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**Research Article**

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# Timing-dependent effects of fine-bubble $CO_2$ supplementation on mixotrophic growth and biomass production of *Euglena gracilis*

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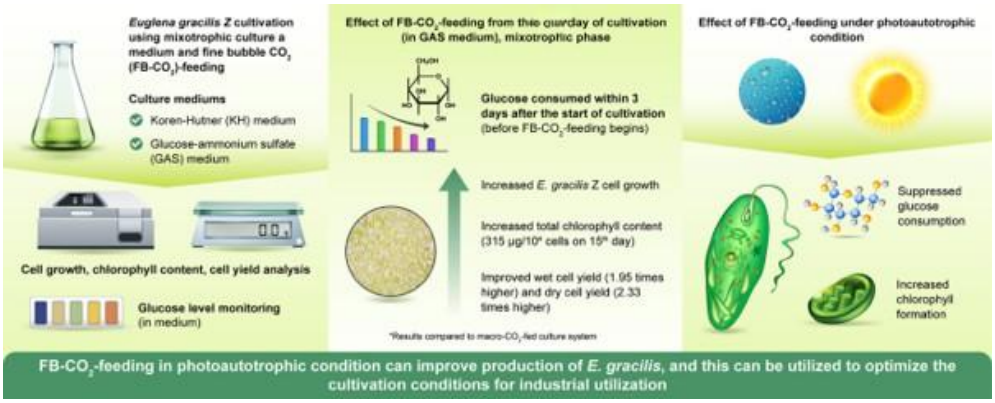
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**Abstract**

Fine-bubble $CO_2$ (FB-$CO_2$) technology has recently been shown to enhance the autotrophic growth of *Euglena gracilis* by improving inorganic carbon delivery. However, its effectiveness under mixotrophic conditions and the importance of supplementation timing remain unclear. This study investigated the effects of pulsed FB-$CO_2$ supplementation on the growth of *E. gracilis* cultured in glucose-ammonium sulfate medium (GAS). FB-$CO_2$ was supplied at 48-h intervals either from the start of cultivation (Method A) or after the initial glucose-utilization phase (Method B). Glucose consumption, cell density, chlorophyll accumulation, and biomass production were compared with those obtained using conventional $CO_2$ delivery (macro-$CO_2$). Early FB-$CO_2$ supplementation delayed glucose depletion, suppressed chlorophyll accumulation, and did not improve cell growth. In contrast, initiating FB-$CO_2$ supplementation after glucose depletion enhanced

cell density and biomass production relative to macro-$CO_2$. Chlorophyll accumulation increased predominantly during the late cultivation phase, indicating a transition from glucose-supported growth to greater reliance on photosynthesis. These results demonstrate that the benefits of FB-$CO_2$ are strongly dependent on supplementation timing under mixotrophic conditions. Aligning FB-$CO_2$ delivery with the post-glucose phase may improve carbon utilization, biomass productivity, and $CO_2$ capture efficiency while avoiding unnecessary $CO_2$ enrichment during heterotrophic growth.

**Keywords:** *Euglena gracilis*, fine-bubble $CO_2$, mixotrophic cultivation, carbon utilization, chlorophyll accumulation, biomass production




# Introduction

Microalgae are widely recognised as sustainable biological platforms for carbon capture and the production of value-added products including biofuels, nutraceuticals, and biopolymers (Jung et al. 2021; Lee 2025; Tripathi et al. 2023). Among these microorganisms, *Euglena gracilis* has attracted increasing attention because of its unique metabolic flexibility, enabling growth under autotrophic, heterotrophic, and mixotrophic conditions (Wang et al. 2018; Zakrys et al. 2017). In addition to its photosynthetic capacity, *E. gracilis* accumulates commercially valuable compounds such as paramylon, lipids, vitamins, and bioactive metabolites, making it a promising organism for industrial biotechnology and sustainable biomass production (Inui et al. 2017; Isegawa 2023; Ebenezer et al. 2022). However, biomass productivity remains a primary limitation for large-scale cultivation, necessitating the development of cultivation strategies that improve carbon utilisation efficiency and cellular growth (Suzuki 2017). Fine-bubble technology (FBT) has emerged as an innovative approach for enhancing gas-liquid mass transfer in biological and environmental systems. Ultrafine bubbles ($< 1\mu$m diameter) exhibit prolonged residence times and high interfacial surface areas

that can improve gas dissolution efficiency and carbon availability (Yabe 2016; Hata et al. 2019). These properties have generated increasing interest in applying fine-bubble $CO_2$ (FB-$CO_2$) delivery to microalgal cultivation systems aimed at improving photosynthetic carbon fixation and biomass productivity.

We recently demonstrated that FB-$CO_2$ supplementation increases chlorophyll accumulation and biomass production in autotrophically cultivated *E. gracilis* (Suzuki-Nagata et al. 2025). However, the applicability of FB-$CO_2$ technology to mixotrophic cultivation remains unresolved. Under mixotrophic conditions, carbon metabolism changes dynamically as cells transition from glucose-supported growth to greater reliance on photosynthetic carbon assimilation (Yamane et al. 2001; Gu et al. 2022). Consequently, the physiological response to FB-$CO_2$ may depend not only on the mode of $CO_2$ delivery but also on the timing of supplementation relative to carbon-source availability.

Therefore, this study investigated the time-dependent effects of FB-$CO_2$ supplementation on the mixotrophic growth of *Euglena gracilis* cultured in glucose-ammonium sulfate (GAS) medium. Specifically, we evaluated whether the timing of pulsed FB-$CO_2$ delivery influences glucose utilisation, chlorophyll accumulation, cell growth, and biomass production. We hypothesised that FB-$CO_2$ supplementation would be most effective after depletion of the organic carbon source, when photosynthetic metabolism becomes increasingly important for continued biomass accumulation.

# Materials and Methods

## Experimental design

This study examined the timing-dependent effects of FB-$CO_2$ delivery on the mixotrophic growth of *Euglena gracilis Z* in glucose-ammonium sulfate (GAS) medium which contains glucose as the sole organic carbon source (Prescott, 1953; Suzuki et al., 1993), allowing glucose utilisation and the subsequent transition toward photosynthetically supported growth to be evaluated. FB-$CO_2$, supplied via the fine-bubble generator described below, was compared with conventional $CO_2$ bubbling (macro-$CO_2$) under the same supplementation schedules. Growth performance was assessed from cell density, glucose consumption, chlorophyll content, and harvested biomass.

## Strain and preculture conditions

*Euglena gracilis Z* was kindly provided by Laboratory of Nutrition, Osaka Prefectural University; now Osaka Metropolitan University in 1993. The strain was maintained in the Food Chemistry Laboratory, Department of Fisheries Sciences, Hokkaido University, and subsequently transferred to our laboratory for further study.

## Culture medium

The glucose-ammonium sulfate (GAS) medium (Prescott, 1953; Koizumi et al., 1993; Suzuki et al., 1993) is consited of following constituents in 1 L: glucose, 10 g; ammonium sulfate, 1.89 g; $KH_2PO_4$, 0.25 g; $MgSO_4 \cdot 7H_2O$, 1.75 g; $CaCO_3$, 1.2 g; $Na_2$-EDTA,

50 mg; $FeSO_4(NH_4)2SO_4 \cdot 6H_2O$, 50 mg; $MnSO_4 \cdot 7H_2O$, 25 mg; $ZnSO_4 \cdot 7H_2O$, 25 mg.; $(NH_4)6Mo_7O_{24} \cdot 4H_2O$, 4 mg; $CuSO_4$, 1.2 mg; $NH_4VO_3$, 0.5 mg; $CoSO_4 \cdot 7H_2O$, 0.5 mg; $H_3BO_3$, 0.6mg; $NiSO_4 \cdot 6H_2O$, 0.5 mg; thiamine·HCl (vitamin $B_1$), 25 mg.; and cyanocobalamin (vitamin $B_{12}$), 0.005 mg. The initial pH was adjusted to 3.5. Glucose was autoclaved separately to prevent Maillard reactions.

## Cultivation conditions and $CO_2$ delivery protocols

Unless otherwise stated, the cultivation vessel, illumination, aeration, and $CO_2$-delivery system followed those used in our previous autotrophic study (Suzuki-Nagata et al., 2025). A finely dispersed $CO_2$ was produced using a fine ceramic nozzle (with an inner diameter of 6 mm and length of 24 mm, featuring a pore size of 1 $\mu$m) connected to a reservoir cylinder containing liquid $CO_2$. Noritake Corporation (Aichi, Japan) generously provided a prototype of fine ceramic nozzles. As a control (Macro-$CO_2$), a sintered glass filter with an average pore size of 15–40 $\mu$m was utilized.

In the present study, GAS medium was used to examine mixotrophic growth, and two pulsed $CO_2$ supplementation schedules were compared to determine whether pulsed $CO_2$ supplementation should be applied during or after the glucose-supported growth phase. FB-$CO_2$ denotes $CO_2$ delivered through the fine-bubble generator, whereas macro-$CO_2$ denotes conventional $CO_2$ bubbling; both delivery modes were applied under the same timing schedule within each method.

## Method A (early FB-$CO_2$ supplementation)

In Method A, $CO_2$ supplementation was initiated at the start of cultivation and repeated at 48 h intervals until two days before culture termination. This protocol tested the effect of pulsed $CO_2$ supplementation during the glucose-supported early phase.

## Method B (delayed FB-$CO_2$ supplementation)

In Method B, cultures were aerated with atmospheric air for the first three days to allow initial glucose utilisation. $CO_2$ supplementation was then initiated on day 4 and repeated at 48 h intervals. This protocol tested the effect of delaying pulsed $CO_2$ supplementation until after the early glucose-utilisation phase.

For both methods, continuous aeration with atmospheric air was maintained throughout cultivation. Cultures were generally terminated after 10 days for growth, chlorophyll contents and biomass measurements.

Cultures were separately continued for 15 days to determine chlorophyll content and examine late-phase chlorophyll accumulation after glucose depletion.

## Cultivation conditions

For inoculation, *Euglena gracilis Z* cells ($4 \times 10^5$ cells in 10 mL of preculture) were added to 500 mL of GAS medium, giving an initial density of approximately $8 \times 10^3$ cells $mL^{-1}$.

Temperature was controlled by immersing culture vessels in a transparent acrylic water bath (10L volume) maintained at 28°C using a thermostat.

Cultures were illuminated for 14 h $day^{-1}$ (06:00–20:00) using a mixed-LED light source (Excelvan 14W 225LEDS, 6000 K; Tokyostore, Tokyo, Japan), as described previously (Suzuki-Nagata et al., 2025). Illumination strength was monitored using a Light Analyzer LA-105 (NK System, Nihon Rika Co., Tokyo, Japan).

During cultivation days 1–3, the photosynthetic photon flux density (PPFD) was 30 $\mu$mol $m^{-2}\cdot s^{-1}$, followed by from cultivation day 4 illumination strength was subsequently increased to 50 $\mu$mol $m^{-2}\cdot s^{-1}$. The photon flux density (PFD) components were: blue, 16.7 $\mu$mol $m^{-2}\cdot s^{-1}$; green, 4.6 $\mu$mol $m^{-2}\cdot s^{-1}$; and red, 24 $\mu$mol $m^{-2}\cdot s^{-1}$.

**Aeration**: Air was fed constantly through a sterile filter (0.45 mm) at 340 $cm^3\cdot min^{-1}$ as air bubbles to supply 20% oxygen throughout the culture period, and cultures were stirred continuously using a magnetic stirrer.

## Analytical measurements

Cell density (cells·$mL^{-1}$) was determined by microscopic counting using a microscope (Olympus CHA) and the images taken was counted with ImageJ/Fiji software. Optical density of cultured cell suspension was measured at 750nm, either. Growth curves were constructed to compare treatments with excel data analyses and Prism 11 Graph Pad system.

Relation of cell density and Optical Density at 750 nm is presented in Supplementary Fig.S1.

## Glucose analysis

Glucose concentration in the culture medium was quantified using an electrochemical test meter based on flavin adenine dinucleotide (FAD)-dependent glucose dehydrogenase and the ferricyanide method (Loew et al., 2017; Sacks, 1994). Measurements were performed using a OneTouch Verio Meter (LifeScan Scotland Ltd., Inverness IV23ED, UK) (see Supplementary Fig. S2).

Before measurement, the sample pH was adjusted to 6–7 with phosphate buffer. For each sample, 1 mL of cell suspension was centrifuged at 1500 × g for 10 min (Denver Force 7 Microcentrifuge; Iwaki Denver Instruments, USA). The supernatant was diluted 2-fold with phosphate buffer (final pH 6–7), and glucose concentration was quantified using a calibration curve (see Supplementary Fig. S2).

## Chlorophyll analysis

Chlorophyll content was determined spectrophotometrically (Beckman-Coulter DU-600) using methanol extraction procedure (Grimme and Boardman, 1972). Optical density was measured at 650 and 665 nm, and chlorophyll a, chlorophyll b, and total chlorophyll were calculated using the equations of Grimme and Boardman (1972).

$$\text{Chlorophyll a (mg/mL)} = 16.5 \times OD_{665nm} - 8.3 \times OD_{650nm} \quad (1)$$

$$\text{Chlorophyll b (mg/mL)} = 33.8 \times OD_{650nm} - 12.5 \times OD_{665nm} \quad (2)$$

$$\text{Total Chlorophyll (mg/mL)} = 4 \times OD_{665nm} + 25.5 \times OD_{650nm} \quad (3)$$

Chlorophyll content is reported as μg/mL, as indicated in the figure legends.

## Biomass harvesting and yield

Cells cultured for 10 days were harvested by centrifugation (500 × g for 15 min) with a Kubota S500T centrifuge. Wet biomass was recorded, and samples were freeze-dried to determine dry weight. Biomass yield was calculated as the packed wet-cell weight (%) relative to the initial culture volume.

## Measurement of Zeta potential with ZetasizerUltra.

Zeta potential is the measure of the electrical charge on the surface of a microscopic particle suspended in a liquid. Specifically, it represents the electrical potential at the slipping plane, which is the boundary where the liquid moves along with the particle. Generally, an absolute value of ±30mV or higher indicates a stability of the system. Values between ±10mV and±30mV are considered unstable and prone to clumping.

To confirm the presence of fine bubble $CO_2$, Zeta potential was measured with Zetasizer in addition to microscopic observation and quantitation with NanoSight which has been examined previously (Suzuki-Nagata et al., 2025).

Zeta potential was measured for an FB-$CO_2$-fed solution prepared by supplying fine-bubble $CO_2$ for 1 min at 0.38 MPa and a flow rate of 1L x $min^{-1}$. As a reference, ordinary size $CO_2$ was supplied for 1 min at 1 atm pressure filtered through glass filter.

The device used for determining the zeta potential was a Zetasizer Ultra (Malvern Panalytical Ltd., Enigma Business Park, Grovewood Road, Malvern, Worcestershire, WR14 1XZ, England, UK). Zetasizer Ultra is a device that uses an electrophoretic light scattering instrument utilizing capillary cells.

## Statistical analysis

Experiments were conducted in triplicate (n = 3 independent biological replicates).

Measurments of glucose consumption, cell density, chlorophyll contents and harvested cell weight were repeated two times for each sample. Comparisons between FB-$CO_2$ and macro-$CO_2$ treatments at individual time points were performed using paired two-tailed t-tests. Statistical significance between macro-$CO_2$ and FB-$CO_2$ supplemented for chlorophyll content determination was accepted at $p < 0.05$. Data are presented as mean ± SD for six measurements unless otherwise stated.

## Safety

All procedures involving $CO_2$ were conducted in a well-ventilated area to minimize the risk of $CO_2$ exposure.

# Results

## 1. Glucose consumption in GAS medium

Glucose dynamics in GAS medium under the two $CO_2$ supplementation schedules are shown in Figures 1a (Method A; supplementation from day 1) and 1b (Method B; supplementation initiated on day 4).

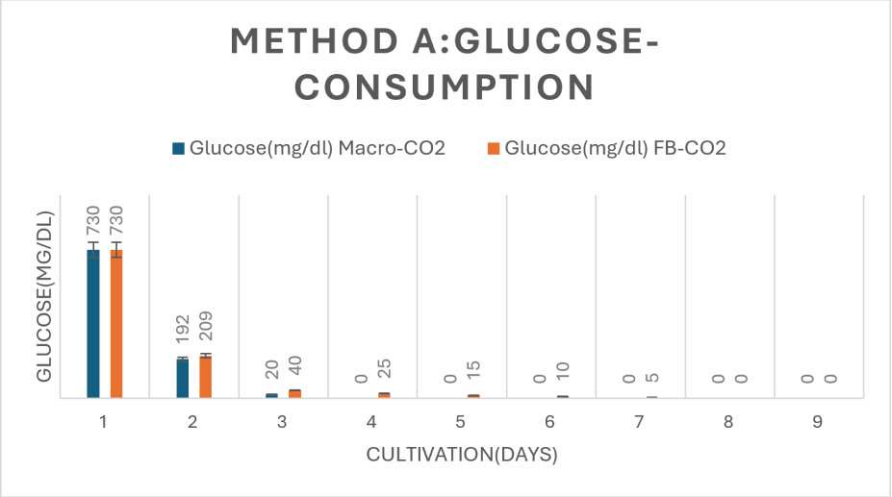



**Fig. 1a** Glucose concentration (or remaining glucose; as plotted mg $dL^{-1}$) in GAS medium during cultivation of *E. gracilis Z* under Method A ($CO_2$ supplementation from day 1).

Dark blue bars indicate macro-$CO_2$ (conventional feeding as control) and orange bars indicate FB-$CO_2$–Y-axis: glucose concentration (mg $dl^{-1}$). Numerals on the top of each bar indicate mean values of glucose concentration for 6 measurements. X-axis: cultivation period (day).

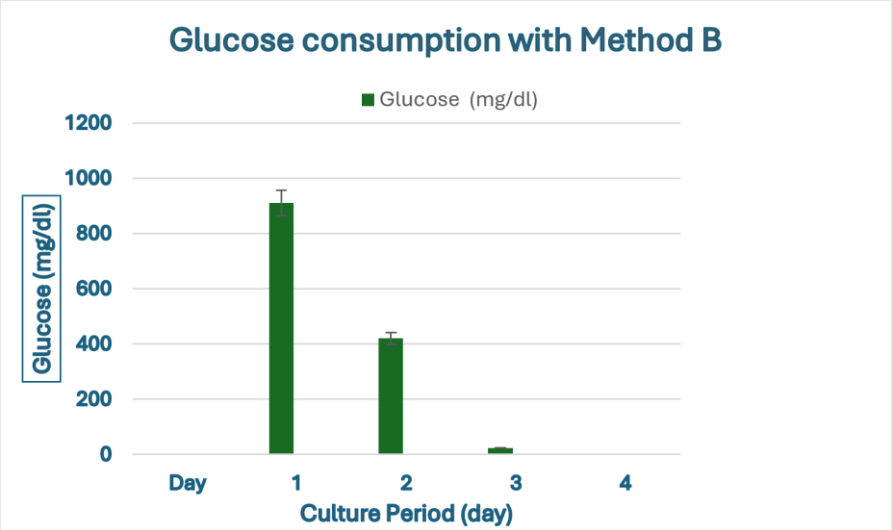



**Fig. 1b** Glucose concentration (or remaining glucose; as plotted mg $dL^{-1}$) in GAS medium during cultivation of *E. gracilis Z* under Method B ($CO_2$ supplementation initiated on day 4)

X-axis: cultivation period (days), Y-axis: Glucose concentration (mg $dl^{-1}$). Dark green bars indicate macro-$CO_2$ (conventional feeding as control). Values are means ± SD (n = 6) with error bars.

Under Method A, early pulsed $CO_2$ supplementation slowed glucose utilisation in both the macro-$CO_2$ control and FB-$CO_2$ treatments (Fig. 1a), indicating that initiating pulsed $CO_2$ supplementation at the start of cultivation may adversely affect glucose consumption and subsequent cell growth (Fig. 2a).

In contrast, under Method B, glucose was largely depleted during the initial aeration period before pulsed $CO_2$ supplementation started on day 4 (Fig. 1b). Overall, these results indicate that the timing of pulsed $CO_2$ supplementation modulates glucose consumption in GAS medium. In other words, as shown in the following section stating cell growth starting FB-$CO_2$ supplementation in the presence of glucose is not beneficial at all but inhibitive.

## 2. Comparison of cell growth in GAS medium under $CO_2$ supplementation Methods A and B

The growth patterns of *E. gracilis Z* cultured in GAS medium under two $CO_2$ supplementation schedules are shown in Figures 2a and 2b. Figure 2a corresponds to Method A ($CO_2$ supplementation from the start of cultivation), whereas Figure 2b corresponds to Method B ($CO_2$ supplementation initiated on day 4). In each panel, FB-$CO_2$ is compared with conventional $CO_2$ delivery (macro-$CO_2$) under the same dosing schedule.

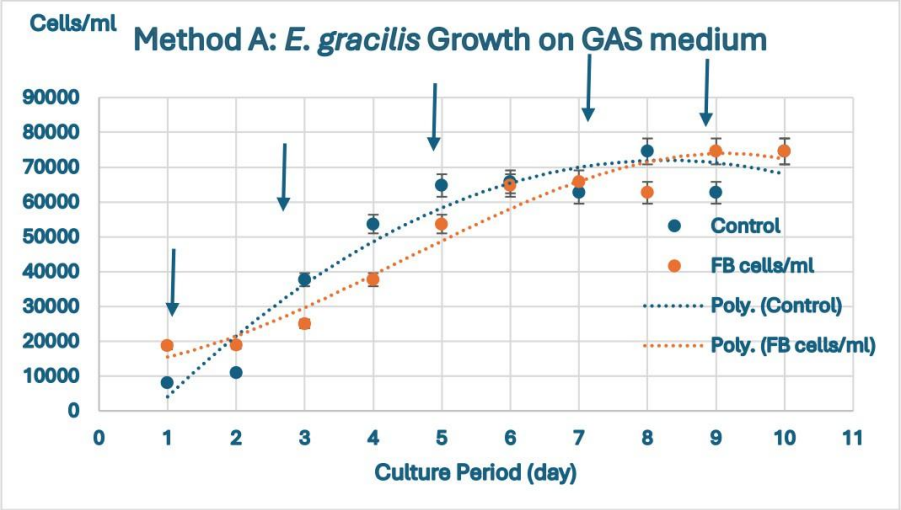



**Fig. 2a** Cell density (cells $mL^{-1}$) of *E. gracilis Z* cultured in GAS medium under Method A ($CO_2$ supplementation from day 1).

X-axis indicates cultivation period in days, and Y-axis cell density (cells $ml^{-1}$). Blue circle symbols (series 1) indicate macro-$CO_2$ (control) supplementation, and orange circle symbols (series 2) indicate FB-$CO_2$. Arrows indicate $CO_2$ supplementation events (48-h intervals). Values are means of six measurements (n = 6) with error bars indicating mean ± SD. Up to day 9, no statistically significant difference was observed.

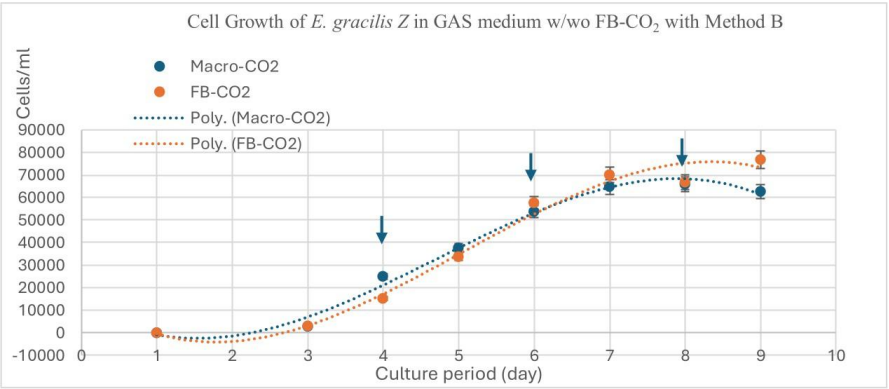



**Fig. 2b** Cell density (cells $mL^{-1}$) of *E. gracilis Z* cultured in GAS medium under Method B ($CO_2$ supplementation initiated on day 4).

X-axis shows cultivation period in days, and Y-axis cell density (cells $ml^{-1}$). Blue circle symbols indicate macro-$CO_2$ (control) and orange symbols indicate pulsed FB-$CO_2$ supplemented. Arrows indicate $CO_2$ supplementation events (48-h intervals).

Values indicate mean ± SD for n=6. Up to day 7, no significant difference was recognized between two treatments, however, on day 9 difference was recognized between pulsed FB-$CO_2$ supplementation and control macro-$CO_2$.

When pulsed $CO_2$ supplementation was started on day 1 (Method A), FB-$CO_2$ did not enhance cell growth relative to macro-$CO_2$, and cell density remained comparatively low throughout the cultivation period (Fig. 2a). In contrast, when pulsed $CO_2$ supplementation was delayed until day 4 (Method B), FB-$CO_2$ resulted in higher cell densities than macro-$CO_2$, particularly in the later phase of cultivation (Fig. 2b). These results indicate that the timing of FB-$CO_2$ supplementation is a key determinant of growth performance in mixotrophic GAS medium to enhance photosynthesis activity resulting in promoting cell growth.

## 3. Chlorophyll content in *E. gracilis Z* grown in GAS medium under $CO_2$ supplementation Methods A and B

Chlorophyll content in *E. gracilis Z* cultured on GAS medium under Methods A and B is shown in Fig. 3a and Fig. 3b, respectively. Measurement of chlorophylls under Method A was run for 15 days, while under Method B cultivation was terminated on 10th day. In both methods, total chlorophyll content was generally higher under macro-$CO_2$ than under FB-$CO_2$ from days 1 to 12 with Method A, but for 6 days with Method B. In Method A, this trend reversed during the late cultivation phase (days 13–15), when FB-$CO_2$ cultures exhibited significantly higher chlorophyll content than macro-$CO_2$ cultures (Fig. 3a).

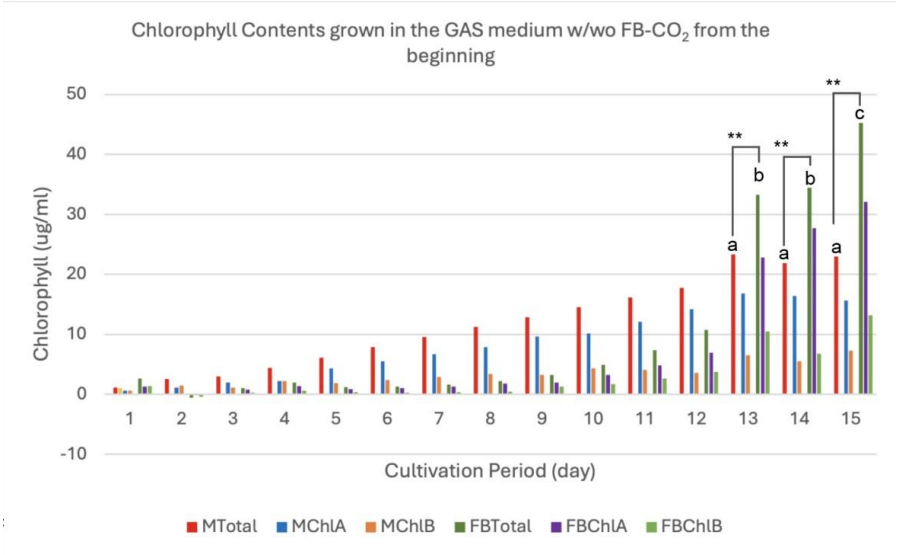



**Fig. 3a** Chlorophyll content of *E. gracilis Z* cultured in GAS medium under Method A ($CO_2$ supplementation from day 1) with macro-$CO_2$ or FB-$CO_2$.

Total chlorophyll and Chlorophyll a ($\mu$g mL$^{-1}$) in *E. gracilis Z* under Method A ($CO_2$ supplementation from day 1). Macro-$CO_2$ and FB-$CO_2$ denote conventional and fine-bubble $CO_2$ delivery, respectively. Macro-total chlo, total chlorophyll contents of macro-$CO_2$; Macro-chlA, chlorophylla contents of macro-$CO_2$. FB-$CO_2$ Total Chlo, total chlorophyll contents of pulsed FB-$CO_2$ supplemented; FB-$CO_2$-ChlA, chlorophyll A contents of pulsed FB-$CO_2$ supplemented. Bars indicate mean chlorophyll content (microgram ml$^{-1}$) of nine measurements± SD. (n = 6)

Throughout cultivation from day 2 to day 12, chlorophyll content in pulsed FB-$CO_2$-supplemented *E. gracilis Z* cultures was lower than in macro-$CO_2$ controls, indicating that FB-$CO_2$ supplementation from the beginning of cultivation did not promote chlorophyll formation during the glucose-supported phase. Only day 13 to 15 chlorophyll contents of FB-$CO_2$ supplemented *E. gracilis Z* were higher than control.

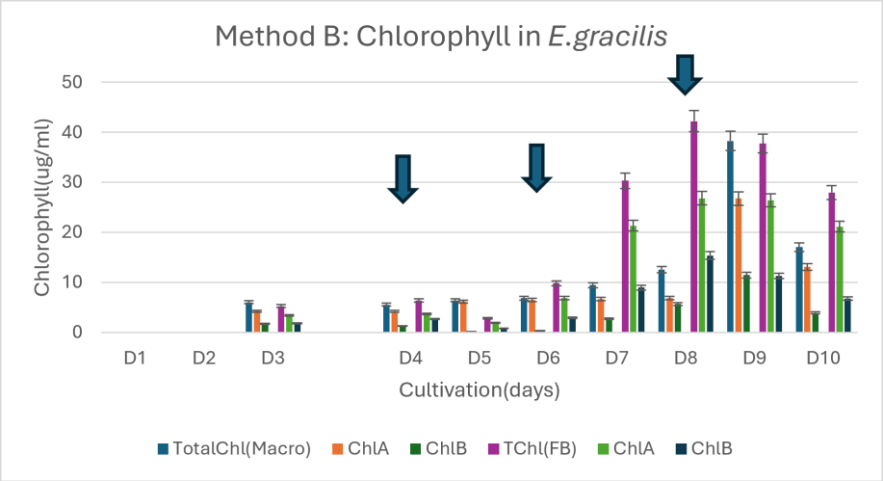



**Fig. 3b** Chlorophyll content of *E. gracilis Z* cultured in GAS medium under Method B ($CO_2$ supplementation initiated on day 4) with macro-$CO_2$ or FB-$CO_2$ with 48 h intervals. Arrows indicate $CO_2$ charge to supplement $CO_2$ with 48 h intervals.
Error bars indicate mean ± SD (n=6); statistically significant differences between macro-$CO_2$ and FB-$CO_2$ were observed on day 7 and 8.

In Method B, differences between macro-$CO_2$ and FB-$CO_2$ treatments were small up to days 6. As reported in our previous paper (Suzuki-Nagata et al., 2025), the effect of FB-$CO_2$ was recognized in 3 days after supplementation with autotrophic culture. The effect in the present study coincided with the previous resut. Total chlorophyll contents were higher under Method B than Method A throughout the cultivation period, consistent with improved late-phase growth when pulsed $CO_2$ supplementation was delayed.

## 4. Harvested cell weight and cell yield of *E. gracilis Z* grown in GAS medium under Method A

Under Method A ($CO_2$ supplementation from day 1), harvested biomass after 10 days was higher in the FB-$CO_2$ treatment than in the macro-$CO_2$ control (Fig. 4a). Packed wet biomass for FB-$CO_2$ supplemented was 6.7g from 500 mL culture medium, and for macro-$CO_2$ as control harvested wet cell weight was 4.7g from 500 mL culture medium, corresponding to wet biomass yields (cell yield %) of 0.95% for macro-$CO_2$ and 1.34% for FB-$CO_2$, respectively.

Dry biomass was 0.67 g and 0.47 g, respectively. Statistic differences between treatments were not recognized.

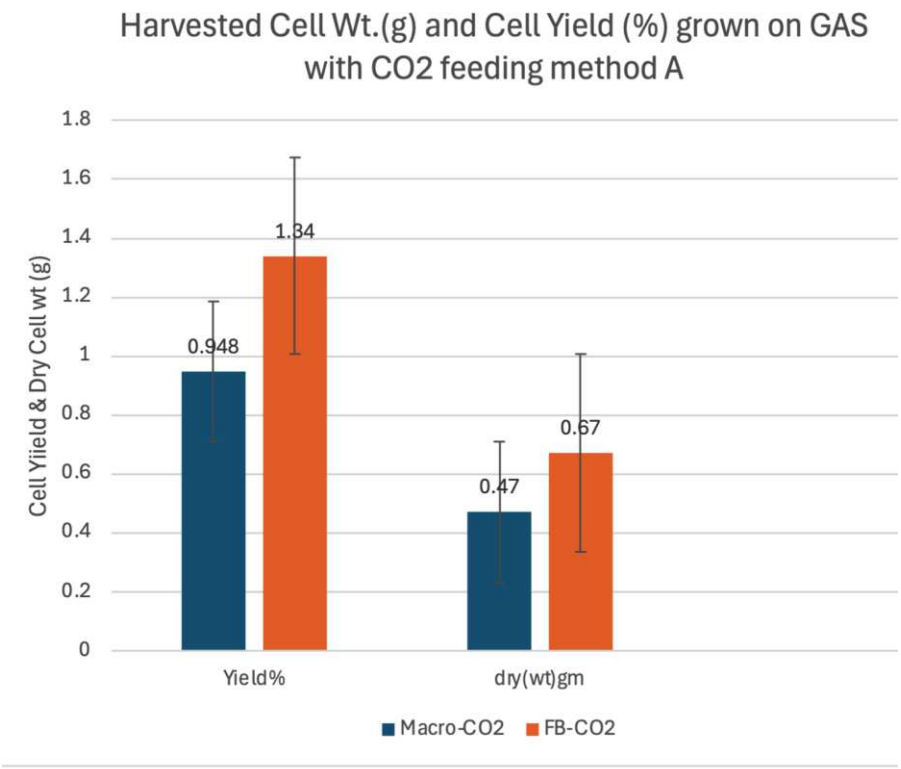



**Fig. 4a** Harvested biomass of *E. gracilis Z* cultured in GAS medium under Method A ($CO_2$ supplementation from the beginning of cultivation, day 1) with macro-$CO_2$ or FB-$CO_2$ 500ml medium. Bars on left indicate packed wet biomass yield % (packed wet-cell weight relative to initial culture volume; far left dark blue bar: 0.948 % for macro-$CO_2$, and second left orange bar 1.34% for FB-$CO_2$) after 10 days cultivation. Bars on right show dry biomass (Blue bar on left: 0.470 g for macro-$CO_2$ and 0.67 g for FB-$CO_2$). Values are means of six measurements with error bars indicating maximum and minimum. Differences were not statistically significant between Macro-$CO_2$ (control) and FB-$CO_2$ supplemented.

## 5. Harvested cell weight and cell yield of *E. gracilis Z* grown in GAS medium under Method B

Under Method B (pulsed $CO_2$ supplementation initiated on day 4), harvested biomass after 10 days was higher in the FB-$CO_2$ treatment than in the macro-$CO_2$ control (Fig. 4b). Packed wet biomass was 10.1 g per 500 mL culture for FB-$CO_2$ and 7.11 g per 500 mL culture for macro-$CO_2$, corresponding to wet biomass yields (cell yield, %) of 2.0% and 1.4%, respectively. Dry biomass was 1.00 g and 0.71 g, respectively. Differences between treatments were not statistically significant.

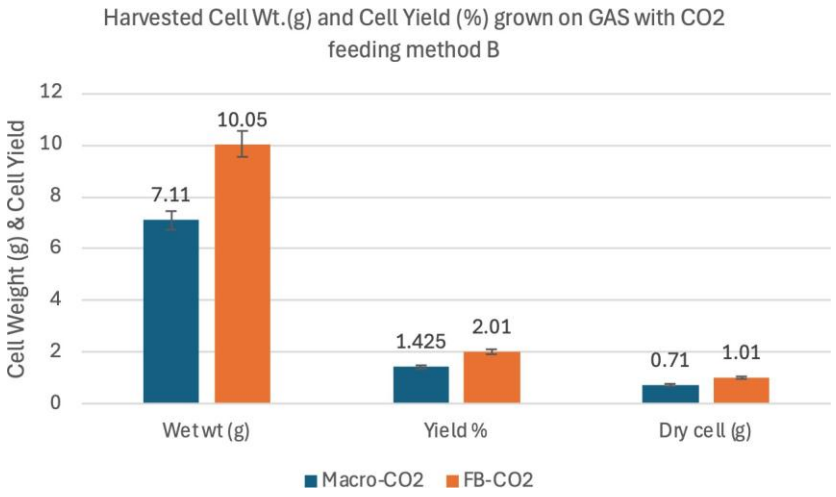



**Fig. 4b** Harvested biomass of *E. gracilis Z* cultured in GAS medium under Method B ($CO_2$ supplementation initiated on day 4) with macro-$CO_2$ or FB-$CO_2$.

Y-axis: Dry cell weight (g) and cell yield (%). (Bars show wet biomass (7.11g & 10.05g; far left panel) and centre bars represent wet biomass yield (cell yield, 1.43 % & 2.01% which were calculated by packed wet-cell weight divided by % of initial culture volume), right bars indicate dried cell mass weight harvested after 10 days cultivation. Values are means of 3 measurements with error bars indicate range of maximum and minimum values.

The wet biomass yields obtained under Method A were 0.95% for macro-$CO_2$ and 1.34% for FB-$CO_2$, whereas those under Method B were 1.4% for macro-$CO_2$ and 2.0% for FB-$CO_2$. These values show a difference between Method A and Method B, suggesting that delaying pulsed $CO_2$ supplementation until after glucose depletion is more effective for biomass production.

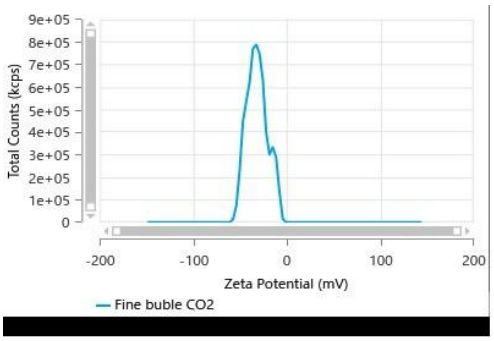
## 6. Zeta-potential of FB-$CO_2$

State of FB-$CO_2$-fed solution measured with Zetasizer Ultra Data measured by Zeta-Sizer Ultra including Zeta potential measured are presented in Table 1 with raw data in Fig. 5a and 5b.

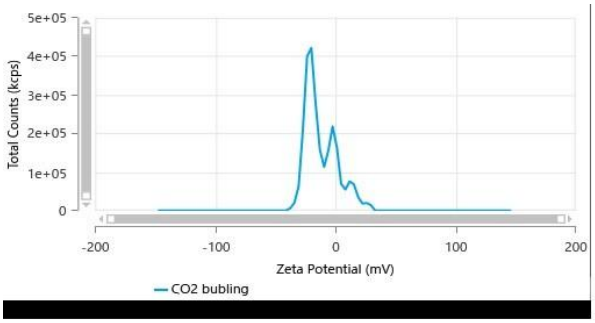
**Table 1** Table 1. Zeta potential of fine-bubble carbon dioxide (FB-$CO_2$)-fed distilled water and macro-carbon dioxide (Macro-$CO_2$)-fed distilled water measured using a ZetaSizer Ultra

| Sample Name | FB-$CO_2$-fed DW | Macro-$CO_2$-fed DW |
|---|---|---|
| Zeta Potential (mV) | -31.2 | -12.12 |
| Conductivity (mS/cm) | 0.01147 | 0.03105 |
| Wall Zeta Potential (mV) | -77.1 | -40.5 |
| Quality Factor | 8.564 | 1.023 |
| Zeta Peak 1 Mean (mV) | -33.92 | -20.59 |
| Zeta Peak 2 Mean (mV) | -13.49 | -2.15 |

The zeta potential of the FB-$CO_2$-fed distilled water is -31.2mV. While zeta potential of solution bubbled with regular $CO_2$ is -12.12 mV. The zeta potential obtained from FB-$CO_2$ fed water was examined its stability that satisfies a requirement for fine bubble $CO_2$.




| Name | Mean | Standard Deviation | RSD | Minimum | Maximum |
|---|---|---|---|---|---|
| Zeta Potential (mV) | -31.2 | - | - | -31.2 | -31.2 |
| Conductivity (mS/cm) | 0.01147 | - | - | 0.01147 | 0.01147 |
| Wall Zeta Potential (mV) | -77.1 | - | - | -77.1 | -77.1 |
| Quality Factor | 8.584 | - | - | 8.584 | 8.584 |
| Zeta Peak 1 Mean (mV) | -33.92 | - | - | -33.92 | -33.92 |
| Zeta Peak 2 Mean (mV) | -13.49 | - | - | -13.49 | -13.49 |




| Name | Mean | Standard Deviation | RSD | Minimum | Maximum |
|---|---|---|---|---|---|
| Zeta Potential (mV) | -12.12 | - | - | -12.12 | -12.12 |
| Conductivity (mS/cm) | 0.03105 | - | - | 0.03105 | 0.03105 |
| Wall Zeta Potential (mV) | -40.3 | - | - | -40.3 | -40.3 |
| Quality Factor | 1.023 | - | - | 1.023 | 1.023 |
| Zeta Peak 1 Mean (mV) | -20.59 | - | - | -20.59 | -20.59 |
| Zeta Peak 2 Mean (mV) | -2.15 | - | - | -2.15 | -2.15 |

**Fig. 5a** and **5b** Raw Data of Zeta Potential measured with ZetasizerUltra. Upper Figure and data indicate raw data obtained from the FB-$CO_2$ fed distilled water. Lower Figure and data indicate raw data obtained from the $CO_2$ bubble fed distilled water.

# Discussion

## Overview of key findings

This study demonstrates that the effects of fine-bubble $CO_2$ (FB-$CO_2$) on mixotrophic growth of *Euglena gracilis* are strongly influenced by the timing of supplementation. When pulsed $CO_2$ supplementation was initiated at the beginning of cultivation (Method A), glucose utilization was slowed and FB-$CO_2$ did not improve cell density relative to conventional $CO_2$ delivery. In contrast, delaying supplementation until day 4 (Method B), after substantial glucose consumption had occurred, resulted in higher cell density and increased biomass production under FB-$CO_2$ treatment.

Chlorophyll accumulation remained low during the glucose-supported phase and increased primarily during the later stages of cultivation, particularly under delayed FB-$CO_2$ supplementation. These findings suggest that FB-$CO_2$ provides the greatest benefit once cultures become increasingly dependent on photosynthetic carbon assimilation rather than organic carbon metabolism.

## Interpreting FB-$CO_2$ properties in acidic, nutrient-rich media

A key uncertainty in applying fine-bubble technology to biological media is the persistence of micro- and nanobubbles after injection. In our previous work, FB-$CO_2$ generation was confirmed by nanoparticle tracking analysis (NanoSight) in a simplified system (Suzuki-Nagata et al., 2025). Furthermore, in addition to NTA analysis with NanoSight, zeta potential measurements using a Zetasizer Ultra supported the presence of a stable, negatively charged $CO_2$ population in FB-$CO_2$-treated distilled water prepared with the same generator ( Table 1 and Figs. 5a and 5b). However, these measurements were performed in a model solution rather than in GAS medium which contains organic and inorganic materials. It is impossible to measure both particle size and zeta-potential of media containing organic and inorganic components directly because those ingredients severely interfere precise determination.

GAS medium is strongly acidic (pH ≤ 3.5) and contains salts and organic compounds that can alter ionic strength, interfacial charge, bubble coalescence, and ammonium sulfate and other ingredients dissolution kinetics; therefore, zeta potential values measured of fine bubbles charged in distilled water may not directly represent the physicochemical state of bubbles in the culture environment. Previous studies of bulk and ultrafine bubbles indicate that bubble number concentration and surface charge can change markedly with pH and ionic composition, and that stability may decrease under strongly acidic conditions (e.g. Nirmalkar et al., 2018; Patois et al., 2012). Accordingly, even if the same dose of $CO_2$ is injected, the fraction that remains as persistent fine bubbles in the glucose-ammonium sulfate medium may decline over time due to dissolution and/or coalescence. This uncertainty motivated the 48-h supplementation interval used here. Future work that directly quantifies bubble size distributions and persistence in GAS medium (or closely matched model media) will be important for linking bubble physics to biological outcomes. Despite disadvantageous information on the determination of zeta potential, it is clear that distict difference of zeta potential between control (distilled water bubbled with $CO_2$) and FB-$CO_2$ distilled water. These differences indicate meaningful difference of FB-$CO_2$ from regularly $CO_2$ bubbled distilled water. Experiments with NanoSight for FB-$CO_2$ bubbled Glucose-Ammonium sulfate medium was impossible due to too much noizes, similarly with ZetaSizer analysis using Glucose-Ammonium sulfate medium.

## Timing-dependent effects on glucose utilisation and chlorophyll accumulation

Mixotrophy in *E. gracilis* reflects its ability to use organic substrates (heterotrophy) while also performing photosynthesis when conditions permit. In the GAS medium used here, glucose is the principal organic carbon source; therefore, changes in glucose depletion and chlorophyll accumulation provide a practical readout of the transition from predominantly heterotrophic growth to a more photosynthetically supported phase (Kempner, 1982).

*E. gracilis Z* can tolerate elevated $CO_2$ and adjust photosynthetic machinery under high $CO_2$ in autotrophic systems (Nakano et al., 1996). Our results show that, under mixotrophic conditions, the response to pulsed $CO_2$ supplementation is modulated by

the availability of organic carbon. While glucose was present, chlorophyll remained low and early pulsed supplementation (Method A) coincided with slower glucose utilisation and weaker growth. After glucose depletion, chlorophyll increased, and delayed pulsed supplementation (Method B) produced higher cell density under FB-$CO_2$ than under macro-$CO_2$, consistent with a more effective transition into a photosynthetically supported phase.

A preference for organic substrates during trophic transitions has also been discussed for other acidophilic photosynthetic microorganisms, where rebuilding photosynthetic capacity can impose substantial biosynthetic and energetic costs (Yamashita et al., 2025). Although the organisms differ, this perspective is consistent with the observation that chlorophyll accumulation in *E. gracilis* was delayed until after glucose depletion in GAS medium.

The glucose profiles suggest that early pulsed $CO_2$ supplementation can interfere with organic-carbon utilisation. Under Method A, pulsed $CO_2$ supplementation from day 1 slowed glucose depletion in both treatments. The FB-$CO_2$ condition showed longer glucose persistence than macro-$CO_2$ (Fig. 1a). In contrast, under Method B, glucose was largely depleted during the initial aeration period (days 1–3), before pulsed $CO_2$ supplementation commenced on day 4 (Fig. 1b). While the mechanism remains to be clarified, several non-exclusive factors could contribute, including $CO_2$-driven changes in intracellular pH regulation, altered redox balance, or shifts in carbon partitioning that affect glucose uptake and metabolism. These results suggest that, in mixotrophic cultivation strategies aiming to leverage FB-$CO_2$, aligning pulsed $CO_2$ supplementation with the post-glucose phase may be critical.

## Implications for biomass production and $CO_2$ supplementation strategy

Across both schedules, harvested biomass after 10 days generally was greater under FB-$CO_2$ than under macro-$CO_2$ (Fig. 4a,b), but total yields were relatively higher when pulsed supplementation was delayed (Method B). This pattern aligns with two-phase cultivation logic: (i) an initial heterotrophic phase supported by glucose, during which excessive early $CO_2$ supplementation may be counterproductive; followed by (ii) a post-glucose phase in which added $CO_2$–especially when delivered as fine bubbles–can more effectively promote chlorophyll accumulation and biomass production. Practically, these results indicate that applying FB-$CO_2$ after organic-carbon depletion may be a more reliable strategy for enhancing productivity than pulsed supplementation from the outset.

From an implementation perspective, FB-$CO_2$ supplementation could support practical, industrial-scale biomass production of *E. gracilis*. More broadly, microalgal cultivation can contribute to $CO_2$ uptake by converting inorganic carbon into biomass; however, the net climate benefit ultimately depends on how the harvested carbon is used or stored, as well as the overall energy balance of the process. Compared with terrestrial forests, microalgae have also been reported to achieve substantially higher areal $CO_2$-fixation rates (often cited as 10–50 times higher under favourable conditions), which is one reason they are frequently discussed as high-efficiency biological platforms for carbon capture and utilisation (Suzuki, 2017). Realising these benefits at scale will

likely require dedicated process engineering (e.g. designing an appropriate production line and integrating suitable glucose-ammonium sulfate-delivery equipment). In our view, FB-$CO_2$ supplementation is best applied in closed cultivation systems, where $CO_2$ use efficiency and contamination control can be better managed.

A further consideration is the broader environmental risk associated with releasing $CO_2$-enriched streams (whether delivered as fine bubbles or conventional bubbles) to natural marine waters. Elevated $CO_2$ can contribute to seawater acidification, which is widely recognised as a key stressor for reef-building corals because reduced carbonate saturation impairs calcification and can exacerbate bleaching-related impacts, including in the Great Barrier Reef region (Fabricius et al., 2020). Accordingly, any large-scale deployment of FB-$CO_2$ technology should prioritise closed or well-contained systems with appropriate glucose-ammonium sulfate capture/neutralisation and monitoring, to minimise unintended $CO_2$ discharge to surrounding aquatic ecosystems.

Mixotrophic cultivation can be integrated with wastewater treatment by using organic-rich effluents as growth substrates while simultaneously removing nutrients; in such systems, the $CO_2$ delivery strategy is a practical lever to shift cultures from organic-carbon utilisation toward a more photosynthetically supported phase once readily available organics are depleted (Molazadeh et al., 2019; Tripathi et al., 2023). Likewise, improving glucose-ammonium sulfate-liquid mass transfer using fine-bubble $CO_2$ may support greenhouse-glucose-ammonium sulfate mitigation by enhancing $CO_2$ capture and conversion into biomass, especially when supplementation is scheduled to coincide with the post-organic-carbon phase (Tripathi et al., 2023). Finally, higher and more stable biomass yields under mixotrophy strengthen the case for Euglena-based production of food, feed and other bioproducts (e.g. paramylon and lipids), offering a pathway that couples resource recovery with biomass generation; notably, *E. gracilis* and related euglenoids have also been investigated for wastewater polishing and for bioremediation of contaminated waters (Nezbrytska et al., 2022; Moreno-Sánchez et al., 2017). Together, these considerations suggest that optimising $CO_2$ supplementation timing is not only a cultivation parameter but also a design variable for coupled wastewater-carbon capture-biomass production systems.

In our previous autotrophic study, FB-$CO_2$ increased chlorophyll content and improved yield relative to conventional $CO_2$ delivery (Suzuki-Nagata et al., 2025). The present results indicate that, under mixotrophic conditions, FB-$CO_2$ benefits are not uniform across the cultivation period and may only emerge once the culture becomes more dependent on photosynthesis. This highlights the importance of matching $CO_2$ delivery strategy to trophic state, rather than assuming that approaches optimised for autotrophy will translate directly to mixotrophy.

## Potential inhibitory effects of early high-dose $CO_2$ under mixotrophy

Previous studies show that *E. gracilis* can acclimate to elevated $CO_2$, and that outcomes depend on culture conditions such as light quality and cellular carbon status (Matsumoto et al., 2007; Nakano et al., 1996; Lv et al., 2024). For example, high $CO_2$ can increase chlorophyll and RuBisCO under some conditions (Nakano et al., 1996),

whereas very high $CO_2$ has also been associated with reduced PSII activity (Nakano et al., 1996). In the present study, the suppressed growth and delayed glucose utilisation observed under Method A suggest that applying pulsed $CO_2$ supplementation from the beginning of mixotrophic cultivation may impose a physiological burden during the glucose-supported phase, even though the organism is acid-tolerant.

Even without high-concentration $CO_2$ supplementation, continuous aeration supplies $CO_2$ at ambient concentration and maintains glucose-ammonium sulfate exchange. In strongly acidic media, dissolved inorganic carbon is expected to be dominated by dissolved $CO_2$/carbonic acid rather than bicarbonate. However, the actual dissolved $CO_2$ concentration and its time course depend on aeration rate, temperature, mixing, and medium composition. Direct measurements of dissolved inorganic carbon (DIC) and pH microenvironments around bubbles would help clarify how $CO_2$ availability differs between macro-$CO_2$ and FB-$CO_2$ delivery in this system.The consistently low chlorophyll content while glucose was present (Figs. 3a, 3b), followed by an increase after glucose depletion, indicates that chlorophyll biosynthesis and/or chloroplast development is downregulated during the heterotrophic phase and recovers later. Early high-dose $CO_2$ supplementation–particularly via FB-$CO_2$–may reinforce this suppression, for example, through carbon-status signalling, intracellular pH regulation, or stress responses. Testing these possibilities will require measurements of photosynthetic performance (e.g., chlorophyll fluorescence/PSII efficiency), key enzyme activities (e.g., RuBisCO), metabolite profiling across the transition, and $CO_2$ availability.

## Mechanistic interpretation of the timing effect

Together, the results can be interpreted as a timing mismatch between carbon source and $CO_2$ delivery. Method A applies pulsed $CO_2$ supplementation during the glucose-supported phase, when photosynthetic capacity is low, and carbon flux is directed to heterotrophic metabolism; under these conditions, additional $CO_2$ may provide little benefit and could impose stress or regulatory inhibition that slows glucose utilisation and growth. Method B postpones pulsed $CO_2$ supplementation until the culture enters a post-glucose phase, when photosynthetic machinery is more readily induced; under this schedule, enhanced $CO_2$ availability–potentially improved by fine-bubble delivery–can support chlorophyll accumulation and higher cell density.

The chlorophyll time course supports this two-phase interpretation. Under both schedules, chlorophyll remained comparatively low in the early period and then increased after glucose depletion, with the most pronounced late-phase increase observed under FB-$CO_2$ (Figs. 3a, 3b). The several-day delay between glucose exhaustion and chlorophyll accumulation suggests that induction of photosynthetic capacity is not immediate and may require cellular remodelling. In this context, supplying FB-$CO_2$ after glucose depletion may be advantageous because it increases $CO_2$ availability during the window in which chlorophyll and photosynthetic capacity are being rebuilt.

Importantly, the timing-dependent differences in cell density and chlorophyll were reflected in harvested biomass, with Method B producing markedly higher packed wet biomass and dry biomass than Method A (Fig. 4a,b). This suggests that optimising

the transition into the post-glucose phase is central to improving overall productivity in GAS medium.

### Limitations and future directions

The present study focused on growth, glucose utilization, chlorophyll accumulation, and biomass production. Consequently, the physiological mechanisms underlying the observed timing-dependent responses remain unresolved. Measurements of photosynthetic performance, chlorophyll fluorescence, RuBisCO activity, dissolved inorganic carbon dynamics, and intracellular metabolite profiles were beyond the scope of this investigation.

Furthermore, fine-bubble stability and size distribution were not measured directly in GAS medium. Future studies should quantify bubble persistence under cultivation conditions and investigate how dissolved inorganic carbon availability changes following supplementation. Such analyses would provide important mechanistic insight into the relationship between FB-$CO_2$ delivery and cellular physiology.

## Conclusion

This study demonstrates that the effectiveness of fine-bubble $CO_2$ supplementation in mixotrophic cultures of *Euglena gracilis* depends strongly on the timing of $CO_2$ delivery. When FB-$CO_2$ supplementation was initiated at the start of cultivation, glucose utilization was delayed and no growth advantage was observed relative to conventional $CO_2$ delivery. In contrast, initiating supplementation after the initial glucose-utilization phase resulted in higher cell density, increased chlorophyll accumulation during the later cultivation period, and greater biomass production.

These findings suggest that FB-$CO_2$ supplementation is most beneficial after depletion of the primary organic carbon source, when cultures become increasingly dependent on photosynthetic carbon assimilation. Optimizing supplementation timing may therefore provide a practical strategy for improving biomass productivity in mixotrophic *E. gracilis* cultivation systems. Future studies should investigate the physiological and physicochemical mechanisms responsible for this timing-dependent response.

## Data availability

The data supporting the findings of this study are provided in the Supplementary Material file: associated with this manuscript.

## Author contributions

S.S.N.: Conceptualisation, methodology, investigation, validation, data curation, writing the original draft, writing the manuscript, preparation of figures, and editing. N.M.: Preparation and supply of fine-bubble $CO_2$, data analysis, and validation. J.C.N.: Performing data assessment and reviewing the manuscript. T.S.: Assist planning

experimental procedures, providing technical assistance, analysing data, and preparing drafts.

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The authors declare no potential conflicts of interest. During preparation of this manuscript, the authors used Co-pilot, Paperpal and GPT for Microsoft Word for grammar checking and refinement of the text's logical flow. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the final content of the publication.

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## Supplementary Files

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- SupplementaryMaterials111326.pdf