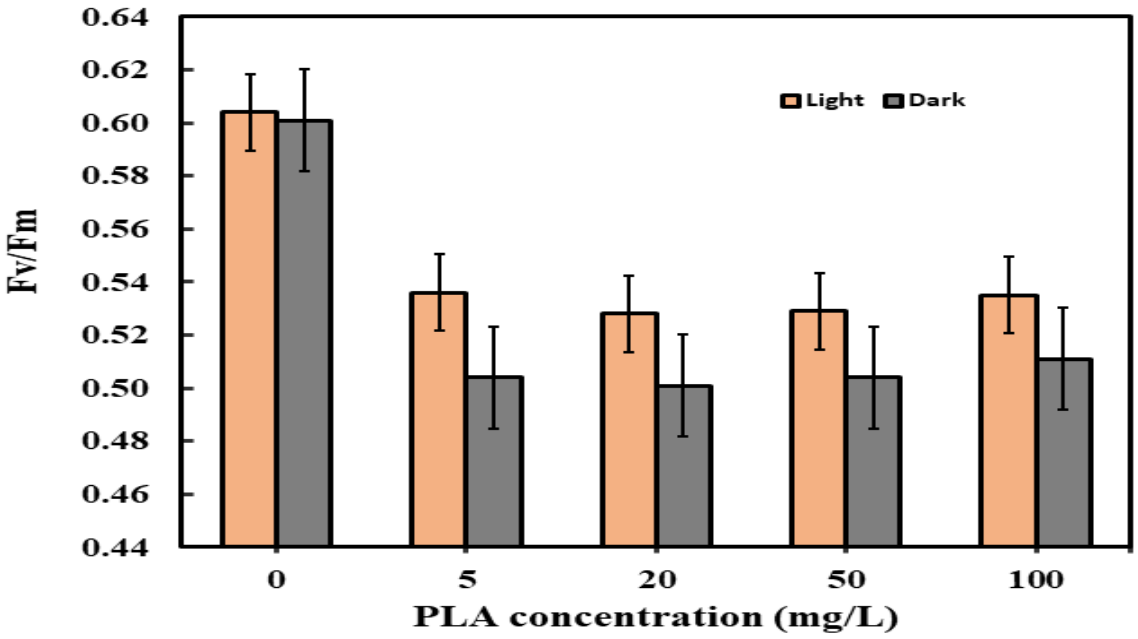

Fig - 6 PLA microplastic effect on microalgal photosynthetic efficiency

3.5 Oxidative stress in microalgae

The presence of pollutants can trigger microalgal cells to produce antioxidant enzymes like SOD, which are essential in eliminating reactive oxygen species (ROS) during the antioxidant defense mechanism[17]. In this study, the impact of BMP (PLA MPs) on SOD levels in microalgal cells (*C. pyrenoidosa*) was examined, revealing a significant decrease in SOD content at a concentration of 100 mg/L of polylactic acid microplastic treatment (Fig -7a and b). Furthermore, the measurement of MDA content can indicate oxidation and lipid damage induced by microplastics on microalgae [37]. SOD, as a critical enzyme in cellular antioxidant defense, responds to oxidative stress triggered by microplastic exposure in microalgae. Monitoring SOD levels provides insights into the antioxidant capability of microalgae and their ability to combat the detrimental effects of ROS generated by microplastic exposure.

On the other hand, MDA is a marker for lipid peroxidation, which is a common consequence of oxidative stress. Elevated levels of MDA in microalgae exposed to microplastics suggest that the cells are experiencing oxidative damage to their cell membranes. By measuring MDA levels, we can assess the extent of lipid peroxidation and the severity of oxidative stress in the microalgae population that may cause by the effects of microplastic to a microalga. Overall, in our current study SOD and MDA measurements, in *C. pyrenoidosa* microalgae toxicity studies caused by PLA microplastic exposure can help us to evaluate the

impact of oxidative stress on cellular health and integrity. These biomarkers provide valuable information about the mechanisms of toxicity and can guide efforts to mitigate the harmful effects of microplastics on aquatic ecosystems as well.

In the presence of PLA microplastics, the MDA levels of *C. pyrenoidosa* exhibited a significant increase. At concentrations of 100 mg/L, 20 mg/L, and 5 mg/L, the MDA levels rose by 95.78%, 57.84%, and 57.74% respectively (see Fig -7 b). This notable increment suggests a substantial elevation in oxidative damage to the cellular membranes of the algae[38]. However, at a concentration of 50 mg/L, there was a decrease of 26.29% in MDA levels compared to the control treatment. Significant variations were observed in the inhibition of SOD activity. In the case of *C. pyrenoidosa*, the findings revealed a noteworthy decrease in the rates of SOD activity at PLA microplastic concentrations of 100 mg/L and 20 mg/L, with rates of 76.8% and 20.55% respectively (Fig –7a). This decline can be attributed to the suppression of the microalgal antioxidant system, which is a consequence of reduced enzyme activity, such as SOD, that effectively scavenges ROS, particularly in stressful conditions [17]. Conversely, at concentrations of 5 mg/L and 50 mg/L of PLA microplastics, there was an insignificant increase in SOD activity inhibition when compared to the control treatment.

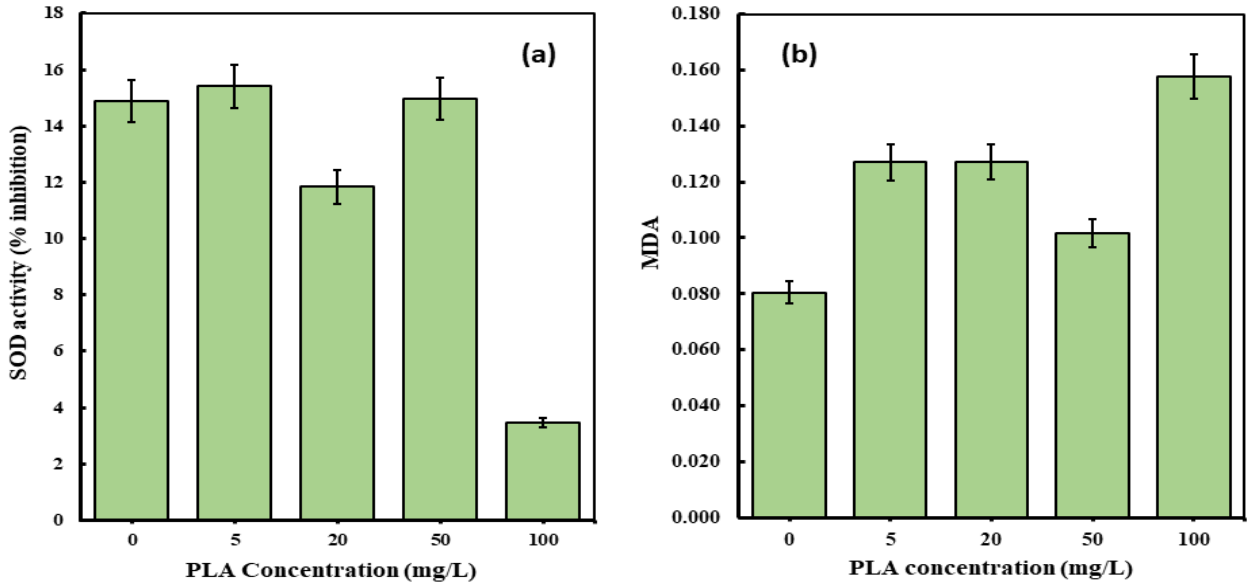

Fig - 7 PLA effect on the oxidative stress of *Chlorella pyrenoidosa* (a) SOD and (b) MDA.

3.6 Effects on microalgae nitrogen uptake

Microalgae heavily depend on nitrogen absorption to support their fundamental growth and metabolic functions, as nitrogen plays a vital role in the synthesis of amino acids, proteins, nucleic acids, and chlorophyll. These minute organisms have the ability to utilize different nitrogen forms, including nitrate, nitrite, ammonium, and urea, with distinct species displaying varying preferences in terms of uptake mechanisms. Nitrate is commonly taken up into the cell via nitrate transporters, where it is transformed into nitrite and subsequently reduced to ammonium within the chloroplasts for integration into amino acids. The presence of nitrogen significantly influences the growth rate, biomass yield, photosynthetic efficacy, and lipid accumulation in microalgae [39]. Intriguingly, heightened nitrogen uptake in microalgae exposed to microplastics can induce physiological reactions, such as compensatory responses, improved growth and metabolism, and modified metabolic pathways, potentially resulting in enhanced overall growth in specific circumstances despite the general harmful impacts of microplastics [40].

The changes in nitrate content at different concentrations of polylactic microplastics (PLA MPs) were examined, and the results are presented in (Fig -8). In the case of *C. pyrenoidosa* microalgae, the nitrate content at 5 mg/L, 20 mg/L, 50 mg/L, and 100 mg/L showed enhancements of 11.17%, 9.77%, 9.58%, and 8.4%, respectively. The fluctuations in results observed during toxicity tests examining the influence of microplastics on microalgae nitrogen uptake can provide valuable insights. A

reduction in nitrogen uptake by microalgae when exposed to microplastics may suggest an inhibition in their capacity to absorb nitrogen, which is essential for their growth and survival. Conversely, an increase in nitrogen uptake could indicate that microalgae are trying to counteract the stress caused by microplastics by absorbing more nutrients.

These findings contribute to understanding the potential repercussions of microplastics on marine ecosystems and formulating approaches to minimize their harmful impacts. The variations in nitrogen levels detected in toxicity tests due to the impact of microplastics on microalgae can be affected by several factors. These factors encompass physical interference, where microplastics hinder the absorption of vital nutrients such as nitrogen by adhering to microalgae cell surfaces or disrupting their feeding mechanisms. Moreover, chemical leaching from microplastics may introduce harmful substances into the environment, potentially influencing the nutrient absorption processes of microalgae. Indirect consequences may emerge as microplastics modify the composition of the microbial community, leading to changes in nutrient availability and competition for resources that can impact nitrogen levels in microalgae. Additionally, exposure to microplastics can induce stress responses in microalgae, resulting in alterations in their metabolic processes, including nitrogen uptake and utilization. Understanding these factors allows researchers to assess the effects of microplastics on microalgae and develop strategies to mitigate their impacts on marine ecosystems.

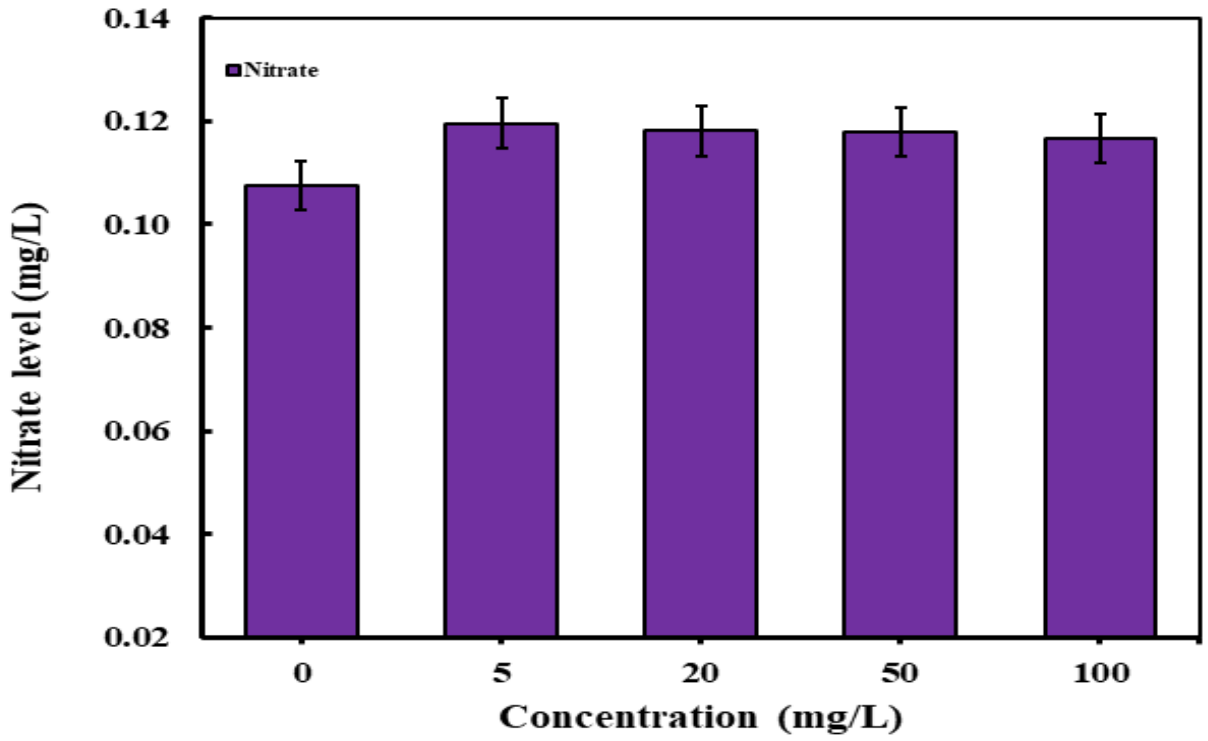

Fig - 8 The effects of PLA microplastics on microalgae nitrate contents C. pyrenoidosa.

4. CONCLUSION

This study focused on examining the influence of polylactic acid (PLA) microplastics on the green microalga *Chlorella pyrenoidosa*. The results demonstrated that the effects of PLA microplastics on algae growth varied over time. Initially, after 24 hours, all concentrations of PLA exhibited similar growth patterns, indicating no immediate negative consequences. However, after 48 hours, slight fluctuations were observed, with the most significant growth enhancement observed at a concentration of 100 mg/L. Subsequently, at 72 hours, a continuous decline in growth was observed at a concentration of 50 mg/L, suggesting potential adverse effects. The protein content decreased as the concentration of PLA increased, with the most

substantial reduction observed at 100 mg/L. Additionally, there was a slight decrease in carbohydrate content across all concentrations of PLA. These findings emphasize the intricate impact of PLA microplastics on the biochemical composition of microalgae, indicating potential stress responses and metabolic changes. The study also revealed lower fluorescence activity in *C. pyrenoidosa* samples treated with PLA microplastics compared to the control group. Notably, under light-adapted conditions, at a concentration of 20 mg/L, the fluorescence parameters Fm/Fo, Fv/Fo, and Fv/Fm decreased by 16.01%, 26.53%, and 12.58%, respectively. Similar reductions in fluorescence parameters were observed in dark-adapted treatments. Monitoring fluorescence parameters is crucial for comprehending the effects of microplastic exposure on microalgae. Furthermore, the levels of superoxide dismutase (SOD) significantly decreased with a treatment of 100 mg/L PLA microplastics, indicating oxidative stress. The levels of malondialdehyde (MDA), a marker for lipid peroxidation, notably increased in *C. pyrenoidosa* exposed to PLA microplastics, suggesting oxidative damage to cell membranes. The nitrate content varied at different concentrations of PLA, with enhanced nitrogen uptake observed in microalgae. These findings offer valuable insights into the potential impacts of microplastics on marine ecosystems and emphasize the importance of mitigating their adverse effects.

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