HAYATI
Journal of Biosciences

Vol. 33 No. 5, September 2026 1208-1223
DOI:10.4308/hjb.33.5.1208-1223
ISSN: 1978-3019
EISSN: 2086-4094

Research Article

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Hormone-Enriched Culture of *Chlorella* sp. as a Practical Strategy to Improve the Nutritional Quality and Survival of *Artemia*

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ARTICLE INFO

Article history:
Received February 27, 2026
Received in revised form May 20, 2026
Accepted June 2, 2026
Available Online July 1, 2026

KEYWORDS:
Artemia,
Enrichment,
Local Microalgae,
Survival rate

ABSTRACT

Artemia sp. is widely used as a live feed in the larval culture of both marine and freshwater species. Inconsistent nutritional content can be overcome by enriching microalgae. This study aimed to evaluate the growth and quality of local microalgae as a source of enrichment to improve the quality of *Artemia*. This study used a randomized block design (RBD) with three replications. The microalgae used were a collection of isolates from the Fisheries Biology Laboratory, UBT. The research procedure consisted of 1) identification of microalgae based on cell morphology using a Labomed LX400 microscope and Scanning Electron Microscope (SEM), 2) microalgae culture with the addition of IAA (auxin), BAP (cytokinin), and a combination of both hormones, and 3) *Artemia* enrichment with cultured microalgae with soaking times of 3, 6, 9, and 12 h. Data were analyzed using analysis of variance (ANOVA), followed by Tukey's test. The results showed that single treatment or a combination of IAA and BAP had no significant effect on cell density, protein, and carbohydrate content of microalgae, but lipid content increased significantly with IAA + BAP treatment. Enrichment with *Chlorella* sp. s significantly increased protein, carbohydrate, lipid content, and the survival rate of *Artemia* increased by 33.42% after 12 h of soaking.

1. Introduction

Larval culture is a critical phase in aquaculture because it strongly influences seed quality and availability, survival rate, and overall production efficiency in hatchery systems (Herath & Atapaththu 2013; Khanjani *et al.* 2024). During early developmental stages, larvae experience rapid morphological and physiological changes, making them highly dependent on nutritionally adequate feed. Consequently, the demand for high-quality larval feed in hatcheries continues to increase to accelerate growth, enhance survival, and reduce physiological stress. At this stage, natural feed (live food) is generally preferred over artificial diets because it provides endogenous digestive enzymes, stimulates larval feeding behavior, and improves feed utilization efficiency (Das *et al.* 2007; Zeng *et al.* 2018). Despite advances in formulated feeds, artificial diets cannot yet fully replace live food during the early larval stages, particularly during the critical transition period from endogenous to exogenous feeding (Silva *et al.* 2004; Sserwadda 2024).

Among various live food organisms, *Artemia* spp. are widely used in marine and freshwater hatcheries due to their suitable size, high palatability, ease of digestion, and active swimming behavior, which effectively stimulate larval feeding response (Sorgeloos *et al.* 2001; Teye-gaga *et al.* 2025). *Artemia* also functions as a biological vector capable of filtering and accumulating nutrients

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from the surrounding water, making it an ideal candidate for nutritional enrichment. However, native *Artemia nauplii* have inherent nutritional limitations, particularly low levels of highly unsaturated fatty acids (HUFA) and inconsistent biochemical composition, which may compromise larval growth and survival if used without enrichment (Carter *et al.* 2015; Teye-gaga *et al.* 2025). Therefore, enrichment strategies are essential to improve the nutritional quality of *Artemia* before its use as larval feed.

Microalgae are among the most promising enrichment sources for *Artemia* because they are rich in essential nutrients, including proteins, lipids (PUFAs such as EPA), carbohydrates, vitamins, pigments, bioactive compounds, and immunostimulants (Andrade 2018; Gui et al. 2022; García-encinas et al. 2025). These biochemical components play crucial roles in larval development, and early growth processes. Numerous studies have demonstrated that nutritional enrichment of live food can improve its biochemical quality, particularly fatty acid composition, thereby enhancing growth performance, survival, and overall larval quality in fish and crustacean aquaculture (Faulk *et al.* 2005; Thai et al. 2014; Martínez Soler et al., 2023).

Chlorella and *Nannochloropsis* are widely utilized in aquaculture owing to their high nutritional value, including high protein content, favorable lipid composition, and rich polyunsaturated fatty acid (PUFA) profiles (Roy & Pal 2015; du Preez *et al.* 2021; Ma & Hu 2023). These characteristics make them suitable candidates for *Artemia* enrichment and for improving the nutritional quality of live feed used in larval rearing. For instance, larvae fed with *Artemia* enriched with *Chlorella* sp. exhibited higher specific growth rates and survival, accompanied by significant upregulation of growth-related genes, indicating improved physiological performance (Joshua *et al.* 2024). Nevertheless, most existing studies rely on commercial microalgal strains or stress-based cultivation approaches to enhance biochemical composition, which often result in trade-offs between biomass production and nutritional quality.

Manipulation of microalgal physiology using plant growth regulators (PGRs) is a promising approach to optimize both cell density and biochemical composition prior to *Artemia* enrichment. PGRs act at low concentrations (Davies 2004) and regulate key physiological processes in microalgae (Han *et al.* 2018; Strik & Van Staden 2020). Recent studies have demonstrated that the application of indole-3-acetic acid (IAA) and 6-benzylaminopurine (BAP), either individually or in combination, can significantly enhance microalgal biomass and lipid productivity (Han *et al.* 2018; Lin *et al.* 2020; Singh *et al.* 2020). IAA supplementation has been shown to enhance biomass productivity, photosynthetic efficiency, lipid accumulation, and PUFA content in *Dunaliella salina* cultures, indicating its potential as a physiological regulator for improving microalgal biomass quality (Seemashree *et al.* 2022). Similarly, Singh *et al.* (2024) reported that exogenous IAA application under nitrogen-limited conditions significantly increased biomass and lipid production in microalgae. Meanwhile, BAP, a cytokinin, promotes cell division and photosynthetic pigment accumulation, resulting in higher cell density and lipid content in green microalgae, both when applied alone and in combination with auxins (Han *et al.* 2018; Singh *et al.* 2020).The combination of IAA and BAP leverages complementary signaling pathways, resulting in synergistic improvements in growth, lipid biosynthesis, and stress tolerance (Sing *et al.* 2020). This approach holds promise for maximizing microalgal productivity in biotechnological applications (Singh *et al.* 2020). Despite these promising findings, the application of phytohormone-induced microalgae for *Artemia* enrichment and its subsequent effects on *Artemia* quality have not been sufficiently investigated. Local microalgae isolated from Kakaban Lake have been reported to exhibit strong potential as a natural feed source for *Artemia* due to their tolerance to salinities of 24–30 ppt and adaptability to local environmental conditions (Kartina *et al.* 2024), offering economic advantages over commercial microalgal products. However, the nutritional value of microalgal biomass, particularly lipid accumulation, is highly dependent on cultivation conditions, as conventional culture systems primarily promote cell proliferation rather than the synthesis of energy-dense compounds required for effective *Artemia* enrichment. However, the use of locally isolated microalgae as *Artemia* enrichment cultured under phytohormonal regulation remains poorly explored, and the effects of combining growth optimization and nutritional enhancement are presently unclear. Most previous studies have focused on commercially important microalgae and on stress-based cultivation strategies, such as nutrient limitation or high-light exposure, to enhance lipid and carotenoid accumulation. Although these approaches can increase the production of target metabolites, they often inhibit cell growth and photosynthetic activity, resulting in lower biomass and protein productivity (Shokravi *et al.*

2022; Figueroa-Torres *et al.* 2021; Song *et al.* 2022). In contrast, limited studies have explored the use of plant growth regulators, such as Indole Acetic Acid (IAA) and 6-Benzylaminopurine (BAP), as an alternative approach to improve microalgal growth and nutritional quality, particularly for live feed-oriented microalgae production.

Therefore, this study presents a novel approach by applying the Phytohormonal Algal Cultivation System (PACS) to optimize the growth and nutritional quality of local microalgae isolates from Kakaban Lake and subsequently evaluating their effectiveness as a bio-enrichment source for *Artemia*. Previous studies have focused on larval enrichment methods using Moina (Rasdi *et al.* 2021) and different microalgal species (Yilman *et al.* 2021; Joshua *et al.* 2024) or enrichment through microalgal supplementation in commercial feeds or bioencapsulation (Supamattaya *et al.* 2005; Saputra *et al.* 2025). Joshua *et al.* (2024) examined the potential of *C. vulgaris* as a dietary supplement to improve the performance and immune response of *T. tombroides* larvae, with positive results.

This study aimed to optimize the growth and quality of local microalgae using the Phytohormonal Algal Cultivation System (PACS) with IAA and BAP. The best-performing cultures were applied as a nutrient source for *Artemia* sp. via bio-enrichment, followed by evaluation of the quality of the enriched nauplii. Microalgae isolates were morphologically characterized using microscopy. This study is the first to report the use of hormone-treated local microalgae to enhance growth and nutritional value, and to apply them as a natural feed to improve primary metabolites and the survival of *Artemia* sp. The results provide new insights into phytohormone-assisted microalgae cultivation and offer a practical, sustainable strategy for improving live feed quality in aquaculture hatchery systems.

2. Materials and Methods

2.1. The Material and Equipment

The equipment used includes a Labomed LX400 microscope and camera, a Scanning Electron Microscope (SEM) with a Dual-Beam FIB Aquilos2 from ThermoFisher, a spectrophotometer (Thermo Orion Aquamate 8100), a centrifuge, and a Gas Chromatography-Mass Spectrometry (GC-MS) system. The sample (isolate KK6) was obtained from the Biology Fisheries Laboratory of the Faculty of Fisheries and Marine Sciences, Universitas Borneo Tarakan, Indonesia. The sample was maintained in a 1,000 mL conical flask using sterilized Walne Medium. Plant growth regulators Indole-3-Acetic Acid (IAA) as Auxin and 6-Benzylaminopurine (BAP) as cytokinin were purchased from MEBEP Bioscience. Stock solutions of plant growth regulators were prepared.

2.2. Method

This study was experimental research using a randomized block design (RBD), with research procedures consisting of 1) identification of microalgae, 2) microalgae culture with the addition of IAA (auxin) 10 ppm, BAP (cytokinin) 10 ppm, and a combination of both hormones in walne medium, with three replications respectively. 3) *Artemia* enrichment with cultured microalgae with soaking times of 3, 6, 9, and 12 h with three replications.

2.2.1. Identification of Local Microalgae

Local microalgae were identified using a Labomed LX400 microscope with a camera and a Scanning Electron Microscope (SEM) with a Dual-Beam FIB Aquilos2 (ThermoFisher), equipped with a field-emission source (FEG) and operated at room temperature (±25-26°C). SEM procedure: The sample was placed on a glass slide on top of the SEM stub and attached using carbon double-sided tape. To increase the surface conductivity, a thin layer of platinum (~5 nm) was sprayed onto the sample for 20 s at a current of 30 mA using an integrated retractable sputter coater. Imaging was performed using an electron beam with a current of 25 pA and a voltage of 5 kV, with a working distance of 9-16 mm. This setup ensured high-resolution observation without the need to remove the sample.

2.2.2. Culture of Microalgae

The inoculum microalgae sample was inoculated in f/2 Walne medium (Peter R. Walne 1966) with an initial concentration of ±0.350 ($OD_{680\ nm}$), at room temperature (25-26°C), 24:0 day/light photoperiod (4,500-6,000 lux), and continuous aeration until it reached the late exponential (log) phase. The treatments used in this study were Indole-3-Acetic Acid (IAA) and Benzylaminopurine (BAP) in powder form. Culture Treatment consisted of control (without adding hormones), IAA 10 ppm, BAP 10 ppm, and IAA 10 ppm + BAP 10 ppm with 3 repetitions. The culture was harvested by centrifugation at 5,000 rpm (rpm) for 15 min (Converti *et al.* 2009). The pellet was washed twice with autoclaved distilled water to remove excess salts from the media (Cha *et al.* 2011). The harvested *Chlorella* sp. was maintained at 20°C for further analysis (Joshua *et al.* 2024). Sampling was performed daily to determine the growth and pigment content of the cells. The number of samples was taken in 5 ml. Sampling was carried out for 13 days, starting from Day-0 to Day-13.

2.2.3. Cell Density Measurement

Optical Density (OD) was calculated daily during the study. Samples were collected at 2 mL per treatment, sterilely in a Laminar Air Flow (LAF) environment, and placed in a ±3 mL microtube. The growth base optical density (OD) was measured using a Thermo Spectrophotometer, Orion Aquamate 8100 (type and brand) at a wavelength of 680 nm. The samples were placed in a cuvette, and OD was measured by spectrophotometry (Hakim *et al.* 2023).

2.2.4. Pigment Content (Chlorophyll *a*, *b* and Carotenoid)

5 mL of the sample was centrifuged at 4,000 rpm for 5 minutes. The pellets were mixed with 2 mL of ethanol, wrapped in aluminum foil, and incubated at 4°C for 24 hours. The mixture was homogenized and centrifuged at 4,000 rpm for 10 minutes. The extract was measured at wavelengths of 470, 665, and 649. Chlorophyll contents and total carotenoids were calculated using the formula (Zu *et al.* 2021):

The concentration of total chlorophyll is calculated from the following equations:

A. Concentration of Chlorophyll*a* (μg mL^{-1}) = (13.95 × A665) – (6.88 × A64)

B. Concentration of Chlorophyll*b* (μg mL^{-1}) = (24.96 × A649) – (7.32 × A665)

Total Chlorophyll concentration = A + B

C. Carotenoid (μg mL^{-1}) = (1,000 × OD (470 nm) - 2.05 × Chl *a* – 114.8 x chl *b*): 245

2.2.5. Biomass Metabolites Content

Microalgae protein assay method using a spectrophotometer (Bradford method). A total of 5 ml of culture was centrifuged at 4,000 rpm for 5 min. The pellet was added to 4 mL of Biuret, shaken, and sonicated at 40 Hz for 5 min. The samples were incubated for 30 min and centrifuged again at 4,000 rpm for 5 min. The supernatant was analyzed at a wavelength of 550 nm. The absorbance value obtained was plotted against the Bovine Serum Albumin (BSA) standard (R = 0.944).

Carbohydrate measurements were performed on day 13. First, carbohydrate levels were calculated using the phenol-sulfuric acid (H_2SO_4) method. Samples (5 mL) were collected under sterile conditions and placed in a 15 mL tube. The tube was centrifuged at 4,000 rpm for 10 min, and then the supernatant was separated. Subsequently, 1 mL of concentrated sulfuric acid (H_2SO_4) and 0.5 mL of 5% phenol were added to the pellet-containing sample. The tubes were then incubated at room temperature for 30 min. The sample was then measured using spectrophotometry at a wavelength of 490 nm, and the absorbance was determined. D-Glucose was used as the standard solution.

Total lipid content was determined on the final day (D13) of cultivation using the modified Bligh and Dyer extraction method (Bligh & Dyer 1959; Hakim *et al.* 2023). Briefly, 5 mL of sample centrifuged at 4,000 rpm for 10 min, and the supernatant was discarded. The pellets were resuspended in a mixture of 3 mL methanol and chloroform, and homogenized using a vortex mixer. An additional 1 mL chloroform and 1 mL distilled water were added, and the mixture was vortexed before centrifuge again. Lower chloroform phase containing the extracted lipids. The chloroform layer was carefully collected for lipid quantification. The lipid content was calculated using the following formula:

$$\text{Lipid content} = \frac{\text{Dt - D0}}{\text{V}}$$

Where:

Dt : Petri dish and sample (Lipid) weight after drying (Day-t) (mg)

D0: Initial weight of Petri dish (mg)

V : Sample volume (mL)

2.2.6. Biomass Total

Biomass calculation was performed on the final day (Day 13). A total of 10 mL of sample was collected in a sterile manner and placed in microcentrifuge tubes. The microtube to be used was weighed to determine its empty weight, and the sample was then centrifuged at 3,300 rpm for 10 min until the pellets and supernatant were distinguishable. The supernatant was collected using a micropipette (Hakim *et al.* 2023). The remaining pellets were then dried in an oven at a temperature of 40°C for 24 h. Biomass content was calculated using the following formula:

$$\text{Biomass} = \frac{\text{Dt - D0}}{\text{V}}$$

Where:
Dt : tube and sample (biomass) weight after drying (Day-t) (mg)
D0: Initial weight of tube (mg)
V : Volume sample (mL)

2.2.7. Preparation of *Artemia* sp.

The cysts of the brine shrimp *Artemia* sp. from O.S.I. Brand Pro 80™ (Ocean Star International, Inc., USA) was used in this study. The *Artemia* cysts were decapsulated according to the method described by Sorgeloos *et al.* (1977) and were hatched following the methods described by Sorgeloos *et al.* (1993) using autoclaved seawater at a temperature of 24-26°C, salinity of 25 ppt, and oxygen levels near saturation, which was aerated from the bottom and illuminated under light for 24 h. The hatching bottle was left undisturbed for 5 min to allow the nauplii to concentrate at the bottom. The nauplii were harvested using a siphon, collected on a 100 µm mesh scoop net, and washed with filtered and autoclaved seawater before enrichment. Freshly hatched nauplii were referred to as unenriched *Artemia*.

2.2.8. Enrichment of *Artemia* sp. with Microalgae

The refrigerated *Chlorella* sp. was allowed to return to ambient temperature of 25°C (Habib *et al.* 2003) before feeding to *Artemia nauplii*. The nauplii were kept at a density of ±200,000 individuals per liter of autoclaved seawater and enriched for 3, 6, 9, and 12 h (Radhakrishnan *et al.* 2020) with 3 repetitions. The enrichment bottles were set up, and the microalgae concentration of 3×10^5 cells/mL was added to autoclaved seawater under continuous aeration before the nauplii were transferred. After enrichment, the enriched *Artemia* were harvested using a siphon, collected with a 100 µm mesh scoop net, and washed with autoclaved distilled water.

2.2.9. Survival Rate (%) *Artemia* sp.

The survival rate (%) of the larvae in every treatment was calculated according to the following formula:

$$\text{Survival Rate (\%)} = \frac{\text{Nt}}{\text{N0}} \times 100\%$$

Where:
Nt : number of final Nauplii
N0: number of initial Naupli

2.3. Analysis of the Primary Metabolite Content of *Artemia* sp. nauplii.

Total lipids were analyzed using the Soxhlet method, total carbohydrates using the Clegg Antrone method, and protein content using the Kjeldahl method.

2.4. Data Analysis

Data were visualized using Microsoft Excel, and a heatmap was conducted using metaboAnalyst.ca. The overall effect of all treatments was analyzed using a repeated-measures one-way ANOVA (analysis of variance) using IBM SPSS. Next, multiple comparisons were analyzed using Tukey's HSD test to assess differences between treatment means at the 95% confidence level.

3. Results

3.1. Characterization of Microalgae using Microscopy Method

Microscopic and SEM observations revealed unicellular and colonial spherical cells measuring approximately 3–5 µm, with thick green cell walls (Figure 1A and B). The morphological characteristics of the isolates were similar to those of the genus *Chlorella* sp. *Chlorella* is a unicellular microalgae with a spherical green shape and a cell size range of 2-10 µm. They generally have one cup-shaped chloroplast, with or without a pyrenoid. They have smooth surfaces and strong cellulose walls. The cell wall appeared thick and compact at high magnification (Figure 1D). However, species-level identification requires additional confirmation through molecular analysis, such as 18S rRNA or ITS gene sequencing.

3.2. Microalgae Cell Density in IAA and BAP Enriched Media

In this study, microalgae were cultured using a Phytohormonal Algae Cultivation System (PACS). The treatments used Indole-3-Acetic Acid (IAA), an Auxin, and Benzylaminopurine (BAP), a cytokinin. The following are the growth and quality results of the microalgae cultured over 13 days of observation.

Based on the microalgal cell density curve over 13 days of culture (Figure 2), the BAP treatment showed faster growth from the first day of culture, a shorter lag phase, and continued to increase, reaching the exponential phase by day 8. However, at the end of the

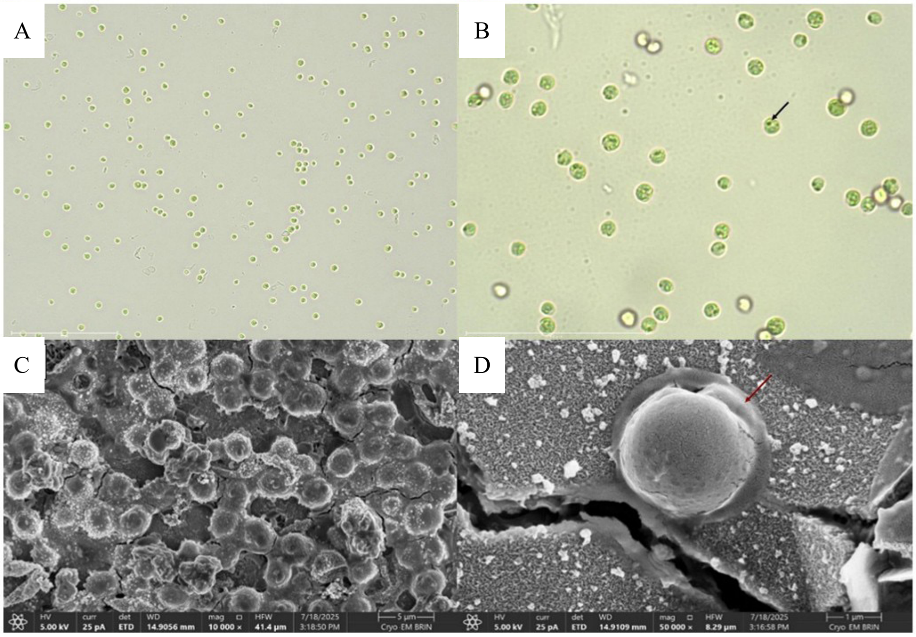

Figure 1. Microalgae cells under a microscope: (A) 100x magnification, (B) 400x magnification. Scanning electron microscopy (SEM) appearance: (C) 10,000 × magnification, and (D) 50,000 × magnification

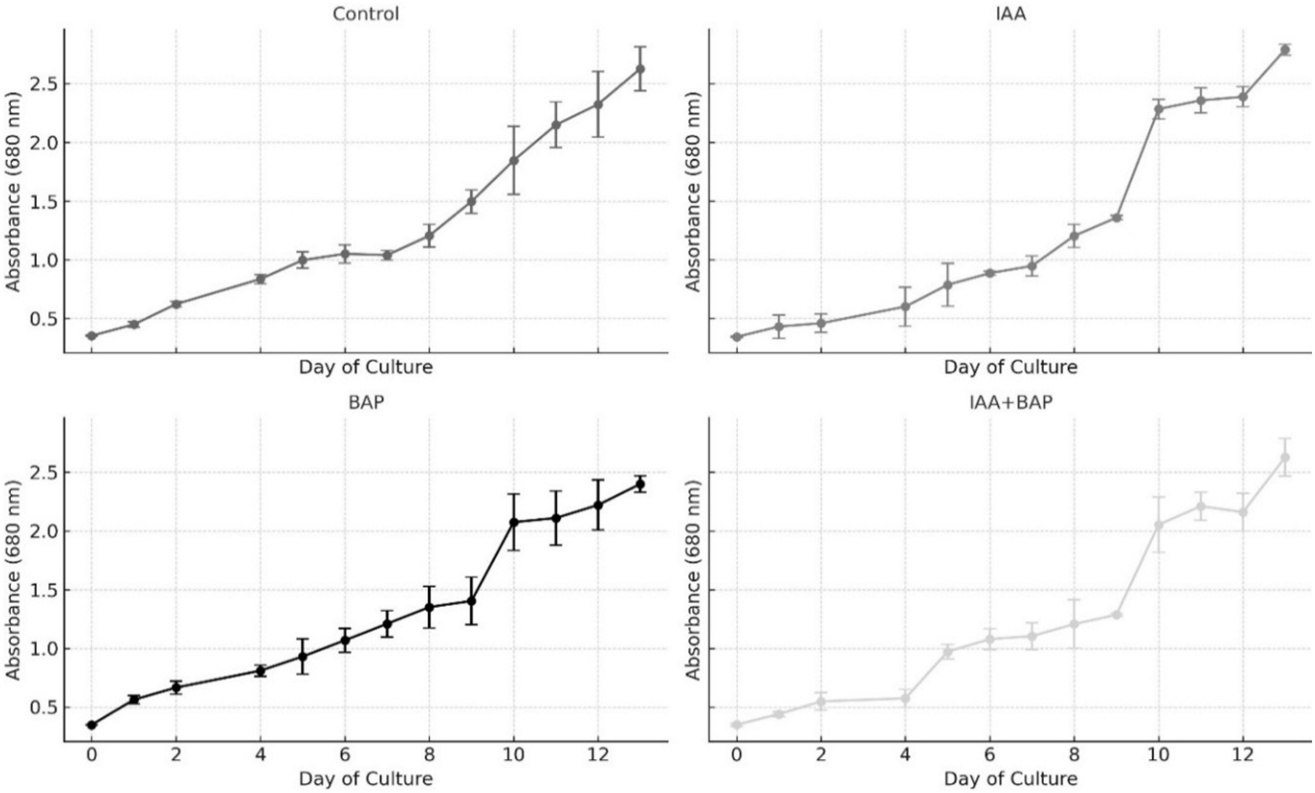

Figure 2. Cell density was measured by spectrophotometer (680 nm) for 13 days of culture. Data were means ± SD (n = 3). There were no significant differences between treatments, as determined by one-way ANOVA ($p>0.05$)

observation period, BAP treatment produced growth values with the lowest cell density. The IAA and IAA + BAP treatments required a longer adaptation period, and growth was slow until day 4. However, it continued to increase until day 5.

3.3. Pigment Content (Chl *a*, *b*, and Carotenoid)

The chlorophyll pigment in microalgae is the primary agent in harvesting light energy for photosynthesis, enabling them to produce energy and nutrients for growth. Based on the results of the analysis of variance, the final total chlorophyll or carotenoid content value did not show significantly different results (P>0.05) (Figure 3).

The daily trend of the total chlorophyll increase showed a similar pattern (Figure 3A) with no significant differences. The chlorophyll content continued to increase during the exponential phase of the cell

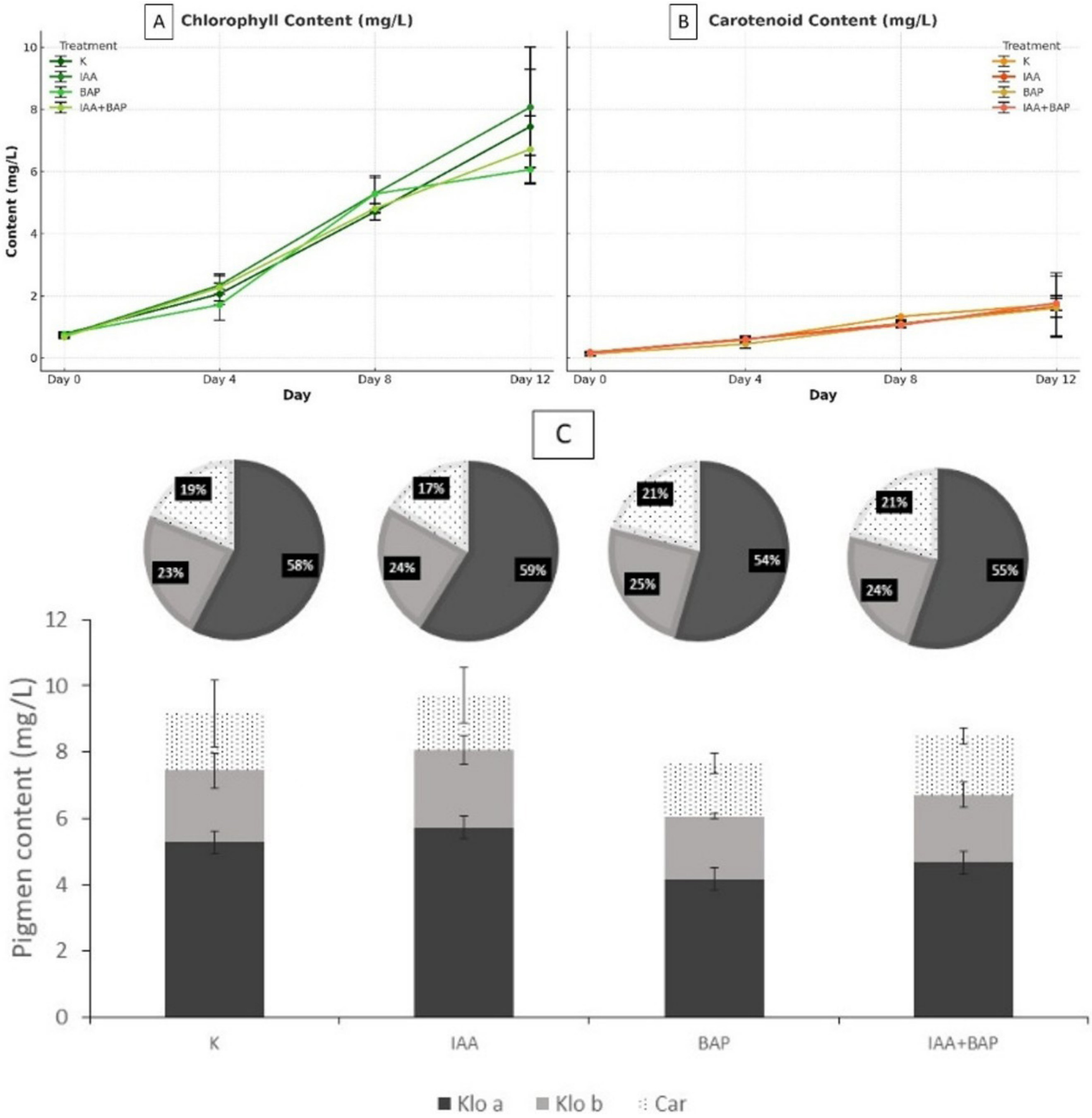

Figure 3. (A) total chlorophyll on days 0-12, (B) carotenoid on days 0-12, (C) composition of chlorophyll a, b and carotenoid content on day 12

cycle. BAP treatment showed a slightly different pattern: on days 0-4, the increase was smaller; on days 4-8, it was lower than that of the other treatments; and on days 8-12, it was lower than that of the other treatments. IAA showed an almost linear increase in total chlorophyll from the first day, reaching its highest value of 8.07±1.93 mg/L, followed by the control at 7.45±1.84 mg/L and IAA+BAP and BAP at 6.71±0.46 and 6.06±1.077 mg/L, respectively. These results correlated with the cell growth data.

3.4. Total Biomass Content

Biomass in microalgae is the total mass of algal cells produced, usually expressed as dry weight. Table 1 shows the highest biomass in the BAP treatment (1.92±0.12 g/L), followed by IAA (1.88±0.29 g/L), IAA+BAP (1.66±0.29 g/L), and the lowest in the Control (1.64±0.20 g/L). Based on the results of the analysis of variance (ANOVA). There was no significant difference in biomass content between the treatments (p-value = 0.329> 0.05).

3.5. Biomass Metabolite Content

Analysis of the primary metabolite content of microalgae after 13 days of culture with the addition of IAA, BAP, and their combination showed varying responses in protein, lipid, and carbohydrate parameters. There is no significant difference in carbohydrate and protein content under hormone induction (Table 2).

Figure 4 shows a significant increase in lipid content in the IAA + BAP combination treatment (0.33±0.026 mg/mL) compared to the control (0.18±0.015 mg/mL), single IAA (0.20±0.015 mg/mL), and single BAP (0.15±0.020 mg/mL). One-way ANOVA revealed that hormonal treatments significantly affected total lipid accumulation ($p<0.05$),while protein and carbohydrate contents were not significantly altered. This indicates that hormonal induction primarily modulated lipid biosynthesis by redistributing metabolic flux rather than increasing overall biomass production.

3.6. Fatty Acid Profile

Based on Figure 5, methyl palmitate (SFA) had the highest color intensity, indicating dominance in all treatments. Methyl linoleate and methyl cis-11-eicosenoate also appeared high but decreased in the IAA and BAP treatments. Gamma-linolenic acid methyl ester levels increased dramatically in the IAA+BAP treatment (5,23), which is a stark contrast in

Table 1. Effect of hormone-enriched media on biomass content

Treatment	Biomass content (mg/mL)
Control	1.64 ±0.19
IAA	1.88±0.30
BAP	1.92±0.11
IAA+BAP	1.66±0.04

Table 2. Results of analysis of variance (One-way ANOVA), microalgae biomass content after 13 days of observation

Variabel	df	F-value	F-table	P-Value (α 0.05)	Note
Lipid	3.8	73.6	4.07	0.001	P<0.05 (S*)
Protein		1.64		0.25	p>0.05 (NS)
Carbohydrate		2.25		0.15	p>0.05 (NS)

NS (not significant), S (Significant) (n = 3)

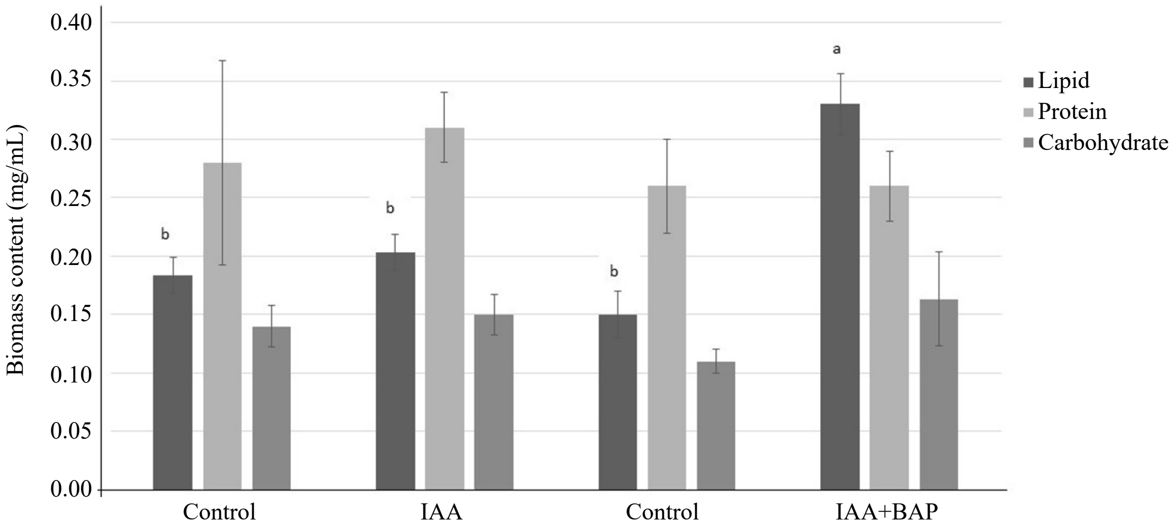

Figure 4. Primary metabolite content of microalgae with different treatments on H-13 observation. (n = 3). Data were means ± SD (n = 3). Different small and capital letters indicated significant differences between treatments and were determined by one-way ANOVA followed by Tukey's HSD test at the 95% confidence level ($p<0.05$)

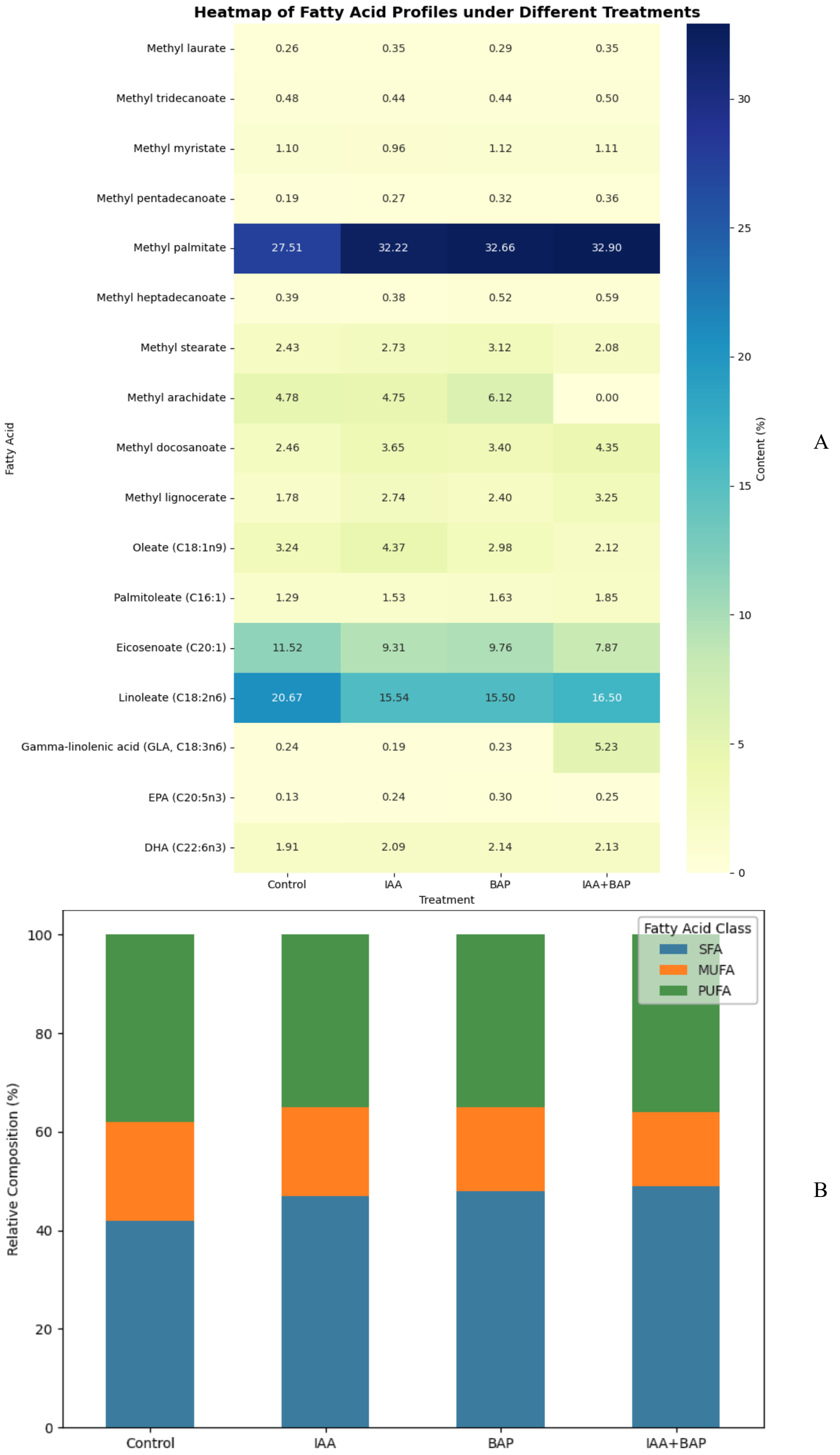

Figure 5. (A) Heatmap of fatty acid profile under different treatments, (B) relative proportion of Saturated Fatty Acid (SFA), Monounsaturated Fatty Acid (MUFA), Polyunsaturated Fatty Acid (PUFA) under different treatments

the heatmap. Hormonal treatments altered the relative fatty acid composition of green microalgae, with SFA increasing from 42% in the control to 47-49% under IAA, BAP, and IAA+BAP treatments. In contrast, MUFA decreased from 20% (control) to 15-18%, while PUFA declined slightly from 38% (control) to 35-36%, with the IAA+BAP treatment maintaining a PUFA proportion closer to the control value.

Based on the evaluation results, the growth and quality of microalgae cultured with hormone-enriched media, the IAA addition treatment was chosen because it was more efficient in terms of results and costs, and was further tested for *Artemia* enrichment.

3.7. Primary Metabolite Content of Microalgae-enriched *Artemia*

Based on Table 3, the enrichment period had a significant effect ($p<0.05$) on the protein, carbohydrate, and lipid content of *Artemia*. The longer the larvae were immersed, the higher the protein content, with the highest value at 12 h of immersion (23.80%), which was significantly higher than that of the control. For carbohydrates, the highest value was achieved at 6 h of immersion, and it decreased again with increasing immersion duration. However, the highest total lipid content was obtained at 9 h of immersion, reaching 30.13%, which was not significantly different from that at 6 h. When the immersion duration was increased to 12 h, the lipid content decreased to 23.74%. The metabolite content data showed that the enrichment of larvae with microalgae increased the protein, carbohydrate, and lipid content compared to no immersion (control).

Table 3. Primary metabolites of microalgae-enriched *Artemia* sp. with different immersion times

Enrichment	Protein (%)	Carbohydrate (%)	Lipid (%)
Control	18.45 ± 0.00^{d}	20.84 ± 0.30^{d}	17.80 ± 1.00^{c}
3 H	20.23 ± 0.77^{C}	27.96 ± 0.13^{c}	27.96 ± 1.98^{b}
6 H	22.09 ± 0.80^{b}	30.13 ± 0.28^{a}	30.13 ± 2.00^{a}
9 H	22.75 ± 0.00^{b}	27.46 ± 0.21^{c}	31.40 ± 1.98^{a}
12 H	23.80 ± 0.00^{a}	23.74 ± 1.27^{d}	23.74 ± 1.13^{b}

Means with different superscript letters are significantly different ($P<0.05$); H: soaked duration in hours

3.8. Survival Rate (SR) *Artemia* Enriched with Microalgae

The survival of *Artemia* cysts that hatched after being given long-term enrichment treatment with live microalgae is illustrated in Figure 6.

Based on the ANOVA results, with a p-value of $0.0001<0.05$, there was a significant difference in average survival rates across the four enrichment treatments (3h, 6h, 9h, and 12h). The 3h enrichment treatment showed a significant difference compared to the 6h, 9h, and 12h enrichments. However, the 6h, 9h, and 12h treatments did not show any significant differences. Comparison with the respective control values indicates that the enrichment of *Artemia* sp. with

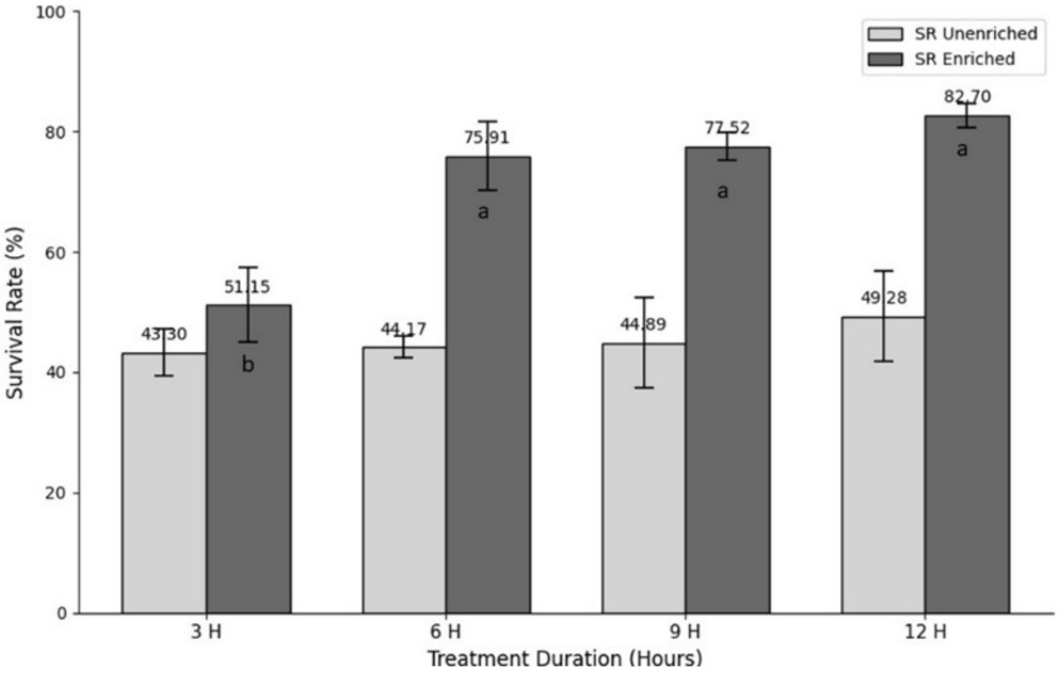

Figure 6. Larval survival rate under enriched and unenriched conditions with microalgae

microalgae increased survival. The longer the treatment duration, the higher the SR value of *Artemia* sp. The best treatment was achieved with a 12-hour enriched time, reaching 82.7%.

4. Discussion

4.1. Effect of Hormone-Induced Cell Density

IAA treatment resulted in the highest cell growth among the treatments on day 13 of culture. This demonstrates its role in stimulating cell growth from days 5 to 13. The presence of IAA in the media acts as a signaling molecule that regulates algal growth and metabolic pathways, thereby enhancing biomass accumulation in several green microalgae species (Piotrowska-Niczyporuk & Bajguz 2014; Piotrowska-Niczyporuk *et al.* 2018). Conversely, BAP accelerated the transition to the exponential phase, reaching a high cell density on day 8, but growth slowed until the end of the observation period. BAP enhanced initial exponential growth but did not maintain growth at the same level as that of IAA. Exogenous BAP treatment alters the cytokinin: auxin ratio and cellular hormone homeostasis, potentially suppressing pathways that regulate the cell cycle, N/C assimilation, and cell division. The effect of BAP on microalgae depends on the species, concentration, and environmental conditions of the microalgae. Previous studies have reported that stimulating biomass increases at low to moderate concentrations, but an inhibitory effect at high concentrations or in the later stages of cultivation (Han *et al.* 2018). The combination of IAA and BAP showed increased growth on day 5, ultimately achieving the same cell growth as the control. This indicates that the combination produces a balancing effect rather than a synergistic increase in plant growth. This suggests that the interaction between IAA and BAP does not synergistically enhance growth. Instead, this combination produces a balancing effect between the individual responses of each hormone. Although BAP accelerates early exponential growth, IAA is more effective in promoting higher final cell growth rates. Previous studies have reported that both IAA disruption and addition alter gene expression related to the cell cycle, N/C metabolism, and photosynthetic pathways in algae. This finding supports the phenotypic observation of increased cell density (Calatrava *et al.* 2024).

4.2. Effect of Hormone-Induced Pigment Content

IAA, which showed the highest cell density at the end, also produced the highest total chlorophyll content, although it was not statistically different from other treatments. IAA increases the growth rate and density of microalgal cells by promoting cell cycle progression, increasing photosynthesis (chlorophyll) and nutrient uptake (Fathy *et al.* 2023). In contrast, BAP consistently resulted in the lowest growth cell density and total chlorophyll values at the end of the observation period. These results indicate a correlation between increasing cell density and increasing chlorophyll-a, but cellular variation between species also influences the strength of this correlation (de Melo-Santos *et al.* 2024). Chlorophyll content per cell can change even as cell density increases. Therefore, the relationship between chlorophyll and growth is not always linear (Aizpuru & González-Sánchez 2024). Chlorophyll per cell can vary with growth phase, nutritional conditions, and light intensity; thus, chlorophyll-a can increase with increasing cell density (Solovchenko *et al.* 2022).

Pigment analysis showed that chlorophyll-*a* accounted for more than 50% of total pigments in all treatments, followed by chlorophyll-*b* (23–25%) and carotenoids (17–21%). The IAA treatment produced the highest total pigment content, consistent with the increased cell density, whereas BAP resulted in lower total pigment accumulation but a higher proportion of chlorophyll-b and carotenoids. The combined IAA + BAP treatment generated a more balanced pigment composition but was less effective than IAA alone in enhancing total pigments. Previous studies reported that IAA enhances chlorophyll accumulation and photosynthetic activity, while BAP contributes to chlorophyll preservation and carotenoid biosynthesis (Piotrowska-Niczyporuk & Bajguz 2014; Ermhavitalini *et al.* 2021). Similar results were observed in *Nannochloropsis* sp., where combined IAA and BAP application had no significant effect on chlorophyll a content (Solovchenko *et al.* 2022). The effects of auxins and cytokinins on microalgal pigments are known to be highly dependent on dosage and species-specific responses (Piotrowska *et al.* 2018).

4.3. Effect of Hormone-Induced on Biomass and Metabolite Content of Microlagae

As shown in Table 1, BAP treatment resulted in the highest biomass production among all treatments, whereas the control exhibited the lowest biomass, although the differences were not statistically significant. Interestingly, despite producing greater biomass, BAP-treated cultures showed lower protein, carbohydrate, and lipid contents than those treated with IAA (Figure 4), suggesting a differential carbon partitioning pattern in which assimilated carbon may have been allocated to cellular constituents that were not quantified in the present study.

The physiological effects of hormone are highly dependent on hormone concentration, species-specific characteristics, and cultivation conditions, resulting in either stimulatory or inhibitory effects on microalgal growth and metabolite accumulation (Piotrowska-Niczyporuk & Bajguz 2014; Han *et al.* 2018). Therefore, the lower accumulation of primary metabolites observed under BAP treatment may indicate that the applied concentration was not optimal for this species. Ermavitalini *et al.* (2021) reported that supplementation with 10 ppm IAA and 10 ppm BAP increased biomass production in *Nannochloropsis* sp. without affecting chlorophyll-a content.

Based on evaluations of several parameters, only lipid content had a significant effect. It is suspected that the treatment concentration was not optimal for increasing the growth and quality of the microalgae. Another possibility is that the direct administration of powdered hormones was less effective. Previous research with the same treatment on *Nannochloropsis* sp. yielded better results. The hormone administration method used a stock solution prepared with 1 N NaOH for IAA and 1 N HCl for BAP. This auxin molecule contains a carboxylate group (-COOH) that is insoluble in neutral water but is deprotonated by a base (NaOH) to form a much more soluble carboxylate salt when used together. The use of HCl protonates BAP, increasing its solubility. BAP contains an amine/heterocyclic group that can be protonated to form a water-soluble salt when treated with an acid (HCl). Adding NaOH facilitates the dissolution of the molecule at neutral pH.

One-way ANOVA demonstrated that hormonal treatments significantly affected total lipid accumulation in Chlorella after 13 days of cultivation ($p<0.05$), while protein and carbohydrate contents were not significantly influenced ($p>0.05$). This result indicates that hormonal induction selectively regulated lipid biosynthesis rather than promoting a general increase in biomass components. Among the treatments, the combination of IAA and BAP resulted in the highest lipid content, suggesting a synergistic effect between auxin and cytokinin in enhancing lipogenesis. In line with Singh *et al.* (2020), the combination of auxin and cytokinin showed a synergistic effect, resulting in approximately 42% lipid accumulation in microalgae. This response was associated with the regulation of ROS homeostasis and antioxidant enzyme activities, which may contribute to enhanced lipid biosynthesis under phytohormone treatment (Singh *et al.* 2020; Guldhe *et al.* 2019). Phytohormones have been reported to increase the expression of acetyl-CoA carboxylase and RuBisCo genes in *C. sorokiniana*, thereby enhancing lipid biosynthesis and photosynthetic carbon fixation. Auxin (IAA) has been reported to stimulate biomass and lipid (TAG) biosynthesis (Singh *et al.* 2024) by enhancing carbon metabolism and activating key enzymes involved in fatty acid synthesis, while cytokinin (BAP) may support cellular growth and photosynthetic capacity, thereby strengthening the lipid-inducing effect when applied in combination. Similar hormonal interactions have been reported to enhance lipid accumulation in *Chlorella* and other microalgae, supporting the observed pattern in this study. Overall, these findings confirm that combined hormonal induction is an effective strategy to enhance lipid accumulation without significantly altering other biomass components.

4.4. Effect of Hormonal Induction on Fatty Acid Profile

Figure 5 clearly illustrates the clustering of fatty acids into SFA, MUFA, and PUFA groups. SFA, particularly palmitic acid (C16:0), dominated all treatments with the highest intensity blocks. Interestingly, arachidic acid (C20:0) was abundant in the control but almost absent in the IAA+BAP treatment, suggesting regulatory suppression of this SFA. In the MUFA cluster, oleic acid (C18:1n9) peaked under IAA treatment, while cis-11-eicosenoate (C20:1) decreased drastically under IAA+BAP, indicating altered desaturation and elongation processes. The PUFA cluster revealed that linoleic acid (C18:2n6) decreased in intensity across treatments, while gamma-linolenic acid (GLA, C18:3n6) showed a dramatic increase under IAA+BAP, emerging as a distinct dark block. Although EPA (C20:5n3) and DHA (C22:6n3) remained relatively low, their intensities were slightly higher in BAP and IAA+BAP than in the control. *Artemia* live feeds often lack highly unsaturated fatty acids (HUFA) such as eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), which are essential for the growth of fish larvae/aquaculture feed (Sorgeloos *et al.* 1987). Hormone-induced culture results showed increased EPA and DHA content of microalgae compared with the control treatment (Figure 6A).

Collectively, the heatmap analysis demonstrates that growth regulator supplementation (IAA, BAP, and their combination) not only increases total SFA (particularly palmitic acid) but also modulates PUFA synthesis pathways, especially the induction of GLA and the slight enhancement of long-chain n-3 PUFAs (EPA, DHA), which are nutritionally valuable for aquaculture feed enrichment.

Based on the evaluation of research results on microalgae cultures induced by IAA and BAP hormones, IAA treatment was chosen as a source of Artemia for subsequent microalgal cultures. This was based on efficiency considerations, as IAA treatment tends to increase growth, biomass, primary metabolites, EPA, and DHA, which are generally not significantly different from those of IAA + BAP. Exogenous IAA has been shown to increase total lipid acidity twofold and alter fatty acid composition in several microalgae (*Synechocystis* and *Chlorella*), a response that is dependent on the dose and culture conditions (including interactions with nitrogen) (Fathy *et al.* 2023).

4.5. Primary Metabolite Content of Microalgae-enriched *Artemia*

The protein content continued to increase with enrichment duration up to 12 h, but the rate of increase slowed. Protein values may increase with longer enrichment treatments, depending on the composition of the algal biomass (Ma & Hu 2024). Microalgae generally have a high protein content (±30-70% dry weight, depending on species and culture phase) with 20 essential amino acids, thus functioning as a "nitrogen donor" that increases the protein composition of Artemia during enrichment (Mosibo *et al.* 2024). This composition is strongly influenced by the growth phase of microalgae. In the stationary phase under nitrogen limitation, protein levels decrease, while carbohydrate levels increase. Conversely, the log phase maintains higher protein and a better amino acid profile, making microalgal biomass more effective for Artemia enrichment (Morales-Plasencia *et al.* 2023). The results indicate that the duration of enrichment time affects the rate and peak of protein accumulation in *Artemia*. The duration of enrichment modulates the rate and peak of protein accumulation in *Artemia*. Experimental studies have shown that during the first ±6 h, adjusting the biomass density of *Chaetoceros muelleri* and lighting can increase the ingestion rate and protein content of Artemia franciscana juveniles (Millán-Almaraz *et al.* 2021).

Carbohydrate levels increased during 3–6 h of enrichment, but decreased after 12 h. The decreased carbohydrate content is suspected to be due to Artemia's energy requirements. The carbohydrate content in microalgae-enriched *Artemia* tends to increase rapidly and peak at approximately ±6 h, in line with the active feeding phase and high carbohydrate supply from microalgae biomass (which increases under N-limiting conditions). However, it decreases at 9–12 h due to its utilization for respiration/osmoregulation and potential leaching into the media (Millán-Almaraz *et al.* 2021). The microalgal growth phase and nitrogen status influence the amount of carbohydrate supplied. The stationary phase is generally richer in carbohydrates than the log phase. A limitation (stationary phase) shifts carbon from proteins to carbohydrates/β-glucans, whereas the log phase decreases the carbohydrate proportion (Morales-Plasencia *et al.* 2023). *Artemia* rapidly accumulate carbohydrates optimally at an enrichment duration of approximately six hours as a quick energy reserve (glycogen/soluble carbohydrates) before metabolic utilization or leaching occurs (Mosibo *et al.* 2024). In addition, feeding density and lighting modulate digestion rates, thereby determining carbohydrate accumulation (Millán-Almaraz *et al.* 2021). Therefore, controlling algal cell density during enrichment is crucial.

Lipid accumulation occurred more rapidly and peaked after 3–9 h of treatment (the highest peak at 9 h). These results are consistent with those reported by Millán-Almaraz *et al.* (2021), who found that short-term enrichment optimizes lipid/HUFA deposition before metabolic utilization and leaching. The duration of enrichment plays a crucial role in determining the PUFA content in juvenile *Artemia franciscana* when enriched with tuna orbital oil (Nieves-Soto *et al.* 2021). *Artemia* sp. can rapidly accumulate lipids and HUFAs during the first few hours of enrichment; therefore, short-term enrichment is often optimal for increasing lipid/DHA/EPA content (Martínez Soler *et al.* 2023). After 12 h, lipid levels decreased, suggesting a possible decrease in metabolic efficiency or the reuse of energy reserves by *Artemia* sp. Microalgal enrichment generally improves the nutritional composition of *Artemia* sp., but the increase in lipids/HUFAs is most effective during short enrichment periods (3–6 h). Long enrichment times can lead to changes (depuration, metabolic utilization, or decreased lipid accumulation efficiency), further reducing lipid content (Ma & Hu 2024). The sustained increase in protein for up to 12 h of treatment suggests that a more extended enrichment period may be necessary if the primary goal is to increase total protein. However, lipid adverse effects monitoring is necessary.

4.6. Effect of Microalgae-enriched *Artemia* on the Survival Rate of Larvae

Figure 6 shows that *Artemia* enriched with local microalgae effectively enhanced the survival rate of larvae. Compared with the unenriched, the SR value increased by 33.42% after 12 h of soaking. Microalgae, such as *Chlorella*, contain long-chain unsaturated fatty acids (omega-3 precursors), vitamins, and proteins that can be absorbed and utilized by *Artemia* sp. during the enrichment process. The enrichment results showed higher protein and lipid content, providing the recipient larvae with energy reserves and essential micronutrients. These results are consistent with the literature (Samat *et al.* 2020). *Artemia* sp. enriched with *Chlorella vulgaris* changes the fatty acid composition of the larvae. Lipid mobilization (β-oxidation) provides the ATP required during hatching and early naupliar movement. A favorable lipid content and FA profile increase the probability of successful egg hatching and nauplius survival until they begin feeding (Lopalco *et al.* 2019). Previous research reported that enriched *Artemia* sp. enhanced the growth performance and expression of immune and growth genes of the larvae, potentially due to its nutritional content and improved ingestion by Tor tambroides larvae (Joshua *et al.* 2024). Polyunsaturated fatty acids (PUFAs, especially n-3 fatty acids such as EPA and DHA) are important indicators of the nutritional quality of *Artemia*. Growth and survival factors depend not only on HUFAs but also on protein profiles, total lipids, and digestibility. This is in line with the test results, which showed that the metabolite content of microalgae-enriched *Artemia*, such as protein, lipids, and carbohydrates, increased compared to the control.

In conclusion, the study results show that the local microalgae isolated from Kakaban Lake exhibit characteristics identical to those of *Chlorella* sp. cells. Induction of culture media with IAA hormones affects the growth, biomass, metabolite profiles, and fatty acid content of microalgae. *Artemia* sp. enriched with IAA-induced microalgae cultured with increased primary metabolite content over time, and survival rates increased in *Artemia* sp. compared to unenriched (control) cultures.

Acknowledgements

The authors wish to express their sincere gratitude to the KEMENDIKTISAINTEK for financial support provided through the Regular Fundamental Research Scheme (Master Contract No. 063/C3/DT.05.00/PL/2025; Sub-Contract No. 006/UN51.9/SP2H-PFR/2025). The authors also acknowledge the Institute for Lembaga Penelitian dan Pengabdian Masyarakat (LP2M), Universitas Borneo Tarakan, Badan Riset dan Inovasi Nasional (BRIN) for the provision of research facilities and continuous institutional support during the course of this study.

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