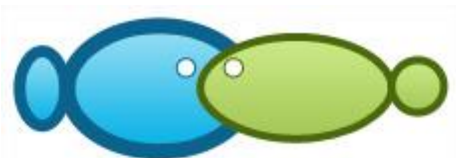

Investigation of the effect of AMPEP concentration in nutrient medium on the cell density, growth response, and pigment accumulation of *Nannochloropsis* sp. culture

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Abstract. Microalgae of the genus *Nannochloropsis* is used for research and business purposes due to their rapid growth and adaptability to a variety of environments. Various nutrients have been used to enhance the growth and pigmentation of *Nannochloropsis* cultures. It is known that Acadian Marine Plant Extract Powder (AMPEP) improves the production of crops and macroalgae. However, AMPEP has not yet been investigated as a method of producing microalgae. Therefore, this study examines microalgae production in a nutrient medium containing AMPEP. Five concentrations of AMPEP were prepared: group A (no AMPEP, as control), group B (25 mg L^{-1} AMPEP), group C (50 mg L^{-1} AMPEP), group D (100 mg L^{-1} AMPEP), and group E (150 g L^{-1} AMPEP). Experiments were conducted for each group for 24 days in triplicate. Results revealed that group D (100 mg L^{-1} AMPEP) in a nutrient medium marked a significant difference ($p<0.05$) by reaching a cell density (cell mL^{-1}) 1.77-fold higher than in group A (control). The specific growth rate of *Nannochloropsis* sp. culture was significantly increased in group C ($p<0.05$), at 0.43±0.04 day^{-1}, compared to the other experimental groups, including the control group. In addition, the maximum dry weight was obtained in the group B (5.09±0.18 g L^{-1}), higher than in the other experimental groups. Moreover, in terms of pigment accumulation, group E (150 mg L^{-1}) reached higher chlorophyll-a levels, at 10.89±4.29 μg mL^{-1}, than the other group experiments, while group B (25 mg L^{-1}) reached higher total carotenoid pigment accumulation levels, at 3.12±0.83 μg mL^{-1} than other experimental groups. Hence, the present study highlights that AMPEP may improve growth and pigment accumulation when used in microalgae production, in particular in the *Nannochloropsis* sp. culture.
Key Words: AMPEP, cell density, growth response, pigment accumulation, nutrient medium.

Introduction. Microalgae, such as *Nannochloropsis*, *Tetraselmis*, and *Chaetoceros*, are essential photosynthetic organisms widely utilized in aquaculture, as feed for larvae and an alternative protein source in formulated feeds, as well as in biodiesel production (Haoujar 2022). Miicroalgae like *Tetraselmis*, *Nannochloropsis*, *Chaetoceros*, and *Chlorella* are cultured in controlled environments for their contributions to aquaculture and other industries (Bhambri et al 2023; Kumar et al 2023; Sarri et al 2024a). Microalgae play a crucial role in the early development of finfish, crustaceans, and mollusks, providing essential nutrients and serving as enrichment for live feed organisms such as *Rotifers* and *Artemia* (Anikuttan 2024). In addition to their role in aquaculture, microalgae produce about one-third of the Earth's oxygen and grow through photosynthesis, requiring sunlight, CO_2, water, and oxygen (Morales et al 2018). Also, algae can be cultured after isolation from their natural aquatic environments (Couto et al 2022). Several factors affect microalgae growth, including physico-chemical parameters like salinity, temperature, and pH (Rai & Rajashekhar 2014; Kumar & Thomas 2019). Growth mediums such as BG-11 are used to boost nutrient levels and enhance biomass production, while proper sanitation minimizes contamination risks (Tandon & Jin 2017; Sarri & Elp 2024).

Nannochloropsis sp. is recognized as a significant source of commercially valuable pigments. Key pigments identified in this microalga include chlorophyll-a (Hossain et al 2022), violaxanthin (Yin et al 2024), vaucheriaxanthin, and beta-carotene (Liu et al 2023). Given the potential of microalgae as a pigment source with broad applications in aquaculture and industry, numerous research efforts have focused on this organism (Silva et al 2020; Yusoff et al 2020; Sarri & Elp 2024; Sarri et al 2024b). Among the pigments found in microalgae, carotenoids and chlorophylls are particularly noteworthy due to their wide range of uses (Ambati et al 2019; Cezare-Gomes et al 2019; Yusoff et al 2020; Sarri et al 2024a). Specifically, chlorophylls present in *Nannochloropsis* sp. have been approved for use as food colorants in products such as ice cream and cold beverages (Koller et al 2014). Moreover, *Nannochloropsis* sp. is considered a promising photoautotrophic organism (Cheng-Wu et al 2021) within marine biotechnology due to its high photosynthetic efficiency and substantial lipid content, ranging from 30-70% (Li et al 2020; Ishika et al 2021). As a cosmopolitan species, *Nannochloropsis* can be easily cultured and thrives in both marine and freshwater environments (Chew et al 2018). The availability of essential nutrients plays a critical role in promoting higher biomass, which is necessary for sustaining growth and productivity in both microalgae and macroalgae (Long et al 2024; Magyar et al 2024). Key nutrients, including phosphorus, nitrogen, carbon dioxide, and trace elements, are vital for the growth of microalgae (Magyar et al 2024). Cultures of *Nannochloropsis* sp. in controlled environments utilize enriched nutrient media, such as BG-11, Conway, and other trace elements, to maximize productivity (Jazzar et al 2016; Khemiri et al 2022; Sanuddin et al 2023).

Acadian Marine Plant Extract Powder (AMPEP), derived primarily from the brown algae *Ascophyllum nodosum* (Hurtado et al 2009), is a protein extract commonly used in food, pharmaceuticals, and feed applications. It has also been applied in macroalgae culture, such as *Kappaphycus alvarezii*, for micropropagation and field cultivation (Hurtado & Critchley 2018; Satriani al 2022). Previous studies have tested the efficacy of AMPEP at concentrations of 0.01, 0.1, and 1.0 g L^{-1}, with immersion durations of 30 and 60 minutes over a three-month culture period in outdoor field conditions. These studies demonstrated improved growth responses at lower concentrations of AMPEP (Hurtado et al 2012), supporting earlier findings (Hurtado et al 2009) that lower concentrations of AMPEP combined with Plant Growth Regulators (PGRs) yield the best results in *K. alvarezii*. AMPEP has thus proven beneficial for macroalgae culture, enhancing growth and propagation. However, there has been limited research on the use of AMPEP in microalgae. Thus, this study aimed to provide baseline data on the potential of AMPEP as a growth promoter for microalga *Nannochloropsis* sp., evaluating its effects on cell density, growth response, and pigment accumulation at varying concentrations in the nutrient medium.

Material and Method

Culture condition of microalgae. Microalga *Nannochloropsis* sp. culture was conducted at the Ministry of Agriculture, Fisheries, and Agrarian Reform (MAFAR), Pangasinan, Bongao Tawi-Tawi, Philippines. 500 mL of improvised glass bottles were used in *Nannochloropsis* sp. culture. The BG-11 was utilized as a nutrient medium (Table 1 and Table 2). Different concentrations of AMPEP were added to the glass bottles with a nutrient medium, as shown in Table 3. The control experiment sample does not contain any AMPEP source. The experiment was done in triplicates. Each solution was autoclaved for 20 minutes at 121°C. After autoclaving, experimental samples were inoculated at an initial density of 1.22×10^5 cells mL^{-1}. Cultures were made with artificial lighting in the laboratory environment. Fluorescent lamps were used for lighting at 24 hour photoperiod. The ventilation of the cultures was carried out with an air motor, and syringe filters with an opening of 0.2 μ were used to prevent contamination (Figure 1). The air conditioner maintained a temperature of 20±1°C. Additionally, the study used a Completely Randomized Design (CRD) with treatments randomly assigned to experimental units. Measurements were taken every three days over a 24-day observation period.

Table 1

BG-11 nutrient medium (Erbil et al 2021)

Solution A	*For 500 mL*
$NaNO_3$	75.0 g
Solution B	*For 500 mL*
K_2HPO_4	2.0 g
$MgSO_4.7H_2O$	3.75 g
$CaCl_2.2H_2O$	1.80 g
Citric acid	0.30 g
Ammonium ferric citrate green	0.30 g
$EDTANa_2$	0.05 g
Na_2CO3	1.00 g

Table 2

Trace element composition

Trace element solution	*For 1,000 mL*
H_3BO_3	2.86 g
$MnCl_2.4H_2O$	1.81 g
$ZnSO_4.7H_2O$	0.22 g
$Na_2MoO_4.2H_2O$	0.39 g
$CuSO_4.5H_2O$	0.08 g
$Co(NO_3)2.6H_2O$	0.05 g

Table 3

Experimental groups and AMPEP concentration

Experimental groups	*Unit*	*AMPEP concentration*
Group A (Control)	mg L^{-1}	0
Group B	mg L^{-1}	25
Group C	mg L^{-1}	50
Group D	mg L^{-1}	100
Group E	mg L^{-1}	150

Figure 1. Experimental set-up. The glass bottles were enriched with different salinity concentrations (g L^{-1}) in a nutrient medium.

Growth response analysis. Each experimental samples of microalga *Nannochloropsis* sp. cultures were collected for cell counting and analysis every three days. A Neubauer hemocytometer was used to count cells daily under the light microscope, and contamination was checked daily visually. An analysis of the biomass of microalgae was conducted on a dry-weight basis. The dried weight of microalgae was determined by drying 5 mL of each experimental samples in an oven at 105°C for 2 hours. The specific growth rate (μ) was calculated by the formula below:

$$\mu = \frac{\ln(X_2) - \ln(X_1)}{t_2 - t_1}$$

Where:
X_2 - the final biomass cell number (t_2);
X_1 - the initial biomass cell number (t_1).

Pigment analysis. The pigment analysis followed the method of Durmaz & Erbil (2020). Spectrophotometric analysis of *Nannochloropsis* sp. was determined by the chlorophyll-a and total carotenoid levels, respectively. Moreover, in experimental culture, a test sample of 5 mL was centrifuged for 10 minutes at 3,500 rpm, and then the supernatants were removed. Afterward, the tubes were filled with 5 mL of methanol, then vortexed for 30 seconds to homogenize the sample. Finally, samples were remixed and centrifuged for 10 minutes at 3,500 rpm. Analyses of supernatants were performed by spectrophotometry. Calculations of the values read at 666 nm for chlorophyll-a, and 475 nm for total carotenoid in the spectrophotometer were made with the formulas given below.

Chlorophyll-a (μg mL^{-1}) = 13.9 A_{666} (Macıas-Sánchez et al 2005)

Where:
A_{666} - the absorbance of chlorophyll-a at a wavelength of 666 nm.

Total carotenoids (μg mL^{-1}) = 4.5 A_{475} (Zou & Richmond 2000)

Where:
A_{475} - the absorbance of carotenoids at a wavelength of 475 nm.

Statistical analysis. IBM SPSS software version 20 was used to analyze the collected data of cell densities, response growth, and pigment accumulation of *Nannochloropsis* sp. culture at $p<0.05$ significance level. Data were presented as mean±standard error of the mean (SEM). Determination of significant differences was computed through the One-way Analysis of Variance (ANOVA). Levene's Test was used to test for homogeneity of variance, and for post hoc comparisons, we used the Duncan Multiple Range Test (DMRT) to rank the treatment means (Hairol et al 2022; Sanuddin et al 2023).

Results

Cell density. The experimental group of *Nannochloropsis* sp. had an initial density of 1.22×10^5 cell mL^{-1} and was maintained for 24 days of culture. Five experimental groups were examined: group A (no AMPEP), group B (25 mg L^{-1} AMPEP), group C (50 mg L^{-1} AMPEP), group D (100 mg L^{-1} AMPEP), and group E (150 mg L^{-1} AMPEP). Figure 1 shows the cell number of *Nannochloropsis* sp. culture at different concentrations of AMPEP in a nutrient medium. Results revealed that the maximum cell density of group D reached $14.46 \pm 0.64 \times 10^6$ cell mL^{-1} and was significantly higher ($p<0.05$) than in groups A ($8.19 \pm 1.09 \times 10^6$ cell mL^{-1}), B ($9.69 \pm 1.30 \times 10^6$ cell mL^{-1}), C ($10.46 \pm 0.64 \times 10^6$ cell mL^{-1}), and E ($9.21 \pm 1.72 \times 10^6$ cell mL^{-1}) after 24 days of culture period. In addition, the mean cell density of group D reached $6.89 \pm 0.82 \times 10^6$ cell mL^{-1} and was statistically higher ($p<0.05$) than in groups A ($6.11 \pm 0.72 \times 10^6$ cell mL^{-1}), B ($6.56 \pm 0.57 \times 10^6$ cell mL^{-1}), C ($4.97 \pm 0.16 \times 10^6$ cell mL^{-1}), and E ($4.44 \pm 0.51 \times 10^6$ cell mL^{-1}) (Figure 2). Data

presented in the figures are means with standard errors (SE). Different lowercase letters in the figures indicate significant differences ($p<0.05$). Group A (no AMPEP), group B (25 mg L^{-1} AMPEP), group C (50 mg L^{-1} AMPEP), group D (100 mg L^{-1} AMPEP), and group E (150 mg L^{-1} AMPEP).

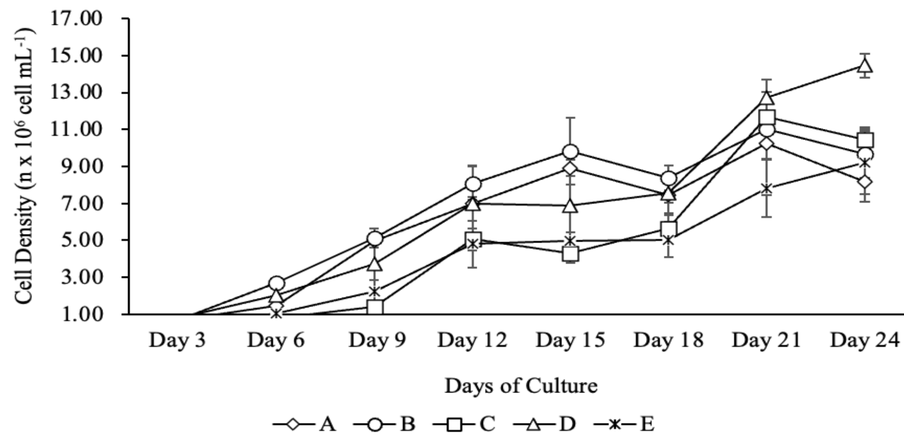

Figure 1. Cell density (n x 10^6, cell mL^{-1}) of *Nannochloropsis* sp. culture at different concentrations of AMPEP in a nutrient medium.

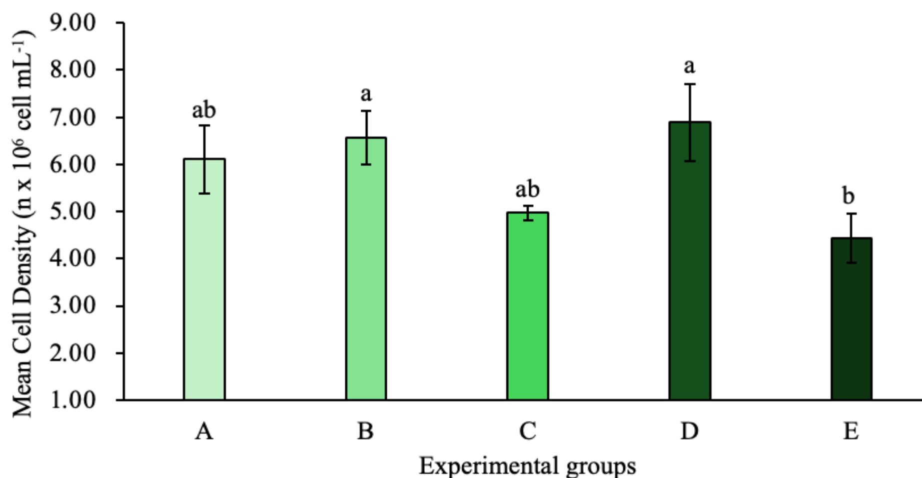

Figure 2. Mean cell density (n x 10^6, cell mL^{-1}) of *Nannochloropsis* sp. culture at different concentrations of AMPEP in a nutrient medium.

Specific growth rate and dry weight. The maximum specific growth rates (SGR) were obtained during the early three days of culture for all experimental samples. SGR of experiments A, B, C, D, and E were 0.55±0.08 day^{-1}, 0.54±0.09 day^{-1}, 0.42±0.09 day^{-1}, 0.58±0.11 day^{-1}, and 0.30±0.15 day^{-1}, respectively (Figure 3). As a result of the study, although not significantly different ($p>0.05$) from the other experimental groups, group D obtained a higher SGR (Figure 3). However, it was also revealed that, at twelve days of the culture period, significant differences were obtained, whereas the SGR in group C (0.43±0.04 day^{-1}) was significantly different ($p<0.05$) from that of other experimental groups. Additionally, in terms of mean values, group D obtained an SGR of 0.20±0.00 day^{-1}, which was significantly different $p<0.05$) than in other experimental groups (Figure 4). Moreover, the dry weight was obtained after the end of the culture period for *Nannochloropsis* sp. culture. It has been revealed in the present study that maximum dry weight was obtained for the group B (5.09±0.18 g L^{-1}) followed by the groups A (4.87±0.36 g L^{-1}), D (4.69±0.21 g L^{-1}), C (3.99±0.04 g L^{-1}), and E (3.92±0.15 g L^{-1}) (Figure 5).

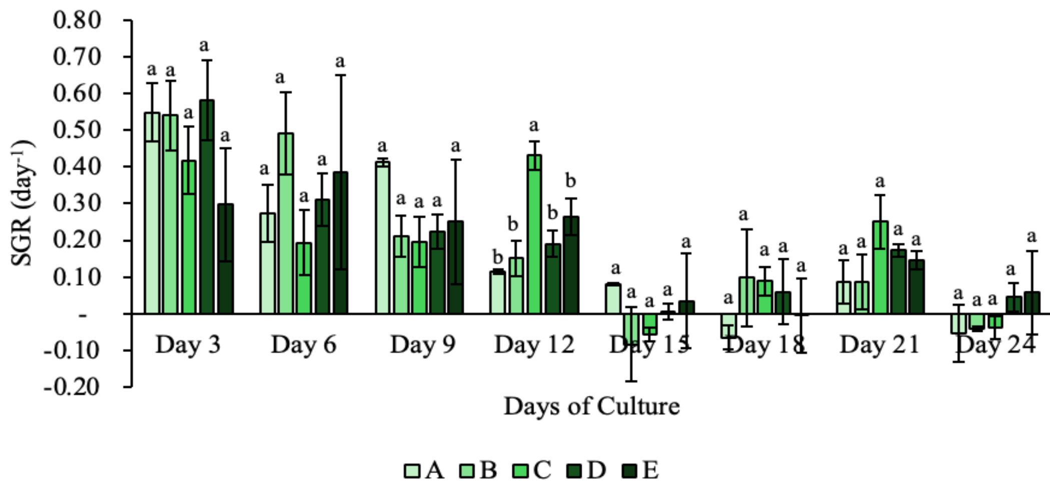

Figure 3. Specific growth rate (SGR, day^{-1}) of *Nannochloropsis* sp. culture at different concentrations of AMPEP in a nutrient medium.

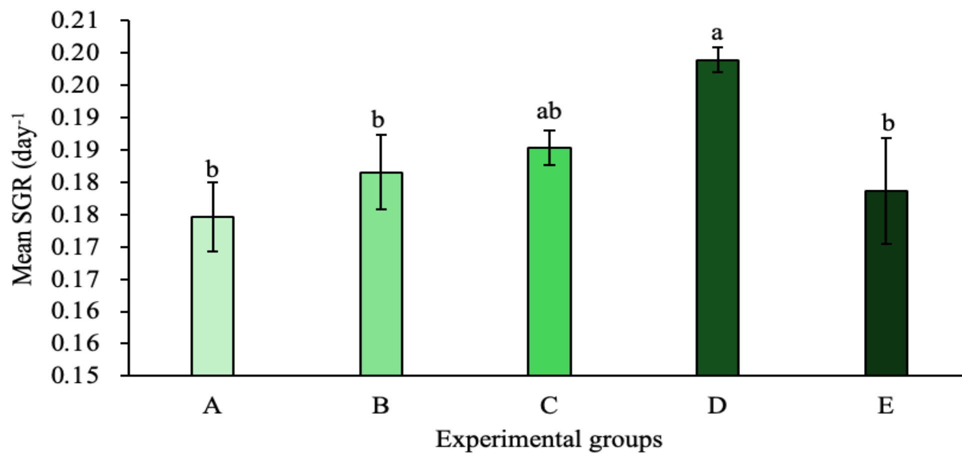

Figure 4. Mean specific growth rate (SGR, day^{-1}) of *Nannochloropsis* sp. culture at different concentrations of AMPEP in a nutrient medium.

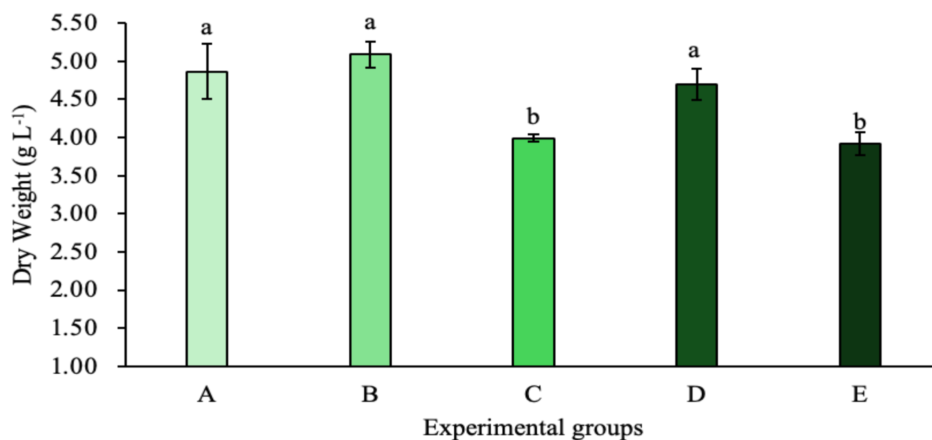

Figure 5. Dry weight (g L^{-1}) of *Nannochloropsis* sp. culture at different concentrations of AMPEP in a nutrient medium.

Chlorophyll-a pigment accumulation. Figure 6 shows the chlorophyll-a pigment accumulation of *Nannochloropsis* sp. culture at different concentrations of AMPEP in a nutrient medium. The measurement of chlorophyll-a pigment accumulation was done in

triplicates. The chlorophyll-a pigment accumulation values of groups A, B, C, D, and E were 5.88±2.41 μg mL^{-1}, 7.35±2.00 μg mL^{-1}, 6.89±1.63 μg mL^{-1}, 5.69±4.48 μg mL^{-1}, 10.89±4.29 μg mL^{-1}, respectively. Although no significant differences ($p>0.05$) were observed in all experimental groups, group E obtained higher chlorophyll-a pigment accumulation than the other group experiments. In addition, in terms of cellular chlorophyll-a pigment accumulation, the values in groups A, B, C, D, and E were 0.88±0.35 pg $cell^{-1}$, 0.74±0.11 pg $cell^{-1}$, 0.69±0.17 pg $cell^{-1}$, 0.38±0.30 pg $cell^{-1}$, 1.26±0.47 pg $cell^{-1}$, respectively. No significant ($p>0.05$) differences have been observed between the experimental groups. However, group E obtained a higher cellular chlorophyll-a pigment accumulation (Figure 7).

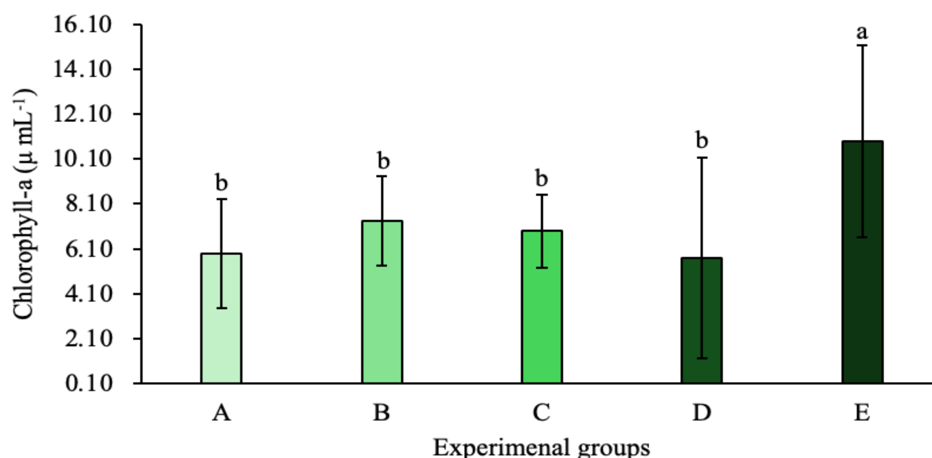

Figure 6. Chlorophyll-a pigment accumulation of *Nannochloropsis* sp. culture at different concentrations of AMPEP in a nutrient medium.

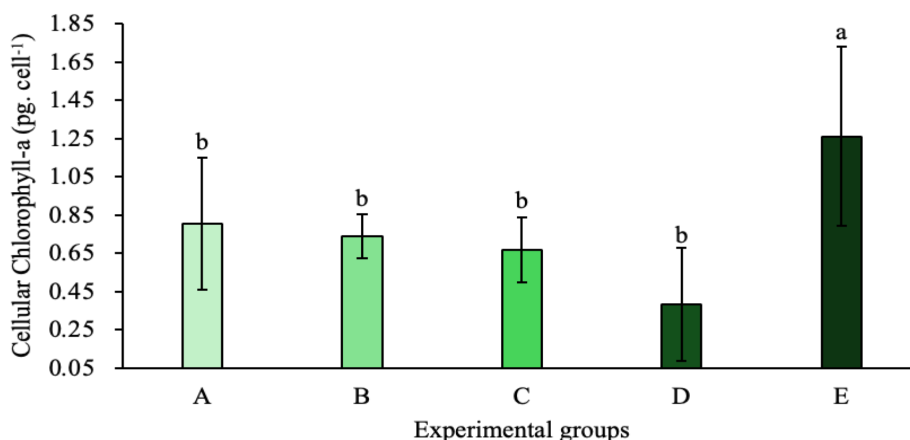

Figure 7. Cellular chlorophyll-a pigment accumulation of *Nannochloropsis* sp. culture at different concentrations of AMPEP in a nutrient medium.

Total carotenoid pigment accumulation. Figure 8 shows the total carotenoid pigment accumulation of *Nannochloropsis* sp. culture at different concentrations of AMPEP in a nutrient medium. It has been revealed in the present study that the total carotenoid pigment accumulation values in groups A, B, C, D, and E were 2.13±1.03 μg mL^{-1}, 3.22±1.07 μg mL^{-1}, 2.89±1.06 μg mL^{-1}, 1.51±1.30 μg mL^{-1}, 3.12±0.83 μg mL^{-1}, respectively, with no significant differences ($p>0.05$) between the experimental groups. In addition, in terms of cellular total carotenoid pigment, the accumulation values for the groups A, B, C, D, and E were 0.30±0.15 pg $cell^{-1}$, 0.34±0.05 pg $cell^{-1}$, 0.29±0.12 pg $cell^{-1}$, 0.10±0.09 pg $cell^{-1}$, 0.37±0.11 pg $cell^{-1}$, respectively. No significant differences ($p>0.05$) were observed between the experimental groups (Figure 9).

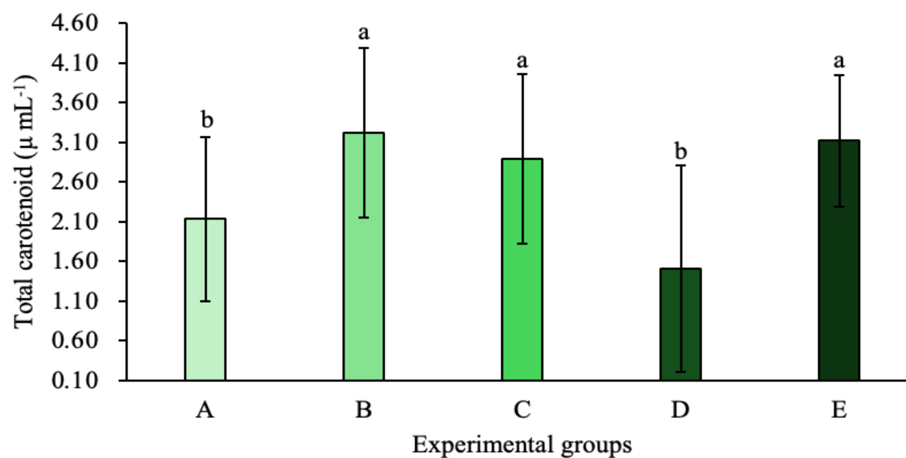

Figure 8. Total carotenoid pigment accumulation of *Nannochloropsis* sp. culture at different concentrations of AMPEP in a nutrient medium.

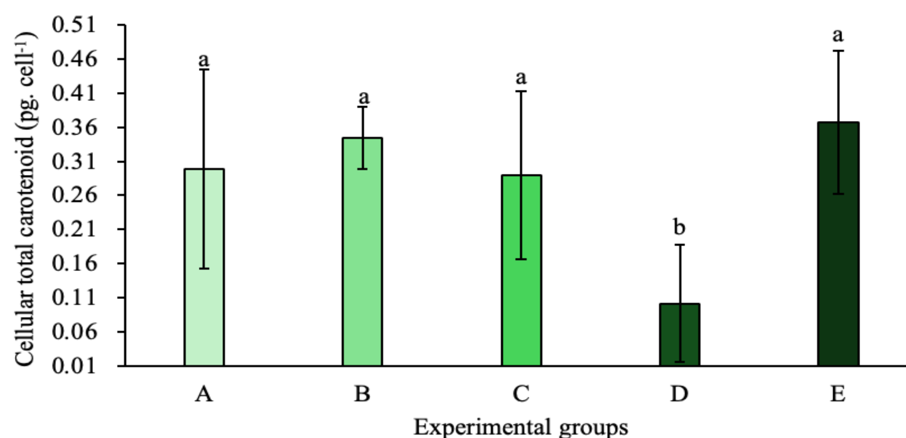

Figure 9. Cellular total carotenoid pigment accumulation of *Nannochloropsis* sp. culture at different concentrations of AMPEP in a nutrient medium.

Discussion. The primary objective in microalgae cultivation is to maintain high cell density in continuous culture systems, as this is essential for efficient reproduction. Continuous culture promotes faster and more effective cell proliferation, with nutrients playing a key role in influencing microalgae growth. In this study, the effect of different concentrations of AMPEP on the growth of *Nannochloropsis* sp. cultures was investigated to better understand its impact on enhancing cell density and pigments, as well as overall growth performance. According to the results of this study, AMPEP addition to the culture medium of *Nannochloropsis* sp. culture provides higher cell densities. The 100 mg L^{-1} AMPEP in the nutrient medium reached 1.77-fold higher cell densities than the control group for the *Nannochloropsis* sp. culture. Various organic and inorganic promoters have been used in microalgae culture, and it has been determined that they induce improvements in culture parameters. For example, the 500 mg L^{-1} concentration of myo-inositol in the nutrient medium revealed a cell density 1.28-fold higher than in the control group for the *Nannochloropsis oculata* microalgae culture (Erbil & Durmaz 2020). Additionally, a study demonstrated that the addition of 500 mg L^{-1} of myo-inositol to a microalga *Dunaliella salina* culture resulted in a 1.4-fold increase in cell density compared to the control group (Cho et al 2015). In line with those findings, our research showed that the introduction of AMPEP into the culture medium significantly enhanced the cell density of microalga *Nannochloropsis* sp. culture.

The specific growth rate (SGR) of *Nannochloropsis* sp. culture in a 100 mg L^{-1} AMPEP concentration in the nutrient medium obtained a higher SGR, at 0.58 day^{-1} for the first three days of the culture period, than in the control group; however, it decreased after 24 days of the culture period. In a study conducted by Erbil et al (2021), the use of

BG-11 medium resulted in an SGR of 0.08 day^{-1} for microalga *Chlorella* sp. culture, which is lower compared to the SGR observed in all experimental groups of the present study. Furthermore, the SGR values obtained in our experimental groups surpass those reported by Liu et al (2021), who investigated the effects of phosphate and iron sources in a nutrient medium and achieved an SGR of 0.29 day^{-1} for microalga *Chlorella vulgaris* cultures. Consequently, the incorporation of AMPEP into the BG-11 medium in the current study significantly enhanced the SGR of microalga *Nannochloropsis* sp. cultures, indicating a notable improvement in growth performance.

The biomass yield of *Nannochloropsis* cells cultured with AMPEP was assessed by measuring the dry weight after 24 days of cultivation. The addition of 25 mg L^{-1} AMPEP to the nutrient medium resulted in a higher dry weight of 5.09 g L^{-1} compared to the control group. This biomass yield is notably higher than those typically observed in open raceway pond systems, where dry weights generally range from 0.1 to 0.5 g L^{-1}, with a maximum of 1.4 g L^{-1} reported by Ketheesan & Nirmalakhandan (2012), Ashokkumar et al (2014), and Kumar et al (2015). The results of the present study are comparable to those obtained by Durmaz & Erbil (2020), who cultivated *N. oculata* in fiberglass-reinforced plastic panel photobioreactors enriched with f/2 medium, achieving a dry weight of 0.81 g L^{-1}. These findings suggest that the incorporation of lower AMPEP concentrations in the nutrient medium can significantly enhance the biomass production of *Nannochloropsis* cultures. Consequently, the use of BG-11 medium supplemented with AMPEP resulted in an increased dry weight of *Nannochloropsis* sp. cultures in the current study.

Pigment accumulation analysis revealed that chlorophyll-a levels reached 10.89 μg mL^{-1} with the addition of 150 mg L^{-1} AMPEP, which was higher than in the other experimental groups and control. Comparatively, previous research by Behzadi Tayemeh et al (2020) reported that the use of a Bold Basal medium resulted in a chlorophyll-a accumulation of 0.7 μg mL^{-1} in *Chlorella vulgaris* cultures. Additionally, Yun et al (2019) found that a BG-11 medium produced a chlorophyll-a level of 9.38 μg mL^{-1} for microalga *Chlorella vulgaris*. Both of these results were lower than those obtained in the current study, where the inclusion of 150 mg L^{-1} AMPEP in BG-11 medium enhanced chlorophyll-a accumulation in microalga *Nannochloropsis* sp. cultures. Moreover, Erbil & Durmaz (2020) reported that the addition of 500 mg L^{-1} myo-inositol to a nutrient medium resulted in a chlorophyll-a accumulation of 6.15 μg mL^{-1} microalgae in the *N. oculata* cultures, which is also lower than the levels observed in the present study with 150 mg L^{-1} AMPEP. These findings indicate that the supplementation of BG-11 medium with AMPEP significantly increased the chlorophyll-a accumulation in *Nannochloropsis* sp. cultures.

A substantial accumulation of total carotenoids, reaching 3.22 μg mL^{-1}, was observed at a lower AMPEP concentration of 25 mg L^{-1} in the nutrient medium for *Nannochloropsis* sp. cultures. This value exceeds those reported in other research studies. For instance, Yun et al (2019) demonstrated that a BG-11 medium produced a total carotenoid content of 11.74 μg mL^{-1} in *Chlorella vulgaris* cultures. Similarly, Pandit et al (2017) recorded a total carotenoid concentration of 22.0 μg mg^{-1} in *Chlorella vulgaris* grown in a BG-11 medium. Both studies reported carotenoid levels lower than those achieved in the current investigation, where 25 mg L^{-1} AMPEP supplementation led to enhanced carotenoid accumulation in *Nannochloropsis* cultures. Additionally, Erbil & Durmaz (2020) found that adding 100 mg L^{-1} myo-inositol to a nutrient medium resulted in a total carotenoid accumulation of 11.93 μg mL^{-1} in microalga *N. oculata* cultures. This value is also lower than the 3.22 μg mL^{-1} observed in the present study with 25 mg L^{-1} AMPEP supplementation. These findings highlight the significant impact of AMPEP on boosting total carotenoid production in *Nannochloropsis* sp., indicating its potential as a powerful growth enhancer in microalgal cultures. The elevated carotenoid levels achieved with AMPEP supplementation suggest that optimizing AMPEP concentrations can improve pigment production, thus offering a promising strategy for enhancing the commercial viability of *Nannochloropsis* cultures.

Conclusions. Nutrients have been used to enhance the growth and pigmentation of *Nannochloropsis* sp. cultures. The present study investigated the use of AMPEP concentrations in the production of microalga *Nannochloropsis* sp. culture. As a result of

this study, 100 mg L^{-1} AMPEP in a nutrient medium significantly increased cell density while 50 mg L^{-1} AMPEP improved specific growth rate. Additionally, the maximum dry weight was obtained in 25 mg L^{-1} AMPEP concentration. Moreover, a higher concentration of AMPEP enhanced chlorophyll-a pigment accumulation, while 25 mg L^{-1} AMPEP improved total carotenoid pigment accumulation in *Nannochloropsis* sp. culture. Hence, incorporation of AMPEP concentration in microalga *Nannochloropsis* sp. culture may improve growth and pigment accumulation.

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Conflict of interest. The authors declare no conflict of interest.

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