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Lipid fluorescence and antioxidant activity in microalgae under polystyrene microplastic stress

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ABSTRACT

Microplastic (MP) pollution, particularly polystyrene (PS), poses a growing threat to aquatic ecosystems and primary producers such as microalgae. Three mangrove-derived diatoms, *Nitzschia navis-varingica*, *Nitzschia palea*, and *Navicula bory*, were used in this work to assess the effects of 2 µm PS MPs on lipid content and antioxidant activity. Clonal cultures were subjected to PS doses of 0, 5, 10, and 50 mg/L. Nile Red fluorescence staining and corrected total cell fluorescence (CTCF) were used to measure lipid content, while IC_{50} values from the DPPH experiment were used to measure antioxidant activity. In all species, the exposure to PS initially decreased lipid fluorescence, especially at 50 mg/L, suggesting transient metabolic stress. Nevertheless, species-specific adaptation responses were noted, with lipid accumulation rising during cultivation and peaking on day 1 in *N. bory* and day 9 in both *Nitzschia* species. While *N. navis-varingica* did not exhibit any significant variations between treatments, *N. palea* and *N. bory* revealed significant effects of PS on lipid content ($p<0.05$). Significantly reduced IC_{50} values in all species ($p<0.05$) indicate that antioxidant activity increased along with PS concentration, with the highest activity seen at 50 mg/L. These findings demonstrate that PS promote oxidative stress and affect lipid metabolism in mangrove microalgae, while also eliciting adaptive antioxidant responses. The observed species-specific reactions point to possible ecological repercussions for trophic interactions and primary productivity in aquatic environments polluted by MPs.

Keywords: antioxidant, lipids, microalgae, microplastics, pollution.

INTRODUCTION

Plastic waste contributes significantly to global pollution, with an estimated 6.3 billion tons generated between 1950 and 2015, over 80% of which has accumulated in the natural environment (Liang et al., 2021). According to the Ministry of Environment and Forestry's 2023 data, plastic waste constitutes 19.3% of the total 35 million tons of waste produced in Indonesia (SIPSN, 2024). Polystyrene (PS) is one of the most commonly produced plastic polymers, accounting for 70% of total polymer production (Salisu and Maigari, 2021). In the fishery industry, PS is frequently used in ice boxes and buoys (Rani et al., 2014). In aquatic environments, including mangrove ecosystems, accumulated plastic waste degrades into microplastics (MPs), which affect various aquatic species. All trophic levels of aquatic biota are potentially at risk of ingesting MPs (Casagrande et al., 2024). Smaller PS particles (e.g. 0.05 –3 μm) are particularly toxic, causing cellular damage and physiological disruptions (Doskocz et al., 2025; Gao et al., 2023; Nikitin et al., 2022). Aquatic organisms, for example zooplankton, have a limited ability to distinguish food and MPs due to their similarity in size range (Cole et al., 2013). A previous study by Nafisyah et al. (2023b) found MPs smaller than 1 mm in mangrove areas, with PS being the dominant polymer. Concurrently, Nafisyah et al. (2023a) reported an abundance of microalgae in these areas, particularly from the *Nitzschia* and *Navicula* genera.

Microalgae are essential to the balance of aquatic ecosystems because they are primary producers and a component of the marine trophic chain. Furthermore, microalgae are much more advantageous for the ecotoxicology assay due to their short growth period and sensitivity to toxic substances (Clément et al., 2013). To investigate the abiotic stress caused by MP, lipid concentration and antioxidant activity were carried out to compare the effect on different microalgal species isolated from mangrove areas. Bacillariophyceae species were reported to be subjected to a high concentration of 2 μm PS which hindered their ability to produce lipids (Raju et al., 2022). On the other hand, MPs may enhance the production of bioresources, such as antioxidants, in microalgae (Ugya et al., 2022). However, according to Prata et al. (2019), the effects of MPs on microalgae vary depending on the type and size of MPs, the microalgae species, and MP concentration. The cultivation conditions of microalgae significantly influence the accumulation of metabolites within their biomass, as different abiotic stressors can trigger the production of specific biomolecules. Microalgae can adapt their metabolic pathways to respond to various stressors (Paliwal et al., 2017). Given these insights, the lipid fluorescence and antioxidant activity (IC_{50}) of three microalgal species isolated from mangrove areas were observed in understanding the impact on microalgae species, which may disturb the ecosystem stability through aquatic food webs.

MATERIALS AND METHODS

Isolation and cultivation

Microalgal strains were isolated from water samples collected from a mangrove area in Surabaya, Indonesia (07° 19.715' S, 112° 50.432' E) in February 2023. Single-cell isolation of each strain was performed using a capillary pipette under an inverted microscope (Olympus CKX31, Japan) at the Biotechnology Laboratory, Faculty of Fisheries and Marine, Universitas Airlangga. Single isolated cells of various species were transferred from the water samples into a well plate containing *f*/2 medium (30 PSU) and incubated at 30 °C with a light intensity of 50 μmol photons m^{-2} s^{-1} under a 12:12 light:dark (L:D) cycle. The cells were kept in an incubator until they had proliferated to a sufficient density in the exponential phase for further experimentation.

The total cultivation period for the clonal strains was approximately 60 days, with the addition of 170 μg mL^{-1} penicillin G, during which the cells underwent gradual adaptation. Initially cultured in a 3 mL well plate, the cells were sequentially transferred to 40 mL and 200 mL culture flasks before being transferred into 100 mL (lipid assay) and 500 mL (antioxidant assay) culture bottles for treatment. Both cultures were maintained under identical experimental conditions throughout the study. The identification of the three successfully isolated strains was based on morphological observation, and the species were identified as *Nitzschia navis-varingica* (119–122 μm), *Nitzschia palea* (70–72 μm), and *Navicula bory* (55–60 μm) according to Eich et al. (2015), Crowell et al. (2019), and Tan et al. (2016).

Microplastic polystyrene (MPs PS) treatment on microalgal cultures

The MPs treatment involved the use of 2 μm polystyrene (78452, Sigma-Aldrich, USA) as non-fluorescent polystyrene in aqueous suspension with a concentration of 10% solids and a density of 1.05 g/cm^3. The size of MP was chosen due to the inhibitory effect on microalgae based on Chen et al. (2020). MP concentration in this experiment was applied by increment from the field, notably as 1.91 mg/L (unpublished), at which, based on Zhang et al. (2017), microalgae showed no response to MP in low concentration (1 mg/L) and were inhibited in the highest concentration of 50 mg/L. It was also confirmed by Wu et al. (2019) who exposed MP to examine microalgae response in a minimum concentration of 5 mg/L. Thus, in this study, MP was exposed at concentrations of 0, 5, 10, and 50 mg/L, labeled as P0, P1, P2, and P3, respectively. Each treatment was conducted using three independent bottles (biological replicates), which were randomized by ensuring the light was diffused evenly. Owing to the limited number of cells available from the clonal culture, sufficient biomass could not be obtained simultaneously from the same culture replicate. Therefore, separate culture sets were established under identical experimental conditions for each assay. For lipid fluorescence analysis was performed in 100 mL bottles with an initial cell density of 1000 cells/mL, while for DPPH antioxidant activity was started with 500,000 cells/mL in a total volume of 500 mL. The experiment was conducted for 14 days with subsamples taken periodically to analyze lipid content to observe their fluctuating conditions. Antioxidant activity was observed on day 7 during their exponential phase in a 500 mL culture set to optimize the antioxidant production in the Bacillariophyceae group as reported in (Rahman et al., 2020).

Lipid content assay

The lipid content of the three microalgal species in this study was measured using the corrected total cell fluorescence (CTCF) method adapted from Kirchner et al. (2022) with slight modifications, such as the exclusion of the monochrome color-change procedure in the imaging process. Sub-samples (1 mL) from each treatment were collected daily and stained using the Nile Red Staining (NR-S) method with Nile Red dye. The samples were left to stain for 30 min, following the protocol described by Zheng et al. (2022). The stained cells were then observed under a fluorescence microscope (Olympus BX-43, Japan) at the Biophysics Laboratory, Universitas Airlangga, using blue light with a wavelength of 450–495 nm (Pham et al., 2024). Observations were made at 200x magnification, and a photograph was taken from a single viewpoint representing the minimum target of five microalgal cells per treatment and repetition (total of 15 cells for each treatment) to obtain an average value following previous research, which was done for at least 10 cells for staining (Elsayed et al., 2017).

Each photograph was processed using ImageJ software, utilizing the "Freehand Selection" tool to outline the morphology of each targeted cell. The microalgal cells were then analyzed using the "Analyze > Measure" function to obtain the "Integrated Density" and "Area of Selected Cell." To account for background fluorescence, the outline of each targeted cell was shifted to an empty area, and the "Mean Fluorescence of Background Readings" was recorded from the same photograph using the same tools. The CTCF was then calculated in Microsoft Excel using the formula in Equation 1.

$$CTCF = Integrated\ density - (Area\ of\ selected\ cell \times Mean\ fluorescence\ of\ background\ readings) \quad (1)$$

Antioxidant activity assay

The antioxidant activity assay in this study was conducted through several key steps: separation, drying, extraction, and concentration of biomass; preparation of stock solutions; and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay to measure antioxidant absorbance. This process was performed once at the conclusion of the experiment (day 7).

Biomass separation followed the procedure described by Sedjati et al. (2020) with slight modifications. A total of 500 mL of the microalgal culture from each treatment was centrifuged for 45 min at 4600 rpm. After centrifugation, the supernatant (aqueous medium) was discarded, leaving behind a pellet (microalgal biomass) with a small residual amount of supernatant. The pellet was homogenized and transferred to a Petri dish.

The biomass was dried using a freeze dryer (Buchi, Switzerland) according to the method of Aljabri et al. (2023), with Petri dishes first tightly

wrapped in perforated plastic wrap. Drying was initiated at 100 mbar for 60 min, with pressure gradually reduced every five min until reaching 3 mbar. This process was continued for 24 h before weighing and extracting the antioxidant content.

The compounds were extracted following the procedure described by Sedjati et al. (2020) by dissolving the dried samples in 96% acetone (1 g: 15 mL) for 24 h. The samples were then filtered through Whatman GF/F filter paper (Cytiva, Cat No. 1825-047). The filtrate was poured into new Petri dishes, weighed, and dissolved in 95% methanol to prepare a stock solution (1000 ppm) for the antioxidant assay.

Antioxidant activity was measured by diluting the stock solution to concentrations of 125, 250, and 500 ppm. Each of these concentrations, along with a blank sample, was reacted with a 0.1 mM DPPH solution in a 4:1 ratio for 30 min. Absorbance was measured using a UV-VIS spectrophotometer at a wavelength of 517 nm.

The half-maximal inhibitory concentration (IC_{50}) value was calculated by correlating the concentration series with the percentage of inhibition. The linear regression equation was $y = ax + b$, where $y = 50$ and x represents the IC_{50} value. The IC_{50} value is inversely correlated with antioxidant activity in microalgae, with phenolic and flavonoid compounds being the major contributors. The IC_{50} values were categorized as follows: very strong (< 50 µg/mL), strong (50–100 µg/mL), moderate (100–150 µg/mL), weak (150–200 µg/mL), or very weak (> 200 µg/mL) (Molyneux Philip, 2004).

Data analysis

Lipid fluorescence intensity and antioxidant activity (IC_{50}) were statistically analyzed. Data normality was assessed using the Shapiro-Wilk test prior to one-way ANOVA. When significant differences were detected ($p < 0.05$), Tukey's HSD post-hoc test was applied to identify differences among treatment groups.

RESULTS

MPs exposure and lipid content in three microalgal species

Lipid content analysis using fluorescence imaging is illustrated in Figures 1 to 3, representing *Nitzschia navis-varingica*, *N. palea*, and *Navicula bory*, respectively. In these figures, the yellow color indicates lipid luminescence owing the staining process. The y-axis corresponds to the number of cultivation days, whereas the x-axis denotes the different treatments (P0-P3). The selected cultivation days were based on the specific response of each species. For example, in the case of *N. navis-varingica* (Figure 1), days 0, 8, 9, and 14 are presented to highlight the visible differences in luminescence across treatments. Overall, the fluorescence observations in Figures 1–3 are in concomitant with the fluorescence values in Figure 4. Initially the lipid fluorescence intensity of three species exposed with the highest MP concentration (Figure 1d,2d,3d) resulting the lowest value with weak fluorescence (33,186 ± 21,077 ~ 69,612 ± 21,801 RFU) (Figure 4). The decrease in lipid fluorescence at P3 on day 0 occurred in 22.65% (*N. navis-varingica*), 16.82% (*N. palea*), and 54.23% (*N. bory*) compared to the control treatment. Each species then shows a different response to the exposure of MP until day 14. On day 2 (Figure 4a), *N. navis-varingica* in P3 treatment showed an increase in luminescence exceeding other treatments, including the control. The highest luminescence intensity was observed on day 9 at 199,267 ± 33,230 RFU in the same treatment (50 mg/L MPs) with more than a half increment from the previous day. Although there were visual differences in luminescence, the quantification of fluorescence revealed that PS treatment did not have a statistically significant effect ($p > 0.05$) on the lipid content of *N. navis-varingica* (Figure 4a). However, on day 14, the lipid content in the P3 treatment showed the highest increase (28%) compared to that in the control.

In the case of *N. palea* (Figure 2), the luminescence of the cells visibly differed as early as day 0, 1, 2, and 9 of cultivation. Lipid content increased by 36% to 57% across treatments on day 1 of cultivation with MPs exposure (P1~P3). This increase continued until day 2, with a further rise of 7% to 17% compared with that on day 1. The luminescence intensity data for *N. palea* are shown in Figure 4b, where the P2 treatment (10 mg/L MPs) recorded the highest value on day 9 at 171,850 ± 27,175 RFU. Statistical analysis revealed that PS contamination significantly ($p < 0.05$) affected the lipid content of *N. palea* on days 1 and 2 of cultivation, with the

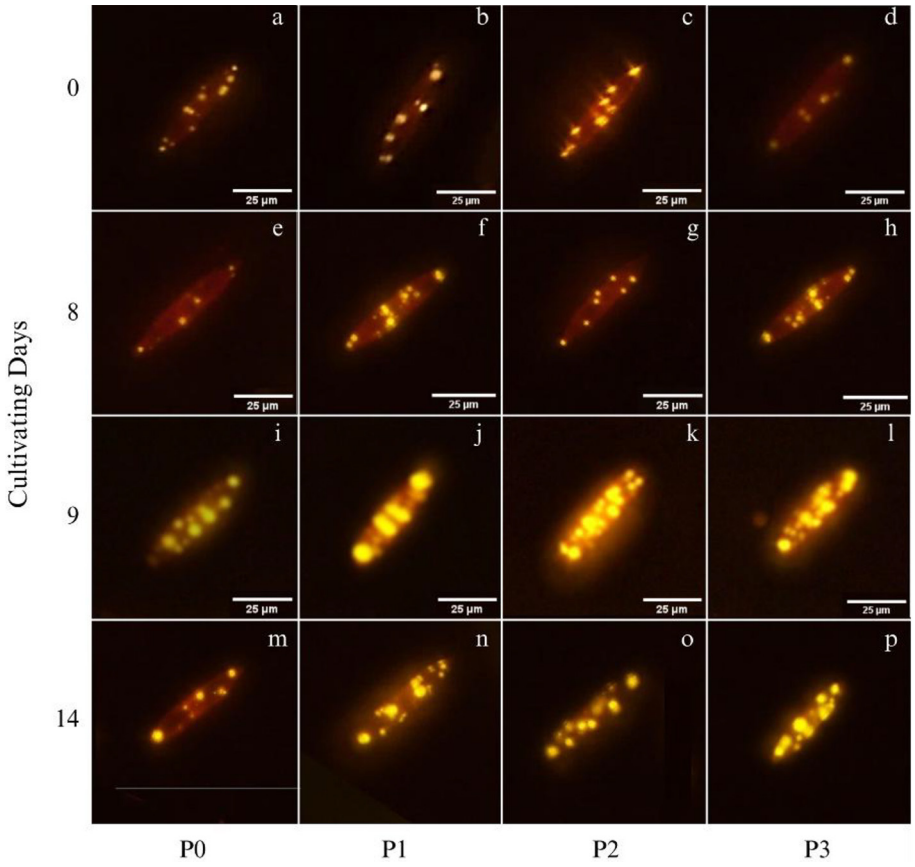

Figure 1. Lipid luminescence of *Nitzschia navis-varingica* under a fluorescence microscope in different cultivation days and treatments; (a-d) culture day 0 of P0-P3, respectively (e-h) culture day 8 of P0-P3, respectively (i-l) culture day 9 of P0-P3, respectively (m-p) culture day 14 of P0-P3, respectively

lowest values recorded in the P3 treatment (50 mg/L MPs) at 93,813 ± 18,186 RFU and 100,569 ± 26,217 RFU, respectively.

N. bory exhibited a markedly different response, with significant changes observed from days 1 to 3 compared to day 0 of cultivation (Figure 3). This species responded rapidly to PS contamination, with the lipid content decreasing drastically within 24 h, as shown in Figure 4c. In the P2 and P3 treatments on day 0, the luminescence intensities dropped to 42,568 ± 29,056 RFU and 33,186 ± 21,077 RFU, respectively, compared with the control (P0 = 72,507 ± 18,053 RFU).

Among the species studied, *N. bory* showed the highest lipid content on day 1 across all treatments except for the highest PS concentration (P3), with P1 exhibiting a peak value of 159,712 ± 118,847 RFU ($p < 0.05$). However, the lipid fluorescence value in *N. bory* (Figure 4c) showed a relatively low value even until the last day of observation with the P3 treatment, which was significantly different ($p<0.05$) from the other treatments, especially on days 1 to 3, with the lowest value. The lipid luminescence of *N. bory* in P3 increased on day 14, which has a similar trend as *N. navis-varingica* (Figure 4a), while in *N. palea* the value has decreased slowly from day 9.

MPs polystyrene exposure and IC_{50} antioxidant activity in three microalgal species

As shown in Table 1, the IC_{50} values of the three microalgal species varied; however, a consistent downward trend was observed (Table 1), with values progressively decreasing from the control treatment (P0) to the highest contamination treatment (P3). For *N. navis-varingica*, the IC_{50} values decreased by 7 to 34%, with the lowest value recorded at 161.46 ± 4.93 µg/mL. The IC_{50} reduction in *N. palea* ranged from 5 to 28%, whereas in *N. bory*, it ranged from 26 to 53%. Statistical analysis confirmed significant differences ($p < 0.05$) in the IC_{50} values across the different PS contamination treatments for all three species.

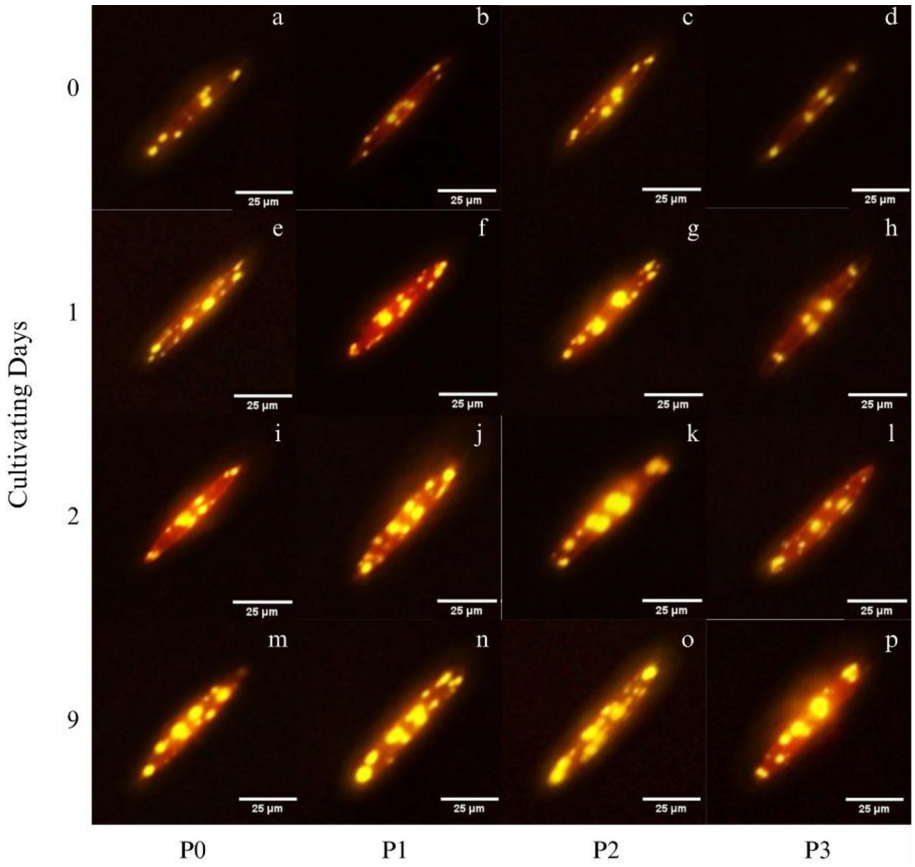

Figure 2. Lipid luminescence of *Nitzschia palea* under a fluorescence microscope in different cultivation days and treatments; (a-d) culture day 0 of P0-P3, respectively (e-h) culture day 1 of P0-P3, respectively (i-l) culture day 2 of P0-P3, respectively (m-p) culture day 9 of P0-P3, respectively

DISCUSSION

Lipid production in microalgae in this study is detected based on its luminescence under Nile Red Staining (NR-S) method, which showed reliability to the gravimetric lipid quantification in other studies (Anam et al., 2021; Balduyck et al., 2015). This method is applicable especially under the conditions of a limited number of cells to be tested because it requires smaller sample sizes (Orr and Rehmann, 2015). In this study, in particular, clonal cultures of three microalgal species were obtained from single cell isolation in the field, making it impossible to obtain high-density cultures in large volumes. The antioxidant activity of each microalgal species in this study has been investigated using the DPPH assay, which measures free radical scavenging and calculates the required concentration to inhibit 50% of free radicals as previously reported by (Afililla et al., 2024; Edelwis et al., 2024; Ermavitalini et al., 2025; Sedjati et al., 2020; Shaima et al., 2022).

Results from this study showed alignment of lipid fluorescence intensity with fluorescence values measured in relative fluorescence units (RFU) in all samples. Relative fluorescence measurements are used to monitor lipid accumulation under stress conditions (Davis et al., 2014; Sharma et al., 2015), which enable rapid and efficient lipid analysis (de Bazan et al., 2023). Low values and weak fluorescence intensities in the highest concentration of microplastics (MP) treatment on day 0 in this study might be triggered due to small particles subjected to the microalgae. Lipid production of Bacillariophyceae species was inhibited after exposure to a high concentration of 2 µm polystyrene (PS) (Raju et al., 2022). The reduction in lipid content may be attributed to excess MPs contamination, which can disrupt microalgal metabolism and affect lipid utilization, as suggested by Wang et al. (2022). These study findings also align with those of Pinpatthanapong et al. (2022), who reported that high concentrations of pollutants could reduce the lipid content in microalgae in less than 24 h. Initial

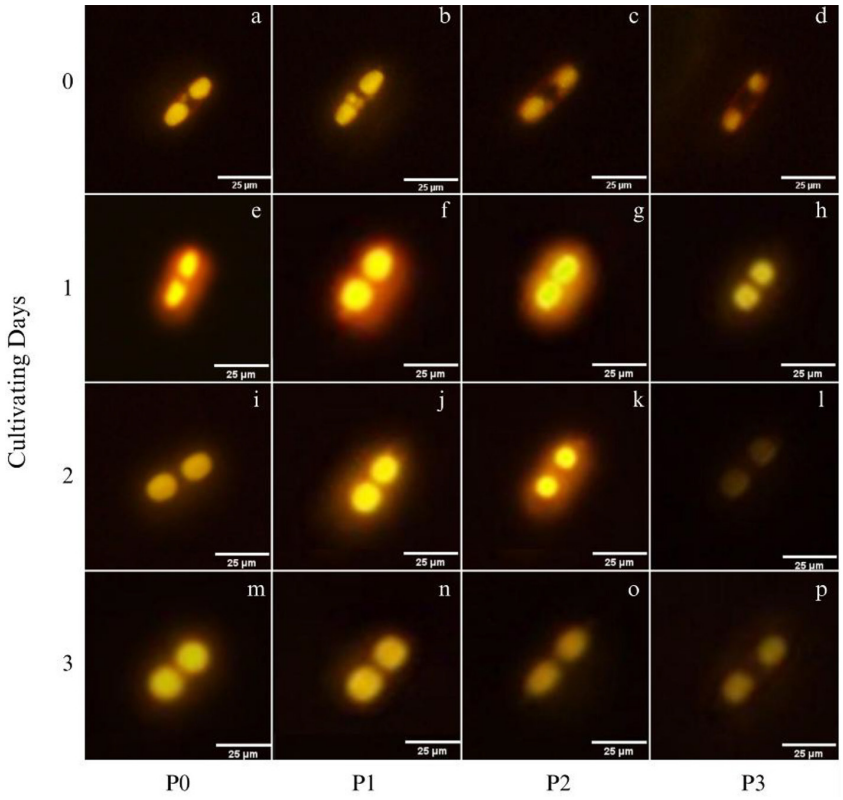

Figure 3. Lipid luminescence of *Navicula bory* under a fluorescence microscope in different cultivation days and treatments; (a-d) culture day 0 of P0-P3, respectively (e-h) culture day 1 of P0-P3, respectively (i-l) culture day 2 of P0-P3, respectively (m-p) culture day 3 of P0-P3, respectively

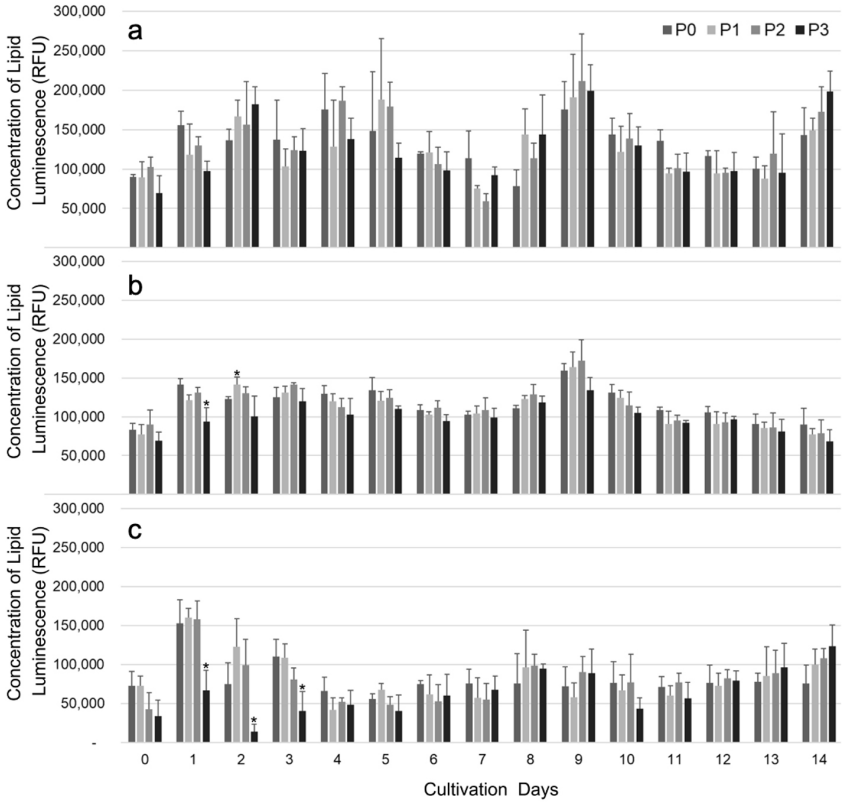

Figure 4. Daily value of the concentration of lipid fluorescence of different clonal cultures on each treatment (P0-P3); (a) *Nitzschia navis-varingica* (b) *Nitzschia palea* (c) *Navicula bory*

Table 1. The IC_{50} values of three microalgal species after being exposed to different microplastic PS treatments (P0-P3)

IC_{50} values (µg/mL)			
P	*Nitzschia navis-varingica*	*Nitzschia palea*	*Navicula bory*
P0	244.21 ± 3.70[a]	220.98 ± 3.33[a]	295.58 ± 0.87[a]
P1	226.41 ± 0.47[b]	210.01 ± 2.45[b]	219.93 ± 0.54[b]
P2	203.82 ± 3.18[c]	182.57 ± 4.01[c]	214.69 ± 1.21[c]
P3	161.46 ± 4.93[d]	159.64 ± 4.54[d]	181.58 ± 0.75[d]

Note: Notation (a-d) shows the significance of the treatment effect ($p < 0.05$).

reduction of lipid concentration in this study was very sharp compared to the previous study that reported in 19% of decrement under the same range of light incubator intensity (40~50 µmol photon/m^2/s) Wang et al. (2022).

The highest values of lipid fluorescence in this study occurred at different times, where in *N. bory* it was detected earlier (day 1), while in both *Nitzschia* species it occurred on day 9. These findings in *Nitzschia* are consistent with Guo et al. (2020), who reported lipid accumulation on day 9 after exposing diatom species to MPs from polyethylene (PE) and polyvinyl chloride (PVC). Meanwhile, lipid accumulation on day 1 in *N. bory* may be attributed to their fast response in encountering the MP pollutant. *Navicula* sp. has been reported to have significant growth inhibition after exposure to PS for a short time within 24 to 48 h (Li et al., 2025). The lipid fluorescence at P3 treatment showed another highest value on day 14, particularly in *N. navis-varingica* and *N. bory*. This trend aligns with the results of Shao et al. (2022), who observed a similar increase in lipid content of microalgae on day 14 of cultivation following exposure to abiotic stress.

The high lipid concentration in microalgae enhances antioxidant potential, particularly during their exponential phase of growth. Banskota et al. (2019) observed high antioxidant potential in high lipid concentration of microalgae. Lipids in microalgae often contain polyunsaturated fatty acids (PUFAs), carotenoids, and tocopherols, which are known for their antioxidant properties that scavenge free radicals and reduce oxidative stress (Millao and Uquiche, 2016). Li et al. (2023) reported that exposure to MPs can enhance antioxidant enzyme activity in microalgae. In concomitant to this study, that report high antioxidant activity with the lowest IC_{50} values (161.46 ± 4.93 ~ 181.58 ± 0.75 µg/mL) in the highest concentration of MP (50 mg/L). Previous studies have also documented reductions in IC_{50} values owing to abiotic stressors. For instance, Nezafatian et al. (2023) reported a decrease of 38 to 41% in IC_{50} values under high-salinity conditions, while León-Vaz et al. (2023) observed a 20–26% decrease in IC_{50} values due to stress from light intensity. Coulombier et al. (2021) found that various abiotic stressors could lower IC_{50} values in microalgae, with reductions ranging from 395.93 to 710.60 µg/mL. These results are considered relatively weak (> 200 µg/mL) when compared to the findings of the current study, which demonstrated IC_{50} values below 200 µg/mL at the highest level of MPs contamination. However, this study shows lower antioxidant potential compared to the report of Xiang et al. (2023) that exposed microalgae to a smaller plastic PS (50 nm) in a high concentration of 50 mg/L and resulting very low value of IC_{50} (19.89 µg/mL). This huge potential of antioxidant activity might triggered by the small particle size, that causing greater membrane damage in cells (Li et al., 2020).

The application of MP in this study affected the lipid fluorescence and antioxidant content of microalgae. All three microalgae in this study were able to adapt to exposure to PS at the highest concentration (50 mg/L). However, the effects of MP will be more severe in aquatic ecosystems, especially those polluted by heavy metals, such as mangrove areas where those three microalgae are isolated (Titah and Pratikno, 2020). Gao et al. (2019) reported that MP have the ability to absorb heavy metals in water. The interaction of MP with heavy metals is very likely in water and can have a more detrimental impact on microalgae. Therefore, the impact of MP will generally be more severe in aquatic ecosystems compared to this study due to the various pollutants that accumulate in the microalgal community.

CONCLUSIONS

Exposure to 2 µm polystyrene (PS) microplastics (MPs) significantly influenced lipid fluorescence and antioxidant potential activity in three isolated microalgal species from the mangrove ecosystem —*Nitzschia navis-varingica*, *Nitzschia palea*, and *Navicula bory*. Lipid fluorescence initially declined under high MP concentrations (50 mg/L) but subsequently increased, indicating species-specific adaptive responses to MP-induced stress. The highest lipid accumulation occurred at different cultivation periods among species, reflecting variations in stress tolerance and metabolic adjustment. MP exposure also enhanced antioxidant activity, as demonstrated by lower IC_{50} values in the highest MP treatment. These findings suggest that MP stress can stimulate lipid production and antioxidant defense mechanisms in benthic microalgae, although the response varies among species. The results further indicate that the ecological impact of MP may be amplified in natural aquatic environments where microplastics interact with other pollutants, such as heavy metals.

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