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Research Article

A simple CO_2 enrichment incubator for investigating physiological responses of harmful algae to ocean acidification

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Abstract

A CO_2 manipulation incubation system using off-the-shelf components was developed to study the effects of ocean acidification (OA) on marine microalgae. The system successfully monitored CO_2 concentrations in real time at the desired levels. The incubation experiment was based on the IPCC's CMIP6 worst-case scenario (SSP5-8.5), with elevated CO_2 concentrations of up to 1000 ppm. Under these conditions, exposure to 1000 ppm CO_2 significantly increased growth rate, cell diameter, and biovolume of harmful microalgae, *Alexandrium tamiyavanichii*. These effects were more pronounced, highlighting the potential for ocean acidification to exacerbate harmful algal blooms. The study also emphasized the importance of accounting for light attenuation in the incubation setup, revealing a 20% loss in light within culture bottles due to uneven light distribution. Correcting light intensity variations caused by the materials of the culture vessels was essential for unbiased growth assessments.

Keywords: Ocean acidification, CO_2 enrichment, experimental setup, harmful algae, physiological studies

Introduction

Ocean acidification (OA) refers to the long-term decline in oceanic pH and shifting of seawater carbonate chemistry, primarily due to increased carbon dioxide (CO_2) in the atmosphere [1]. A projection suggested that the surface ocean pH may decline by 0.2 to 0.4 units by the year 2100 [2]. There have been growing concerns about OA effects on the frequency, duration, and intensity of harmful algae bloom [3–7]. Gaining insights into the physiological, molecular, and community responses of microalgae to environmental changes such as pH holds paramount importance in grasping their holistic ecological influence, particularly considering that certain rapid-growing harmful algae species can exhibit a negative impact on the ecosystems [8].

There are several approaches to manipulating seawater CO_2 to investigate the future response of marine organisms to ocean acidification. Adding strong acid and base is one of the simpler methods to monitor the effect of pH changes on the microalgae [9, 10]. However, this approach alters the total alkalinity (A_T) while keeping the dissolved inorganic carbon (DIC) concentration unchanged, which differs from conditions expected in the future (lower pH and CO_3^{2-}, higher HCO_3^-) [11]. Therefore, most OA-related studies have chosen bubbling the experimental seawater at target CO_2 [4, 12, 13]. Plant growth cabinets using commercial pre-mixed gases may seem convenient for OA studies [30], but they require a significant quantity of CO_2-enriched gases. For instance, a size K tank (1.5 meters, 7100 L) at a flow rate of 1 L/min would supply CO_2 for only five days, which is insufficient for the typical 14-day duration of a harmful algae growth study encompassing the lag, exponential, and stationary phases [14].

In this study, we designed a CO_2-induced OA stimulated system using off-the-shelf materials to study harmful algae (**Figure 1**). The goal of the study

is to provide a controlled and reliable methodology for investigating the physiological responses of harmful algae to future ocean acidification scenarios. This paper describes the design and application of the system and evaluates its performance in maintaining precise control over carbonate parameters. The physiological responses of *Alexandrium tamiyavanichii* to different OA conditions were assessed, demonstrating the utility of this system for OA-related research.

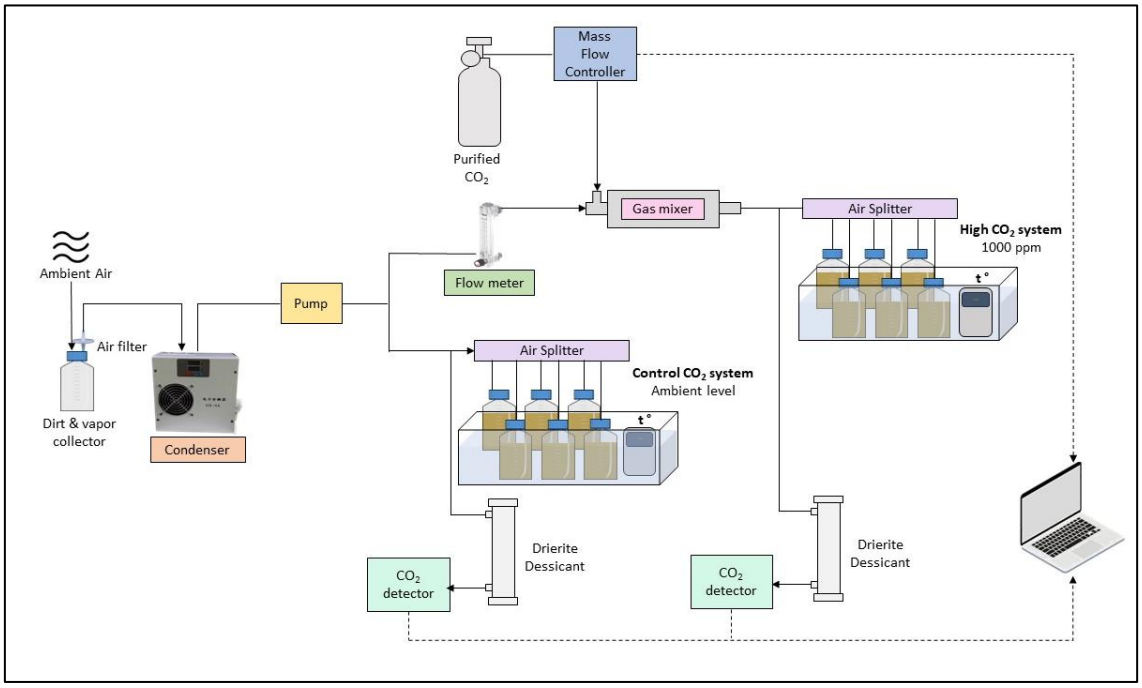

Figure 1. The schematic of the CO_2-induced OA stimulated system. The solid lines indicate the gas flow tubes whereas dotted lines represent electrical connections

Materials and Methods

Chamber design

There were several aspects of chamber design for the CO_2 enrichment incubation system. Firstly, the chamber must maintain a stable and uniform temperature, which is ideal for algae growth. Therefore, the incubation chamber was designed as a water batch configuration, utilizing an acrylic aquarium tank measuring 15 cm in height, 50 cm in length, and 30 cm in width. The experiment was conducted in a temperature-controlled laboratory, and an aquarium heater/chiller was employed to maintain the incubation tank temperature.

Besides temperature, light is also a necessary environmental factor that significantly influences algae growth, photosynthesis, and physiological responses. The lighting system of the chamber comprised four T8 LED 6500K daylight tubes (Philips, Malaysia). The incubation chamber was consistently covered with a black cloth to prevent interference from stray light. For the light variability test, 10 HOBO temperature/light loggers (Onset Computer Corporation, USA) were initially calibrated using an underwater spherical quantum sensor connected to a light sensor logger (LI-1500, LI-COR Biosciences, USA). This calibration enabled the conversion of light units from lux to photosynthesis photon flux density (PPFD). The loggers were then systematically positioned at 30 locations within the incubation chamber, encompassing positions directly under the light source, submerged within the water bath, and immersed within the bottles placed in the water bath. The light intensity was recorded at five-second intervals over 10 minutes.

Lastly, the culture bottle was designed to prevent gas exchange with the external environment and contamination-free collection of samples during the experiments. The culture vessel consisted of a 500 mL borosilicate bottle equipped with a screw cap featuring three ports. The first port was designated for culture sampling, while the other two served as entry and exit points for ambient air or pre-mixed air/CO_2 gas (**Figure 2**). The sampling port was kept closed using a sterile three-way stopcock to prevent contamination and was only opened during sampling.

Seawater carbonate chemistry manipulation

To evaluate the performance of CO_2 enrichment

Figure 2. The designed culture flasks in this study. The red and green arrows indicate the air inlet and outlet respectively, while the blue arrow represents the sampling port outlet.

incubation system in maintaining precise control over carbonate parameters, we treated the seawater under two CO_2 conditions: ambient CO_2 and high CO_2 (1000 ppm) for several days. The carbonate characteristic of seawater was then determined. The pH was determined using a UV-VIS spectrophotometer, and the indicator dye m-cresol purple was used according to the procedure proposed in [16]. The water samples were titrated with 0.1 M of hydrochloride acid to measure the total alkalinity (A_T) [16]. We calculated the HCO_3^-, CO_3^{2-}, CO_2 and total dissolved inorganic carbon using CO_2SYS with the carbonic acid dissociation constant proposed by [17].

The application of CO_2 enrichment incubation system in harmful algae physiological studies

A 12-day experiment was conducted to study the effect of ocean acidification on the growth rate of *Alexandrium tamiyavanichii* and assess the effectiveness of the designed CO_2 enrichment incubation system in harmful microalgal physiological studies. The experiment duration was based on a previous work [18], which found that the exponential growth phase of this species occurs between days 6 and 10. The experiment was conducted at the Institute of Oceanography and Environment, Universiti Malaysia Terengganu, where colonies of *A. tamiyavanichii* were obtained from Bachok Marine Research Station, the Institute of Ocean and Earth Sciences, University of Malaya. The cultures were grown in ES-DK medium [19], prepared using 0.2 µm-filtered, autoclaved natural seawater with a salinity of 30 ppt. The cultures were maintained at 25±0.5 °C under a 12: 12 h light dark cycle.

Approximately 500 cells per milliliter (cell mL^{-1}) of cultures from the culture stock were transferred into a fresh medium that have been acclimatized at two different CO_2 levels: ambient and 1000 ppm, the latter represented the predicted atmospheric CO_2 concentration based on the IPCC's CMIP6 and modeled the worst-case scenario SSP5-8.5 [2]. The culture samples were collected every two days and preserved using acidified Lugol's iodine solution. The cell density was quantified using a Sedgewick Rafter counting chamber under a compound microscope. The specific growth rate (µ, d^{-1}) was calculated using the following equation [20]:

$$\mu = \frac{\ln N_1 - \ln N_0}{t_1 - t_0} \quad \text{(Eq. 1)}$$

where N_1 and N_0 are the cell densities (cells mL^{-1}) at time t_0 and t_1 (days), respectively.

To measure the cell diameter, culture samples on day 8 were collected and the measurement is made using an Olympus CX23 microscope (Olympus, Japan) under 200× magnification. To calculate the cell volume, the following equation is used [21]:

$$V = \frac{\pi}{6} d^3 \quad \text{(Eq. 2)}$$

where *d* is the mean of cell diameter from 30 cells.

Statistical analysis

A statistical analysis assessed the relationship between independent and dependent variables in this study. Turkey's HSD of One-Way ANOVA was carried out to compare the PPFD differences under three logger positions. Independent samples t-test was conducted to compare the growth rate of *Alexandrium tamiyavanichii* under ambient and high CO_2 conditions. All statistical analyses were performed using IBM SPSS Statistics 25 with a

significant level of α = 0.05 (confidence level of 95%).

Results and Discussion

Accuracy and stability of CO_2 concentrations

The primary goal was maintaining a stable CO_2 concentration of approximately 1000 ppm, corresponding to IPCC predictions for ocean acidification scenarios by 2100. The mass flow controllers successfully regulated the flow rates of ambient air and CO_2 flow rate. The target CO_2 concentration was consistently maintained within ± 10% of 1000 ppm. **Figure 3** shows the pCO_2 values delivered in the system that were continuously monitored. Overall, the CO_2 incubation system had been successfully operated for over 12 days, with 489 ± 20 ppm and 934 ± 59 ppm for the ambient CO_2 and high CO_2 settings, respectively. Diurnal fluctuation of pCO_2 was observed in both settings, demonstrating that our system closely mimics realistic atmospheric CO_2 conditions [22, 23]. Ruling out the diurnal variation, the system was stable throughout the study. The stability of CO_2 delivery in the system ensured a constant air-water exchange of CO_2.

During the first 6 days of the experiment, the pCO_2 readings exhibited noticeable fluctuation, which was likely due to the presence of humidity. Improvements were made by introducing a desiccant before CO_2 analyzers measured the air to address this issue (**Figure 1**). Moreover, several improvements were made after the initial observation. Although a 0.2 µm hydrophobic filter was equipped to filter the air pumped into the system, it was not entirely effective in preventing water vapor and dirt, such as insect bodies, leading to the rapid clogging and reduced filter efficiency. Therefore, a vapor and dirt collector, made by an air-tight borosilicate gas washer bottle (Duran®, DWK Life Science, Germany), was used to prevent the water and contaminants from entering the system. Additionally, an electrical condensing dehumidifier (CS-7A; Xianzhuokeji Co. Ltd., China) was employed to minimize moisture from the inlet air. **Figure 3** demonstrates that the pCO_2 readings returned to the expected values once the humidity issue was resolved after day 5.

Three aeration methods were assessed for CO_2 delivery into seawater with a salinity of 30 ppt: 1) bubbling with CO_2 diffuser with at a flow rate of 0.1 L min^{-1}, 2) bubbling with CO_2 diffuser at a flow rate of 0.2 L min^{-1} and 3) bubbling without CO_2 diffuser with a flow rate of 0.2 L min^{-1} (**Figure 4**). The result showed that using CO_2 diffusers promotes faster CO_2 dissolution, allowing us to achieve the desired target pH (decrease of ~ 0.2-0.4 unit as IPCC's projection [2]. The higher the CO_2 flow rate, the faster the pH of water reaches its equilibrium. Approximately 40 minutes were taken to equilibrate by bubbling with flow rate of 0.2 L min^{-1}. However, when approaching the subsequent CO_2 treatment experiment involving harmful algae, consideration was given as some microalgae, especially dinoflagellates, might be affected by small-scale water turbulence [24]. Therefore, a cautious approach was suggested to mitigate potential adverse effects. Initially, the experimental medium was aerated at a high flow rate until target pH was reached. Then, gradually reduce the flow rate to around 0.1 L min^{-1} after the microalgae cultures were transferred into the experimental medium. This approach effectively balanced the need for efficient CO_2 equilibration with the need to protect the integrity of the algae cultures.

Temperature and light intensity control in the chamber

The design of the incubation chamber was efficient, maintaining stable and uniform temperatures, with a precision of ±0.3°C. **Figure 5** shows the photosynthetic photon flux density (PPFD) measured at three logger positions where 1) the loggers were directly exposed to the light source, 2) loggers were submerged under the water bath and 3) loggers were inside the bottles immersed in the water bath. The corresponding PPFD values were recorded at 359 ± 31, 351 ± 22, and 280 ± 24 µmol $m^{-2}s^{-1}$, respectively. The comparison study showed there was no significant difference between loggers directly exposed to the light source and those in the water bath (n = 30, p >0.05). However, approximately 20 % of light supplied was lost when comparing the mean of PPFD directly under the water and in the incubation vessels (n = 30, p <0.05). The findings suggested that there might be absorption, scattering and reflection of light by the glass wall of the borosilicate culture bottles, limiting the effective amount of light reaching the culture in the bottles. These important findings provide an essential base for defining the light intensity within the culture vessels before the experiment. In the following experimental investigation of OA effect on harmful algae growth, we standardized the light intensity supplied to the culture with those measured in the culture bottles.

Moreover, there was uneven light distribution at different water bath positions (n = 10, p <0.05) within three logger positions (**Figure 5**). The light inconsistency led to algae cells experiencing varying zones of light intensity. To address the inconsistency, we repositioned two out of four lamps from top to the sides of the acrylic incubation tank. Positioning

the light sources at different angles has created a more balanced light environment. This adjustment aimed to counteract the localized intensity variations and promote a more uniform light distribution. Furthermore, these findings underscore the importance of increasing the number of experimental replicates to enhance the reliability of the results.

Assessment of seawater carbonate chemistry manipulation

Table 1 shows the carbonate characteristics of seawater for both CO_2 treatments. The results demonstrate that the designed incubation system effectively controlled the carbonate parameters, including pH, AT and other carbonate species. The observed changes in pH and carbonate ion concentrations under high CO_2 treatment align with expectations for ocean acidification scenarios. These findings confirm that the system provides a reliable platform for studying the effects of ocean acidification on harmful algae and their physiological responses.

Application of CO_2 enrichment incubation system in harmful algae physiological studies

The CO_2 enrichment incubation system successfully demonstrated the growth responses of *Alexandrium tamiyavanichii* (**Figure 6**). The harmful dinoflagellate *A. tamiyavanichii* successfully grew under both CO_2 condition treatments. The cell densities of the CO_2 treatments showed an observable lag phase during the first two days and started to grow exponentially until reaching the stationary phase (day 12). There was no significant difference (n=6, p >0.05) in the cell densities during the first six days after inoculation. Subsequently, higher cell densities from high CO_2 treatments were observed compared to ambient CO_2 treatments. The mean growth rate was higher (μ=0.28 day^{-1}) when subjected to elevated CO_2 conditions (~ 1000 ppm).

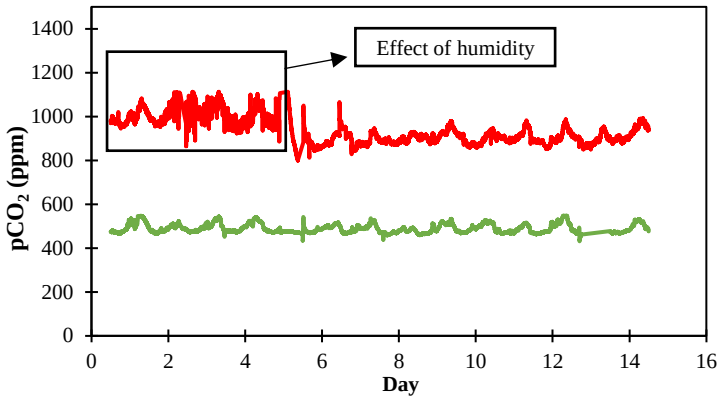

Figure 3. pCO_2 values delivered in the system. The red line indicates high pCO_2 condition and the green line stands for ambient pCO_2 condition

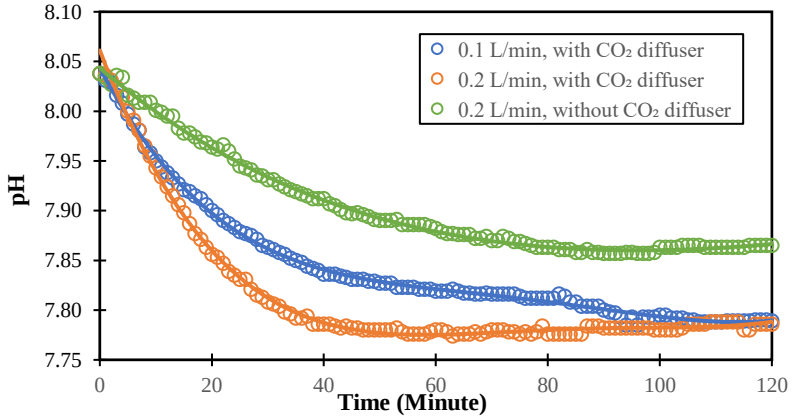

Figure 4. pH changes versus the time, where 1. bubbling with CO_2 diffuser at a flow rate of 0.1 L min^{-1}, 2. bubbling with CO_2 diffuser at a flow rate of 0.2 L min^{-1} and 3. bubbling without CO_2 diffuser at a flow rate of 0.2 L min^{-1} respectively

Table 1. Carbonate chemistry for both CO_2 treatments. pH values are displayed on the total scale. The units of total alkalinity (A_T), HCO_3^-, CO_3^{2-}, CO_2 and dissolved inorganic carbon (DIC) are µmol kg SW^{-1}

Treatments	pH	A_T	HCO_3^-	CO_3^{2-}	CO_2	DIC
Ambient CO_2	8.06 ± 0.01	1792 ± 317	1400 ± 259	154 ± 29	9 ± 1.6	1563 ± 289
High CO_2	7.59 ± 0.01	1551 ± 41	1415 ± 36	52 ± 2	27 ± 0.3	1494 ± 39

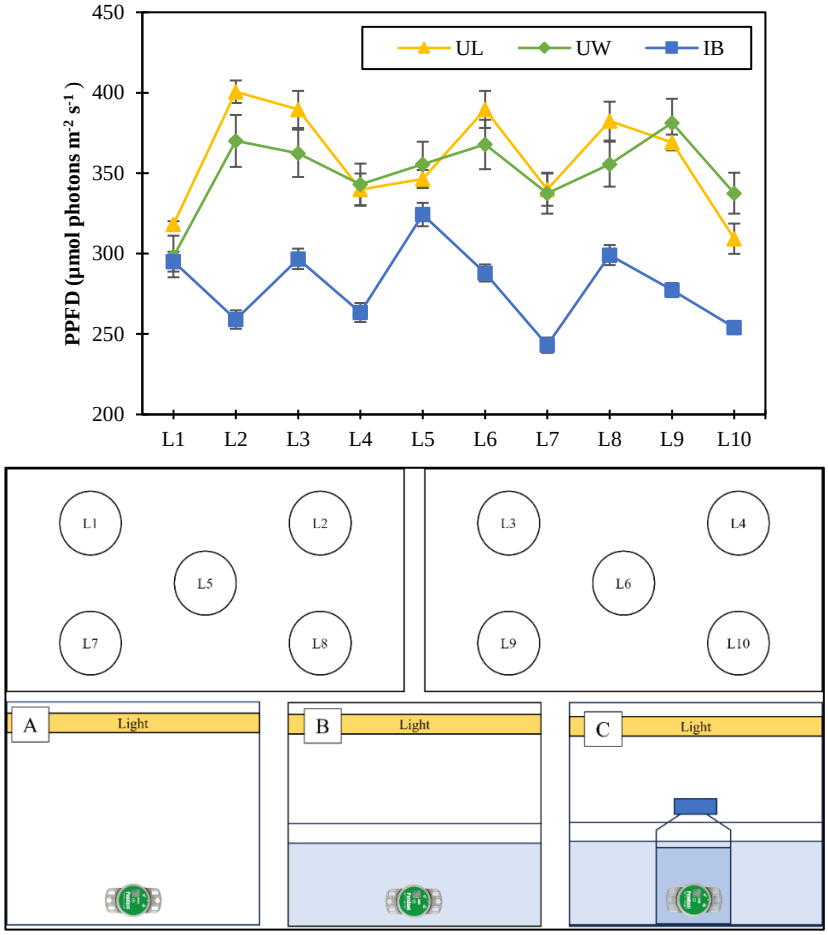

Figure 5. Upper panel: The photosynthetic photon flux density (PPFD) measured at three different positions as shown in (A) loggers directly exposed to light source (UL), (B) loggers submerged under the water bath (UW) and (C) loggers inside the bottles immersed in the water bath (IB). L1-L10 represents the position of incubation bottles within the water tank as shown in the lower panel

The finding highlights the potency of *A. tamiyavanichii* increasing its growth in future elevated CO_2 conditions. This observation is consistent with the outcomes of previous OA studies conducted on various HAB species [12, 13, 25]. Scientists were likely to link the response to carbon acquisition of the species and their ability to regulate their carbon concentrating mechanism (CCM) [27]. The evolution of dinoflagellate can be traced back to 150 to 400 million years ago when the atmospheric CO_2 concentrations were around 1000 to 3000 ppm [28]. Most of the dinoflagellates have evolved into a low-affinity form of the CO_2-fixing enzyme, ribulose-1,5-bis-phosphate carboxylase-oxygenase (type II Rubisco) [29]. Due to the lower affinity, their Rubisco enzyme is not as efficient in capturing CO_2 from their surroundings, especially when the CO_2 concentration is low. However, they might benefit from increased CO_2 concentration because high CO_2 levels help them to overcome the limitations of Rubisco II. In this case, elevated CO_2 conditions allow them to enhance their photosynthetic efficiency and rate of carbon fixation rate, increasing growth.

Table 2. Growth rates, cell diameter, and biovolume measured at different CO_2 treatments (mean ± standard deviation)

Treatments	Growth Rate (Day^{-1})	Cell Diameter (µm)	Cell Biovolume ($µm^3$)
Ambient CO_2	0.21 ± 0.01	36.9 ± 4.4	27571 ± 9961
High CO_2	0.28 ± 0.03	39.2 ± 4.1	32612 ± 9516

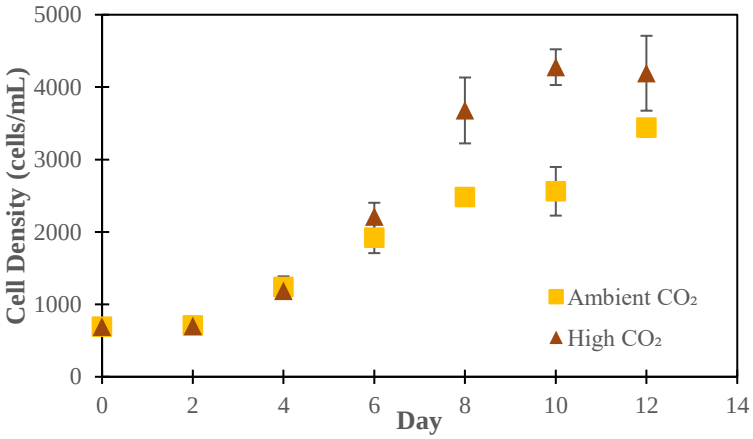

Figure 6. The growth curves of *Alexandrium tamiyavanichii*, ACKP12 under two different CO_2 conditions

In addition to growth, the results in **Table 2** suggest that an increase in cell diameter and biovolume might be one of the consequences of elevated CO_2 levels in future environments. In natural settings, the cell size of phytoplankton plays a crucial role in various physiological processes, including growth, metabolic rate, nutrient uptake and diffusion, light absorption, and cellular composition. It also influences ecological processes such as sinking velocity and ecological interactions [14]. The complex mechanisms by which phytoplankton respond to rising CO_2 have captivated the attention of scientists, driving further exploration into biological and ecological effects on harmful species, including their ability to produce toxins.

Several CO_2 studies on toxicity of harmful microalgae have suggested that many of them can accumulate toxin compounds or transform the toxins into more potent forms with higher toxicity equivalents [13,30]. Study on two harmful dinoflagellate species, *Scrippsiella trochoidea* and *Alexandrium catenella* also had revealed that there was a relocation of energy from C to N assimilation under the elevated pCO_2 conditions, due to lower costs in C acquisition [7]. However, there is limited investigation on future increasing CO_2 toxicity in this study. Previous study on this species revealed that its cellular toxin content can reach up to approximately 180 fmol $cell^{-1}$ when it exposed to optimum conditions [18]. This highlights the necessity to continue exploring the toxicity potential that could be affected by the rising CO_2 in the future.

Conclusion

In this study, we developed and optimized a CO_2-induced OA stimulated system to study the impact of ocean acidification on harmful microalgae. The system successfully maintained precise control over environmental parameters such as CO_2 concentrations, pH, and temperatures, which are crucial for studying the physiological responses of harmful microalgae to elevated CO_2 levels. The results demonstrated that *Alexandrium tamiyavanichii* exhibited a significantly higher growth rate under high CO_2 conditions, highlighting the potential for ocean acidification to promote the proliferation of harmful algal blooms in the future.

Although the developed system provides a significant and accessible solution for studying ocean acidification and its effects on harmful microalgae, the current implementation was designed as a batch culture system, which involves a shorter exponential phase for the most harmful microalgae. Further improvements are necessary for long-term monitoring of marine microalgal growth responses towards different CO_2 levels.

Future research should focus on developing continuous culture systems that allow for extended observation periods, enabling the study of long-term effects on target algae under varying CO_2 concentrations. Additionally, incorporating multi-stressor conditions, such as ocean acidification and hypoxia combined, could provide a more comprehensive understanding of the environmental factors influencing harmful algal blooms. Implementing such systems would enhance the ecological relevance of laboratory findings and contribute to more accurate predictions of future marine ecosystem dynamics.

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