Effects of Polyethylene on Chlorophyll *a* and the Utilization of Microalgae as Bio-Indicators for Polyethylene Contamination in Water

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ABSTRACT

Contamination of polyethylene (PE), one of the most common microplastics detected in water, adversely affects aquatic microorganisms, particularly microalgae. This study examined the effects of PE particles on chlorophyll *a* synthesis and the sensitivity of the two tested strains, *Chlorella* sp. and *Scenedesmus obliquus,* by measuring the inhibition rate (IR). The possibility of using the tested strains as bio-indicators for PE contamination in water was investigated during the batch test. The synthesis was conducted at room temperature between 26-31°C and with light intensities of 1,700-2,200 lux. PE particles of 40-48 µm were mixed with BG-11 at different concentration levels: 50, 100, and 200 mg/L. A batch test to determine PE influences on the two tested strains was carried out for twenty-five days.

Scanning electron microscope images revealed the tested strains' attachment to the PE surface. Meanwhile, Fourier Transform Infrared Spectroscopy illustrated some structural changes from damage caused by the PE. The maximum PE concentration of 200 mg/L resulted in the lowest chlorophyll *a* synthesis of the two tested strains. A significant difference in chlorophyll *a* synthesis between the tested strains and the control was observed on the 20th and 25th day of exposure for *Chlorella* sp. and *Scenedesmus obliquus* ($p<0.05$). Although there was no significant difference in specific growth rate among the treatments of PE 50, 100, and 200 mg/L, the tested strain of *Chlorella* sp. had an induced lower specific growth rate after ten days of exposure. Notably, the tested strain, *Chlorella* sp., showed high growth inhibition on the 10th day of exposure in the 200 mg/L PE treatment in autoclaved natural water. As a result, *Chlorella* sp. showed potential as a bio-indicator for PE contamination in water because it provided a high IR with limited harmful effects.

Keywords: Microalgae/ Microplastics/ Polyethylene/ Chlorophyll *a*

1. INTRODUCTION

Microplastics (MPs) contamination in the environment is one of the ongoing pollution problems worldwide [1, 2]. They are released into the aquatic environment in various types, including Polyethylene (PE), Polypropylene (PP), Polystyrene (PS), and Polycarbonates (PC) [3]. PE is the most produced polymer, which can be utilized in various applications and has lower production costs. In the aquatic environment, PE is located mostly at relatively low depths due to its low density [4]. In a study by Rossatto et al. [5], PE was found to be present in the aquatic system of the continent of Asia at approximately 18.52%. The microorganisms in water, particularly bacteria, initiate the process of biodegradation by adhering to the surface of plastic materials and releasing enzymes that catalyze the polymerization of plastic polymer chains [6]. The short-term impacts of PE exposure at 300 mg/L on scleractinian coral *G. columna* significantly changed the dynamics of the bacteria in the aquatic system [7]. Meanwhile, the main producers in the aquatic ecosystem, phytoplankton and microalgae, can accumulate MPs by adsorption, which can result in adverse effects, especially on pigment production, photosynthesis [8, 9, 10], and growth inhibition rates accordingly [9, 11, 12, 13]. Moreover, the higher adsorption of MPs-PE and Polyvinyl chloride (PVC) by algae leads to higher capacities for the adhesion of other organic micropollutants [14]. Various factors of the MPs related to their effects on microalgae include the sizes, the concentrations, and the exposure times - all of which showed interesting results (i.e., *Chlorella* and *Scenedesmus* can adsorb to polyethylene/polystyrene (PE/PS) and can affect the synthesis and growth rate [15, 16, 17]. MPs such as PVC, PP, PS, and PE are also effective in chlorophyll *a* synthesis and in photosynthesis [10, 11, 18, 19, 20]. It was revealed that the size of MPs

is related to the level of the adverse effects that occur in the microalgae [12, 21, 22]. It has also been shown that higher concentrations and smaller particle sizes of MPs lead to lower growth rates for both marine and freshwater microalgae, especially *Chlorella* spp. [23, 24]. However, the adaptation of microalgae to exposure to MPs has been mentioned in some studies, but perhaps the relative increase in contamination over the tolerance may limit such microalgae.

Recently, scientific studies, which were conducted on MPs and microalgae have been concerned with assessing the global risk to aquatic life and with also serving the ecosystem, which focuses on the occurrence of microplastics, their links to toxicity, and the removal of MPs from aquatic environments [1, 3, 25, 26]. However, the potential removal of MPs by microalgae has been limited, and only a few studies have been performed. For example, *Scenedesmus abundans* removed more than 84% of multiple kinds of MPs and 98% of polymethyl methacrylate (PMMA) microparticles [27]. The effects of the MPs on microalgae seem to be temporary, with an initial period of vulnerability, which is followed by adaptive responses that lead to recovery and utilization of MPs as their habitat, which is especially true for the smaller microalgae [9, 11, 28]. These studies demonstrated the possibility of indirectly monitoring the concentrations together with the removal of the MPs by employing a bio-indicator microalgae [3, 5, 29, 30]. However, prior to that stage, more information on the clarification of the impact of microplastic type and the selection of the index representing the impact on the proposed model species is still required. To address these issues, a batch assay was performed to compare the influence of different PE concentrations on chlorophyll *a* synthesis and growth rate of the two selected model microalgae strains. The potential to further apply the selected test strains as a bio-indicator for PE contamination in water was also evaluated in the current study.

2. METHODOLOGY

2.1 The tested microalgae strains and types of MPs

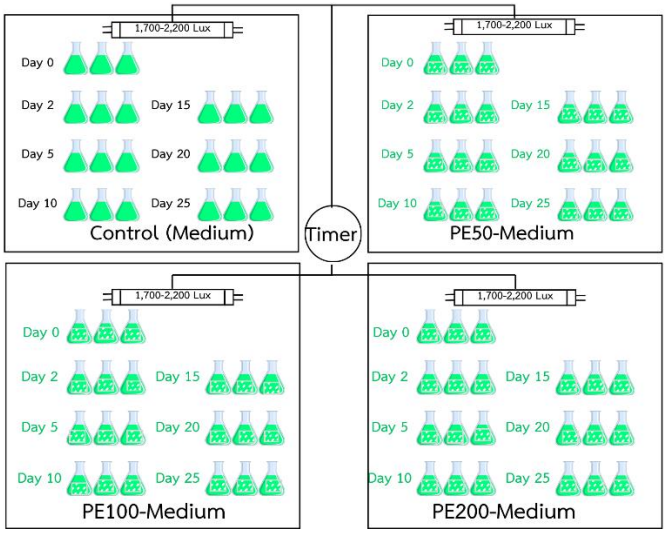
It was reported that MPs caused a relatively high negative effect on the growth of green algae, especially *Chlorella* sp. and *Scenedesmus* sp. [30, 31, 32]. As a result of the important properties of *Chlorella* sp., especially its sensitivity to toxic substances, a short growth cycle, and the ability to grow in small amounts of nutrients [30], and also a unicellular phytoplankton, *Scenedesmus obliquus*, was chosen as a bio-indicator for a toxicity test of PS [33]. Hence, the two microalgae strains of *Chlorella* sp. (TISTR 8262) and *Scenedesmus obliquus* (TISTR 8546), which were obtained from the Thailand Institute of Scientific and Technological Research (TISTR), were designated as the tested strains for this study. The cultivation of the tested strains was conducted following the TISTR guideline, with the optimum temperature for growth of 28 ± 2°C under a light intensity of 2,000-3,000 lux for one week using sterile BG-11 culture medium [34].

The selected type of MPs was a laboratory-grade powdered PE with a size of 40-48 µm, which had been obtained from SIGMA-ALDRICH, USA. Prior to the batch experiment, it was mixed with the BG-11 medium.

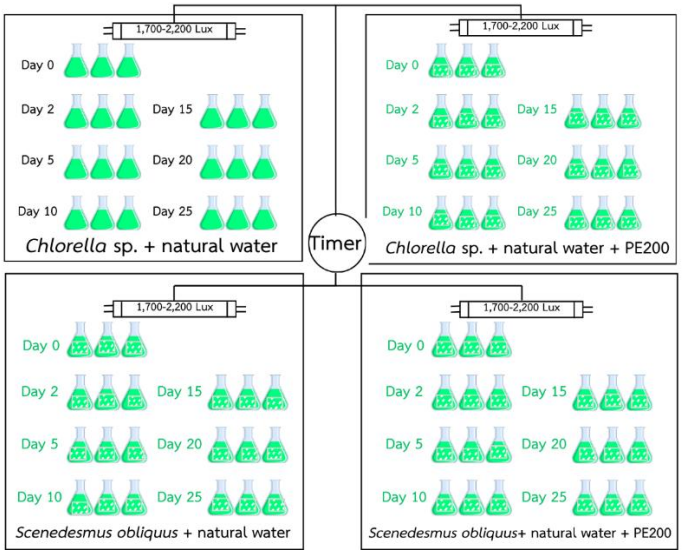
2.2 Experimental Set-up

There were two phases of the experiment. It began with determining the effects of PE on chlorophyll *a* synthesis of the tested strains, followed by conducting tests on the sensitivity levels of the tested strains during PE exposure in autoclaved natural water, all of which were carried out on a laboratory scale. In the first phase of the study, PE was mixed into the autoclaved cultivated microalgae medium (BG-11). According to previous studies, the concentration of MPs was tested in the range of 10-100 mg/L and 100-1,000 mg/L [30]. Different concentrations of PE mixed with BG-11 in the current study were used at 50, 100, and 200 mg/L. Tested strains of 3 mL and 50 mL PE-medium were mixed in a 125 mL Erlenmeyer flask in triplicate treatments (Fig. 1) and were manually shaken 3 times per day. Batch testing and the growth temperature of the tested strains were conducted at room temperatures between 26-31°C with top illumination provided by a combination of natural and artificial light at

intensities of 1,700- 2,200 lux for 12 hrs. Fluctuations in the recorded room temperature were due to the variation of moisture content in the air during the batch experiments, and they were in accordance with the air temperature measured by the Upper Northeastern Meteorological Center in Khon Kaen Province of Thailand. The processes of sampling chlorophyll *a* and taking measurements of the phototrophic growth based on observations of the tested strains were carried out during the twenty-five days of PE exposure. Clarification of the ongoing changes regarding the shapes and structures of the exposed microalgae was observed via scanning electron microscope (SEM). Structure change of the tested PE was examined using Fourier transform infrared spectrometer (FTIR) analysis.



(a) The Initial phase: Exposure of the PE medium



(b) The Second phase: Exposure of PE in autoclaved natural water

Figure 1. The experimental set-up for the PE effects on the tested strains

The second phase of the experiment emphasised examining the sensitivity of the tested strains to PE exposure. The test was conducted under the same environmental conditions as in the first phase. In this phase, water samples were collected from a recreational reservoir in Khon Kaen Province, Thailand, namely Bueng Si Than. The water samples were autoclaved for 15 min at 121 °C. After that, 200 mg/L PE concentrations were added to the water samples (Fig.1). The processes of observing the sensitivities of the tested strains due to PE exposure were achieved by monitoring the reduction in the rate of chlorophyll *a* synthesis and the growth inhibition ratio.

2.3 Data collection and analysis

Experimental samples of 10 mL were collected for chlorophyll *a* extraction and measurements. During sampling, the atmospheric temperature, the light intensity, and the pH values of the medium were all measured. Samples for chlorophyll *a* measurements were extracted using 95% ethanol, and they were kept without any exposure to light for 24 hours before the absorbance measurement at 663 nm via a spectrophotometer (Hitachi; UH-5300). Calculation of chlorophyll *a* was achieved by using the equations mentioned in previous studies [35]. The structural changes on the PE surfaces were studied, and the morphology of the tested microalgae during PE exposure was examined using FTIR spectra (Bruker TENSOR 27, Germany) and SEM images (Tescan, Mira series, Greece).

The specific growth rates (μ) and the growth inhibition rates (IR) of the tested strains during the 25 days of PE exposure were calculated using an equation from Li et.al. [19] with some of the following modifications:

$$\mu = \ln (N_2/N_1)/(t_2 - t_1)$$

in which … N_1 and N_2 are the biomass at time 1 (t_1) and time 2 (t_2)

$$IR\ (\%) = (1 - T/C) \times 100\%$$

in which … C and T are the OD_{663} in the control and the treatments

Statistical analysis was performed using the computer software package, One-way ANOVA, to confirm significant differences in growth response to the following: chlorophyll *a* synthesis, the specific growth rates, and the growth inhibition rates of the tested strains during PE exposure compared to the control. A t-test was utilized to determine the percentage differences in the estimated specific growth rates of the strains tested.

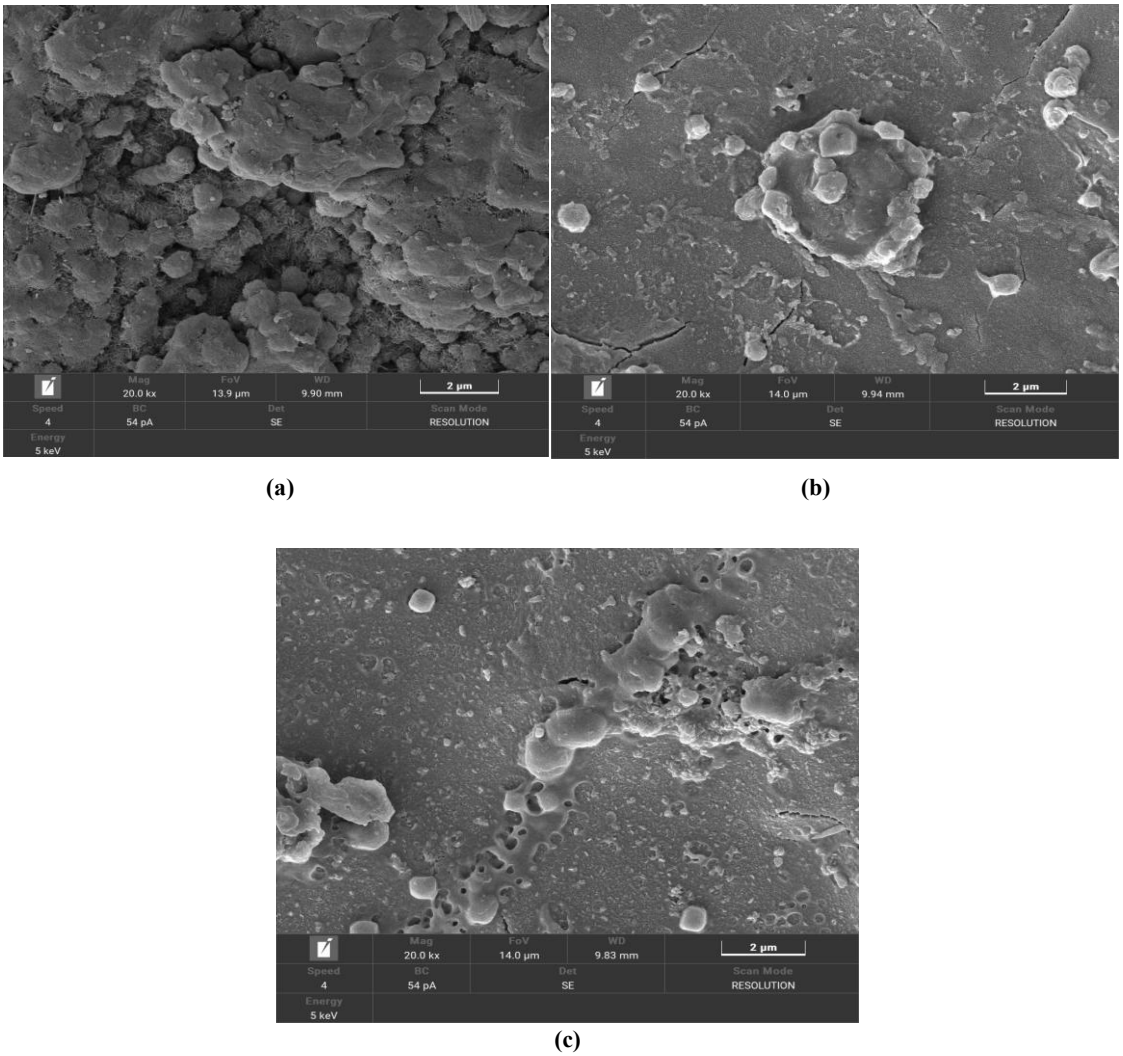
3. RESULTS AND DISCUSSION

3.1 SEM images and FTIR spectrum

SEM images of the surface morphology of the strains tested after 25 days of PE exposure showed surface damage of the cells and also showed that the adhesion mechanism had probably occurred in both strains (Fig. 2). It revealed that the attachment of the tested strains to the PE surfaces, which was similar to the evidence found in previous studies [23, 31, 36], could result in having harmful effects on their growth and metabolism [15]. The accumulation of *S. cerevisiae* yeast on the surface of other MPs, PS particles, was also highlighted in the study of Anbarani et al. [37]. Meanwhile, the current observation was confirmed by the results of the growth inhibition rates, which occurred at the beginning of the exponential phase [11, 13].

According to the findings from previous studies, there are negative effects, occurrences of growth inhibition, and the utilization of MPs, which refers to MPs that are not harmful to microalgae. Hence, these MPs could serve as substrates for algal growth and could promote the growth of microalgae [9, 11]. However, MPs can also facilitate the dispersal of harmful microalgae [28] and can, as a

consequence, pose a danger to aquatic ecosystems. The impacts on the exhibited Hg-sorption capacities of PE have previously been noted by Pinto et al. [38].



(a) **(b)**

(c)

Figure 2. SEM analysis of the surface morphology of PE and the tested strains after 25 days of PE exposure: (a) PE particles, (b) the tested strain of *Chlorella* sp., (c) the tested strain of *Scenedesmus obliquus*

Figure 3 shows the characteristic absorbance bands for the presence of PE in the batch samples of *Chlorella* sp., which are located at 2915 cm^{-1}, 2848 cm^{-1}, 1470 cm^{-1} & 716 cm^{-1}. The same range of FTIR profiles of PE-based polymers was reported [38, 39, 40, 41]. The similarity of the range of absorbance bands detected obtained from the batch assay of *Scenedesmus obliquus* was recorded in the current results. The peaks at 2919.39 cm^{-1} and 2850.41 cm^{-1} were reported for the corresponding asymmetric and symmetric stretch of CH_2 [42]. Meanwhile, four major peaks at 721, 1463, 2850, and 2920 cm^{-1} were highlighted in the study of the PE aging process [14]. In our study, the increment of %T from the stretching of alkyl groups (C–H and CH_2) was found at the wavelengths of 2950 to 2800 and 716 cm^{-1}, which was observed after PE interaction with the tested strains. At the wavelength of 1470 cm^{-1}, the presence of alkenes (C=C) indicated a degradation process of PE during batch tests (Day 10th of exposure) (Fig. 3b). The peak represents the bending deformation, which was consistent with the study by Nasrabadi, Eydi, and Bonyadi [42] and Hadiyanto et al. [40]. Concurrently with SEM

examination, the attachment of the tested strains to the PE surfaces may support the ongoing degradation of PE, as reported in Chen et al. [39]; biofilm development is an important factor in MPs degradation in natural water bodies. The adhesion process between MPs and large biotic surfaces could also influence the abundance and bioavailability of MPs in the aquatic environment [43].

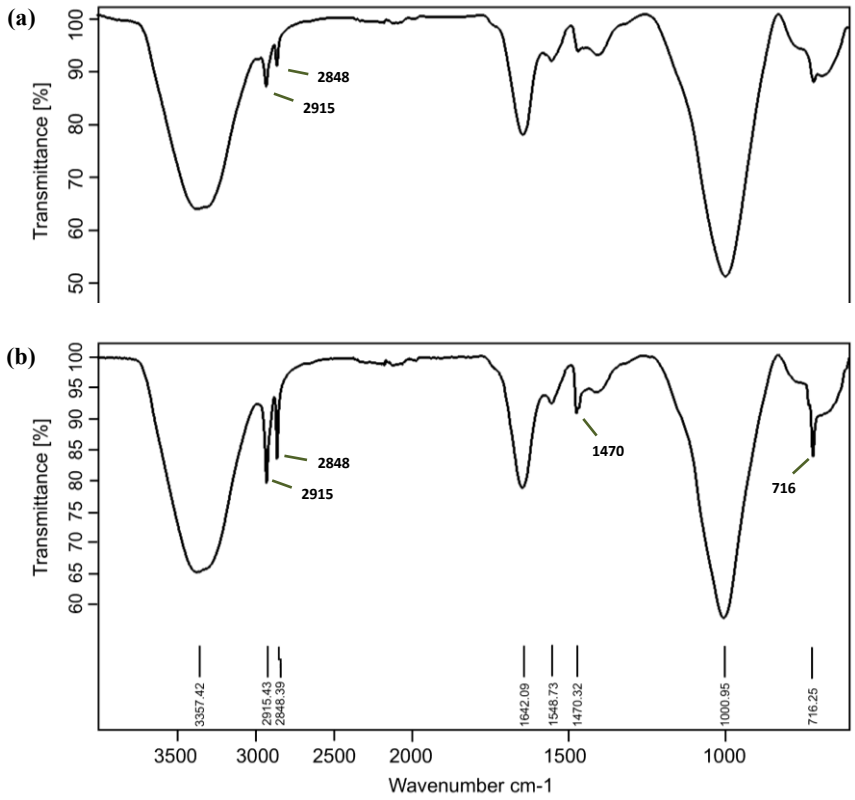


Figure 3. FTIR spectrum of PE in the batch of the tested strain of *Chlorella* sp. (a) Initial day of exposure (b) Day 10th of exposure

3.2 The effects of PE on the tested strains

3.2.1 Chlorophyll a synthesis

The batch assay of chlorophyll *a* synthesis in the tested strains of *Chlorella* sp. (TISTR 8262) and *Scenedesmus obliquus* (TISTR 8546) was conducted during the 25-day PE exposure period. With regard to chlorophyll *a* synthesis compared to the control, the significant adverse effect of PE on the tested strain of *Chlorella* sp. was observed on the 20th day of exposure (One-way ANOVA; $p<0.05$) (Fig. 4a). Meanwhile, the treatment with PE 200 mg/L provided the lowest chlorophyll *a* synthesis. The results indicated that the effect of PE on the tested strains was dependent on PE concentration, which was also found in the studies by Cheng et al. [24] and Xu et al. [23]. The results from some other studies also indicated that the greatest effects on the algae were detected after the highest concentrations of MPs had been added [10, 13, 44]. There were two reasons why MPs can affect the synthesis of the algae's chlorophyll *a*: 1) the cell structure of the algae had been damaged, and 2) the strong adsorption ability of the MPs had caused the microalgae to become inactive [10]. In a study by Cheng and Wang [27], it was revealed that long-term exposure to MPs had promoted the formation of bound extracellular polymeric substances (EPS), while short-term exposures had led to an enhanced adsorption onto solid surfaces by the microalgae.

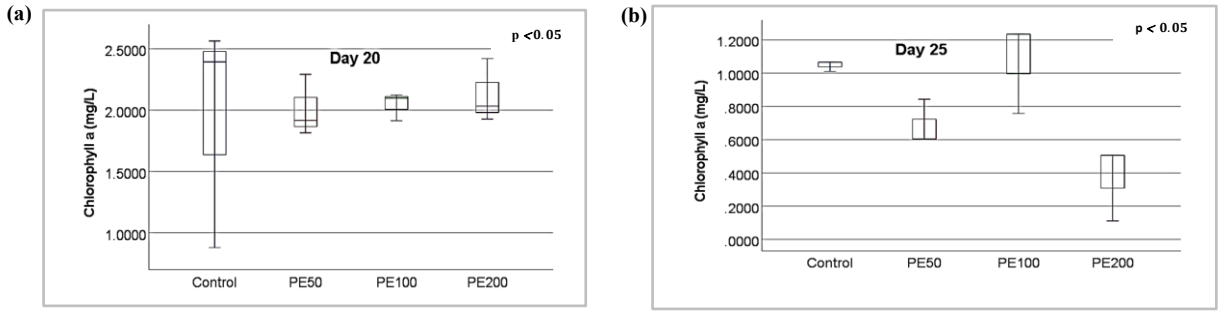


Figure 4. Chlorophyll *a* synthesis of (a) *Chlorella* sp. and (b) *Scenedesmus obliquus* during PE exposure

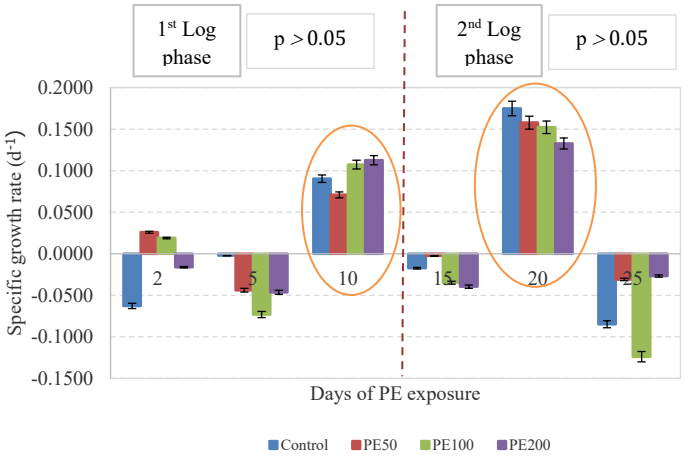
Conversely, the evidence on the impact of PE exposure on *Scenedesmus obliquus* showed no significant difference in chlorophyll *a* content from the control during the log phase (days 5-10). However, it was found in the treatment of PE 200 mg/L on the 25th day of exposure (One-way ANOVA; $p<0.05$) (Fig. 4b). The exponential phase of this strain was unclear and was better demonstrated using a specific growth rate expression. Lower chlorophyll *a* synthesis was recorded than the amount obtained from the tested strain of *Chlorella* sp. Nevertheless, after twenty days of exposure, the amount of chlorophyll *a* synthesis had slightly increased, even higher than the control. These results were in accordance with the research of Malinowski et al. [45], who found that greater amounts of chlorophyll *a* were observed in high MPs treatments. This suggests the possibility that the algae utilize MPs as substrates for growing as well [46]. Concurrent with another type of MPs, PS, it was reported that this type could enhance algal growth [47]. However, in a study by Khatiwada et al. [31], Polyethylene terephthalate (PET) (300 µm with a concentration of 200 mg/L) showed a significant inhibitory effect on *Scenedesmus spp*.

As mentioned earlier, PET 300 µm was shown to have a significant inhibitory effect on *Scenedesmus spp.* [31]. In contrast, PE 40-48 µm in the current work at the same concentration of 200 mg/L seemed to have had no impact on the same species. Nevertheless, adverse effects on growth rates were found in *Chlorella* sp., as indicated in a study by Cheng et al. [24] and in another type of microalgae, *Spirulina* sp., which was reported by Hadiyanto et al. [40]. This illustrates that the sizes of the MPs, which remain in the water source, have an impact on microalgae. Moreover, the sensitivity of the microalgae to various types of MPs depends on the strains of microalgae.

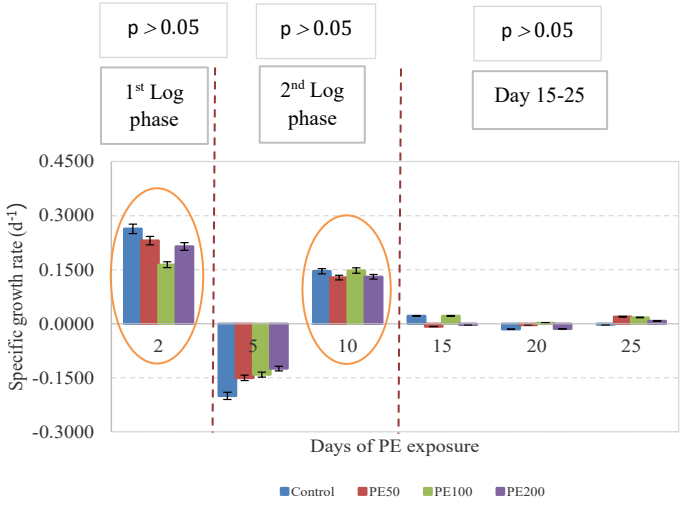
3.2.2 Specific growth rates

From the results, it was demonstrated that under batch test conditions, with light intensities of 1,700-2,200 Lux, at room temperatures ranging from 26-31°C, and at pH values of the PE mixed medium in the range of 6.5-8.4, there were no significant differences in specific growth rates among the treatments of PE 50, 100, and 200 mg/L compared to the control. This was observed during the first and second log phases (One-way ANOVA; $p>0.05$) (Fig. 5). However, the tested strains, especially *Chlorella* sp., which had been exposed to PE mixed with the medium, had induced lower specific growth rates than those in the control during the exponential phase after ten days of exposure, on day 15th (Fig. 5a). This implied that the impact of PE depends on the concentration range, with the lowest specific growth rate having been observed in the PE-200 mg/L exposure treatment. The higher concentrations and smaller particle sizes of MPs resulted in lower growth rates of *Chlorella* spp., as mentioned in Cheng et al. [24]. This pointed to the fact that growth inhibition might have occurred in the tested strain of *Chlorella* sp. during the exponential phase, which was also identified in previous studies [11, 40, 48]. In addition, PE exposure could affect the efficiency of the light energy that is utilized by the algal cells [10, 13], as the adhesion between algae and PE resulted in induced shading,

blocking algal pores or gas exchange [28], which accordingly brings a reduction in chlorophyll *a*. Meanwhile, a lower specific growth rate was recorded for the tested strain of *Scenedesmus obliquus* throughout the experimental period.



(a) The tested strain of *Chlorella* sp.



(b) The tested strain of *Scenedesmus obliquus*

Figure 5. The specific growth rates of the tested strains during the exponential phase

3.3 The sensitivity of the tested strains to PE exposure

In our study, the sensitivity of the tested strains of *Chlorella* sp. and *Scenedesmus obliquus* were compared using the growth inhibition rate (IR) throughout the 25 days of PE exposure. A maximum IR of 29.47% was found in the treatment of the tested strains of *Chlorella* sp. with PE 200 mg/L on the last day of exposure. Initial observations of IR were made at the beginning of the 2nd day, with IR percentages of 9.86%, 13.47%, and 14.37% for PE exposures of 50, 100, and 200 mg/L, respectively. After that, the IR was continuously observed except on the 20th day. On the 10th day, the ordering inhibition rate from low to high PE concentration exposure was depicted with IR values of 2.27%, 3.66%, and 7.51% (Fig. 6a). In another study, low-density PE showed higher inhibition rates of 85%

on the marine microalgae, *Chaetoceros calcitrans* [49]. However, in our study, no significant differences in the obtained IR were shown among the PE concentrations of 50, 100, and 200 mg/L (One-way ANOVA; $p > 0.05$).

A high IR value of the tested strain of *Scenedesmus obliquus* was clearly observed on the 2nd day of exposure in the treatment with PE 100 mg/L. A maximum IR of 18.83% was recorded in the treatment with PE 100 mg/L on the 20th day of exposure (Fig. 6b). However, fluctuations in the IR values were observed in this batch. In the study of Khatiwada et al. [31], a PET with a high concentration (200 mg/L) was reported with a significantly high inhibitory effect on *Scenedesmus* sp. Whereas the IR values, which were obtained from the treatments of this strain that had been exposed to PE concentrations of 50-200 mg/L, did not show any significant differences (One-way ANOVA; $p > 0.05$)

The addition of a PE concentration of 200 mg/L in autoclaved natural water was conducted to continue observing the PE sensitivity of the two tested strains, and the results are depicted in Fig. 6c. It was found that the tested strains interacted with PE and altered cell growth and photosynthesis, possibly via adhesion. A study by Ge et al. [50] highlighted that MPs could alleviate the harm of pollutants by their absorption capacity. Also, the effect could be transferred up the food web, including to humans [30, 44]. In our study, the inhibitory effect of PE on the chlorophyll *a* of *Chlorella* sp. and *Scenedesmus obliquus* was found at the beginning of the experiment (the 2nd day) and clearly shown in an exponential phase on the 15th day of exposure. On the last day of exposure, the tested strains of *Chlorella* sp. showed an extremely high IR value of 40.53% (Fig. 6c). A study by Demir-Yilmaz et al. [51] indicated that the algal cells could directly interact with plastic particles through hydrophobic interactions via direct contact or by inducing the production of exopolysaccharides by the cells. However, the effect on the tested strains in our study was most likely weakened over time due to a slight increase in the growth of both strains. These findings agree with those of previous studies; the growth rate of the tested strains, *Spirulina* sp. and *Chlorella vulgaris,* showed the adaptability of the utilization of MPs as a carbon source for promoting growth and for providing continued increases in growth rates along with a decreasing pattern of inhibition rates over time [40, 52]. Long exposure of algae to micro- and nano-plastics leads to their adaptation and resistance [53]. The mechanisms of adaptive changes in microalgae after long-term exposure to PE occur due to the formation of bound extracellular polymeric substances (EPS) [27]. Meanwhile, Sanchez-Fortún et al. [54] suggested that the formation of PE combined with aquatic organic contaminants could reduce the adverse effects on microalgae. The observed results on the tested strain of *Scenedesmus obliquus* in the presence of PE showed a lower IR and also a lower specific growth rate. This probably arose from the different sizes and structures of the algal cell walls, which influence the adsorption of PE based on the different microalgae strains.

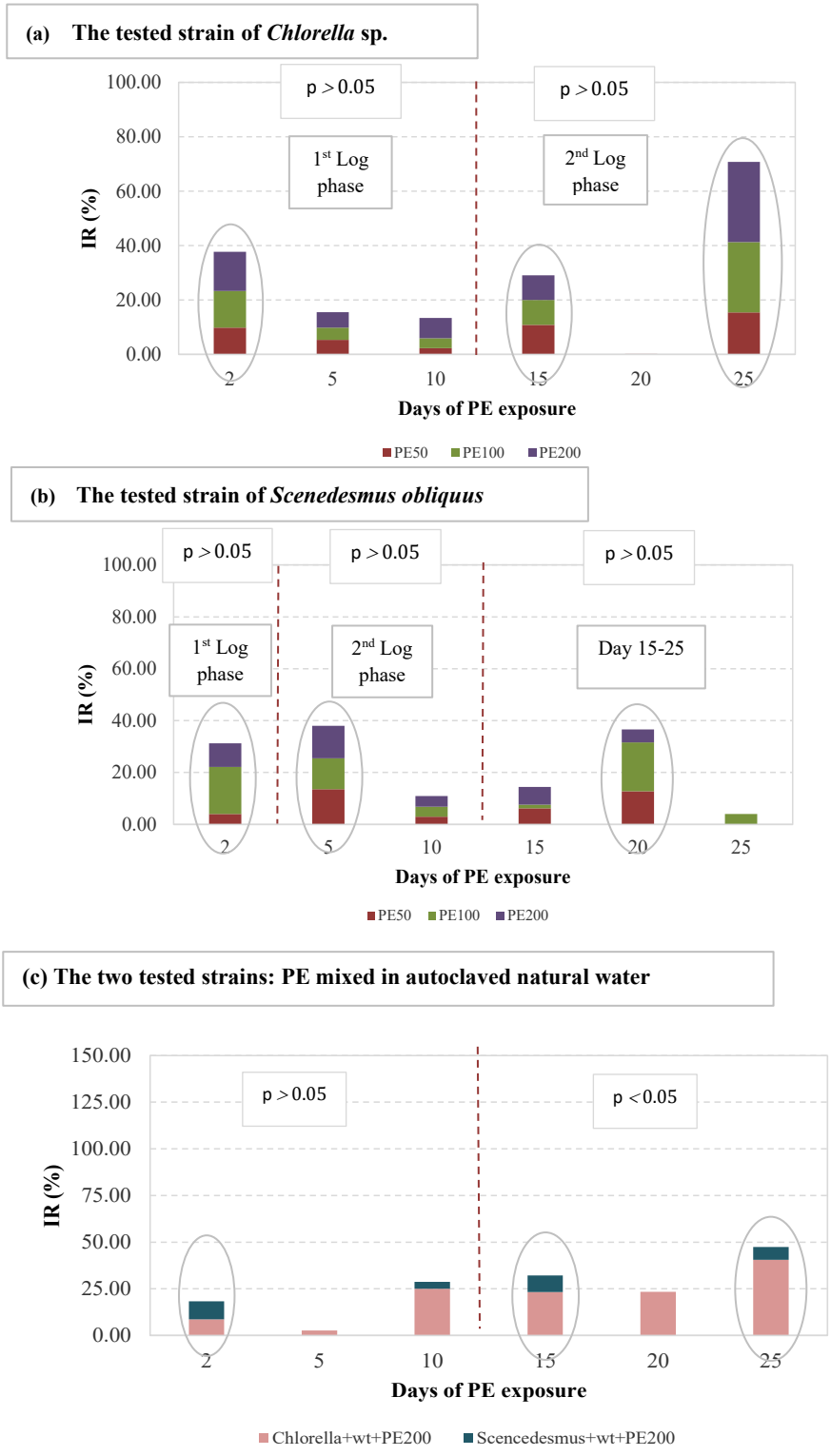


Figure 6. The growth inhibition rates of the tested strains during exposure to PE mixed in medium (a, b) and in autoclaved natural water (c)

Notably, significant differences between the IR values of the two tested strains were observed on the day 15th and 25th of exposure ($p<0.05$) (Fig. 6c). The differences in the IR values between the two tested strains showed different results of adsorption interaction, and shading interruption from PE exposure was found. This could have also arisen from the compositions and synthesis processes of the MPs [11]. In a study by Xie et al. [55], it was mentioned that both the particle sizes and the surface areas of the MPs were other important factors that affect microalgae growth. In this study, the growth inhibition effects obtained for the tested strains of *Chlorella* sp. were more pronounced than in the tested strain of *Scenedesmus obliquus*, which provided an initial growth inhibition ratio of 8.61% on the 2nd day of exposure (Fig. 6c). Meanwhile, a recent study [15] indicated that there was no observed effect on the growth of *C. vulgaris* after 11 days of PE exposure, even though the photosynthetic efficiency was remarkably decreased with increased concentrations of PE exposure. Additionally, the findings from a study by Su et al. [32] showed that *Scenedesmus obliquus* revealed a high degree of aggregation for microalgae and MPs, which led to a high inhibition of algal cell growth. Das et al. [56] also reported that *Scenedesmus obliquus* was more sensitive to PP from disposable surgical face mask leachates than *Chlorella* sp. was. This suggests that there are different sensitivities of microalgae strains to different types of MPs. In our study, the interaction of the tested strains of *Chlorella* sp. with the occurrence of limited harmful effects showed that it could be applied as a bio-indicator for detecting PE in natural water, particularly in freshwater sources, since the effects of MPs on freshwater microalgae are more severe than on marine microalgae [23]. However, in considering microalgae as a potential model species, the role of the ecosystem as well as other factors should be taken into account. These are included in the proposed framework for selecting the bio-indicators of nano-plastics in marine environments [57].

4. CONCLUSIONS

This study aimed to investigate the influence of PE on microalgae strains of *Chlorella* sp. and *Scenedesmus obliquus*. SEM images of the surface morphology of the tested strains revealed the attachment of the tested strains to the PE surfaces, which could lead to harmful effects. Meanwhile, FTIR analysis confirmed a degradation process of PE during batch tests. The maximum PE concentration of 200 mg/L showed the lowest chlorophyll *a* synthesis during the log phase of the tested strains of *Chlorella* sp. It induced lower specific growth rates than those in the control during the exponential phase on day fifteen of PE exposure. Nevertheless, the adaptive ability of the tested strain of *Chlorella* sp. in the environment was observed in this study, as the rise in specific growth rate appeared again on day twenty of exposure. Moreover, a maximum IR of 29.47% was found in the treatment of the tested strains of *Chlorella* sp. with PE 200 mg/L. Fluctuations in the IR values were observed in the other tested strain. An explanation for the batch of the tested strain of *Chlorella* sp. is that the higher the PE exposure, the lower the specific growth rate that was obtained. Also, the growth-inhibitory effect obtained from the tested strain of *Chlorella* sp. was better clarified than that of the tested strain of *Scenedesmus obliquus*. The interaction of the tested strain of *Chlorella* sp. occurred with limited harmful effects. Therefore, its ability to be further applied as a bio-indicator for the screening and detection of PE in natural water has been demonstrated.

Acknowledgement

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