Development of Photobioreactors and Approach for Large-Scale Production of Marine Algae *Chrysotila* and *Nannochloropsis* in Inland Environment

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Abstract of the Dissertation

Due to energy, environment, and food problems, research on microalgae is increasingly gaining attention. Microalgae can utilize photosynthesis to synthesize and accumulate various valuable bioproducts such as lipids, proteins, polysaccharides, pigments, and can also absorb and fix carbon dioxide. Therefore, efficiently, and cost-effectively cultivating specific algae species has become a crucial goal. However, various challenges hinder the development of algae during cultivation. This study focuses on the indoor and outdoor cultivation of two marine microalgae, *Chrysotila (Pleurochrysis) dentata* and *Nannochloropsis oceanica*.

For the cultivation of *Chrysotila (Pleurochrysis) dentata*, optimal indoor cultivation conditions were determined with a light cycle of 18 hours light / 6 hours dark, pH of 8.5, and salt concentration of 2.5 %. When *Chrysotila (Pleurochrysis) dentata* formed a symbiotic system with the bacterium *Nitratireductor aquibiodomus*, a ratio of 8:2 (algae to bacteria) resulted in optimal accumulation of dry weight, chlorophyll α, and calcium carbonate, while ratios 6:4 was more suitable for lipid accumulation. In the experiment on the impact of microplastics polyethylene terephthalate (PET) on *Chrysotila (Pleurochrysis) dentata*, it was observed that low concentrations of plastic (5-15 mg/L) promoted algae growth. However, high concentrations of microplastics (20-50 mg/L) slowed algal growth.

In the case of marine microalgae *Nannochloropsis oceanica*, chlorophyll fluorescence analysis indicated that 0.5 g/L urea was suitable for *Nannochloropsis oceanica* cultivation. However, it is advised to avoid using ammonium chloride as a nitrogen source due to its strong inhibitory effect on *Nannochloropsis oceanica*. Maintaining the salt concentration

between 2.5 % and 3.5 % during cultivation is recommended. Outdoor cultivation experiments revealed that increasing the initial inoculation amount of *Nannochloropsis oceanica* effectively mitigated growth inhibition during cloudy weather. *Nannochloropsis oceanica* shows slower growth in winter. However, it can still grow in low temperatures, and overcast conditions with an initial inoculation concentration exceeding (OD_{680}： 1.0).

Additionally, the results of 185 days of semi-continuous cultivation showed that the cultivable temperature range for *Nannochloropsis oceanica* was 5-30 °C, with the optimal cultivation temperature range being 14-25 °C. *Nannochloropsis oceanica* can accumulate lipids at low temperatures. The study also utilized a low-cost 150 L linear low-density polyethylene (LLDPE) plastic tank for outdoor cultivation, and an analysis of the results revealed that the low-cost, small-volume cultivation equipment exhibited significant advantages in terms of growth rate and biomass productivity compared to the 700 L tank, making it more competitive. However, it is crucial to note that the 150 L tank is more susceptible to environmental influences, leading to significant fluctuations in chlorophyll fluorescence efficiency during semi-continuous cultivation, indicating stress. In contrast, such stress conditions were almost nonexistent in the 700 L tank.

Moreover, a novel in situ real-time oxygen release rate measurement method was developed. This approach provided real-time information on algae oxygen release rates. Whether cultivating *Nannochloropsis oceanica* in a 700 L tank or a 150 L tank, shading during the summer was proven effective in mitigating the inhibitory effects of strong light and high temperatures, thus extending the year-round outdoor cultivable time. *Nannochloropsis oceanica* exhibited a certain degree of cold tolerance, allowing for a

potential extension of cultivation time in winter. Additionally, when temperatures dropped below 5 °C, reducing the frequency of semi-continuous cultivation was recommended. From results, the cultivation cost of *N. oceanica* ranged from $3.35 to $11.89 per kilogram, the price is lower than the current market price (Alibaba 2024 April price: USD $20.19 to $35.34 per kilogram).

Overall, this study delved into the challenges and solutions of indoor and outdoor cultivation of marine microalgae, refining the cultivation conditions for two marine microalgae and identifying potential application areas. A new microalgae photobioreactor design was created, and a novel real-time oxygen release rate monitoring method was established, providing new perspectives for future algae research.

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Introduction of the Thesis

The successive occurrences of the petroleum crisis, food crisis, and climate crisis have prompted people to actively seek solutions. Microalgae, due to their ability to fix carbon dioxide through photosynthesis, rapid growth, and the accumulation of valuable products such as lipids, proteins, polysaccharides, and pigments, have garnered significant attention. However, the reality is that our understanding of algae is still in its early stages, and even within the research community, it remains a niche species. Cultivation techniques pose numerous challenges that require ongoing refinement and improvement.

In algal cultivation, research on indoor cultivation and the cultivation of marine microalgae in inland areas is relatively limited. Most studies on marine microalgae rely on coastal regions (Boruff et al., 2015; Barakoni et al., 2015). However, coastal cultivation of marine microalgae also faces challenges, particularly susceptibility to contamination by other microorganisms or algae in marine environments, which can impact production efficiency. In practical production processes, the majority of commercial cultivation employs open systems, inevitably leading to contamination by bacteria or other algae, affecting product quality. Hence, research on the symbiosis of bacteria and algae and their mutual interactions becomes significant. In some cases, the presence of an appropriate number of bacteria can even promote algal growth and development, and can be set as symbiotic systems, such symbiotic systems have been utilized for wastewater purification such as farm wastewater (Ramanan et al., 2016; Saravanan et al., 2021; Sun et al., 2022; Humenik et al., 1971; Johnson et al., 2020).

Furthermore, marine pollution, especially the presence of microplastics, has an impact

on the growth and development of microalgae. Scholars have reported that microplastics have entered human bloodstreams, indicating their entry into the food chain cycle (Leslie et al., 2021). Whether primary producers such as microalgae are affected by microplastics remains unclear.

In outdoor cultivation, the weather and the location of cultivation systems have a more pronounced impact on algae cultivation. Designing low-cost, efficient light cultivation systems has been a focal point of this research. Currently, most algae cultivation systems are set up in open areas without shading (Osama et al., 2021). However, even in shaded areas, the slow diffuse sunlight intensity is higher than indoor artificial lighting. In densely populated areas, where land is precious, efficiently utilizing various lands to produce microalgae not only enhances land use efficiency for producing high-value-added products but also contributes to carbon sequestration and air purification, yielding multiple benefits.

Additionally, in outdoor environments, fluctuating conditions necessitate effective monitoring of algae growth status and the development of corresponding strategies for different weather conditions. The use of chlorophyll fluorescence technology to detect plant stress has been widely studied. Analyzing the intensity and specific parameters of chlorophyll fluorescence provides crucial information about algae' s growth status, photosynthetic efficiency, stress response, and other important physiological aspects (Xing et al., 2007; Samuelsson et al., 1977).

Experiments were conducted using two characteristic marine microalgae, *Chrysotila (Pleurochrysis) dentata* and *Nannochloropsis oceanica*. *Chrysotila (Pleurochrysis) dentata* is of interest due to its ability to absorb environmental carbon sources, forming calcium

carbonate shell, and its high lipid content, making it a focus in recent years for carbon sequestration applications. *Nannochloropsis oceanica*, on the other hand, is a commonly used feed algae (Li et al., 2020; Gong et al., 2020; Ashour et al., 2019).

In summary, this experiment aims to address challenges faced by microalgae, such as symbiosis with bacteria, microplastic pollution, the design of novel photobioreactor, and considerations for large-scale outdoor cultivation. The goal is to refine solutions to potential challenges in practical production and contribute to the advancement of algology, explore the feasibility of cultivating marine microalgae in inland areas.

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Chapter 1. Literature review：Overview of marine microalgae

1 Introduction

Microalgae also called unicellular algae, is one of the earliest organisms on earth. Their simple structure, single cell or single cell population, contained chlorophyll α can through photosynthesis autotrophic life, widely distributed in fresh water and seawater, individuals generally in a few microns or even dozens of microns. The earth currently known more than 30, 000 kinds of microalgae, accounting for 70 % of the global known algae species (Guschina et al., 2006). Microalgae in the ocean are the main primary producers in the marine ecosystem. Due to their small individuals, fast growth rate, short reproductive cycle, and strong adaptability to the environment, they play an extremely important role in the energy flow and material cycle of the marine ecosystem. Among all marine microalgae, *Chrysophyta, Rhodophyta*, *Cyanobacteria*, and *Chlorophyta* are the most commonly cultivated algae species.

Microalgae contain chlorophyll, phycobiliprotein, and other light-absorbing pigments, they possess an effective photosynthetic biological system. They can efficiently utilize solar energy, reduce carbon dioxide, and convert inorganic salts into organic compounds through photosynthesis. It is precisely due to their high efficiency in fixing and utilizing carbon dioxide that makes them one of the effective ways to reduce the Earth's greenhouse gases.

In addition to these attributes, they also have many more valuable features (Figure 1). 1. Algae, due to their shorter cell cycle, are easier to cultivate on a larger scale. 2. Algae demonstrate good adaptability and can be cultivated in large quantities in artificial seawater

through artificial cultivation. Some single-water algae can also be cultivated in saline water after artificial cultivation. 3. Microalgae cells contain proteins, fats, and carbohydrates (Markou et al., 2013; Parjikolaei et al., 2013; Manirafasha et al., 2016). They can also synthesize and accumulate specific bioproducts in large quantities under special induced environments, making them important resources for food and bioenergy in the future. 4. Moreover, because of the unique living environment of marine microalgae, it can synthesize many bioactive substances with unique structures and physiological functions (such as fucoxanthin). This makes them a promising source for future medicines, health products, and chemical raw materials (Ma et al., 2020; Markou et al., 2013; Skjånes et al., 2013; Cardozo et al., 2007).

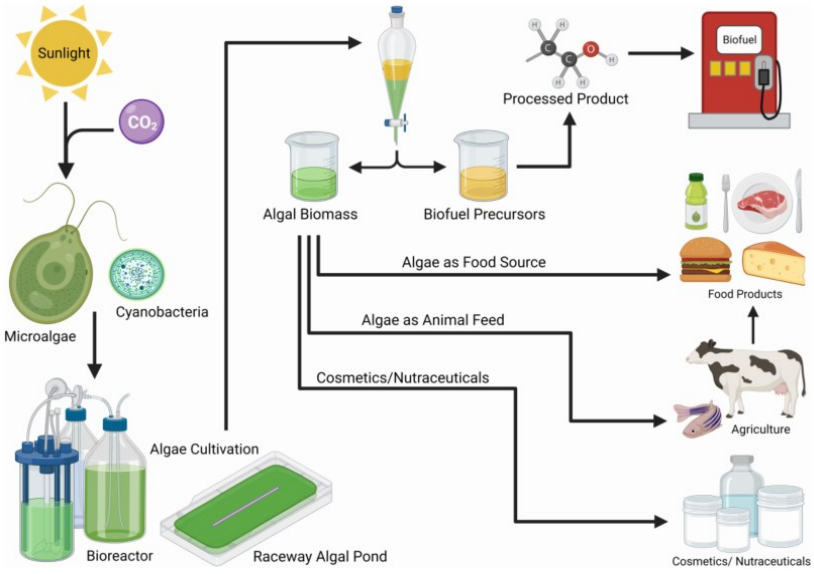

Figure 1 Several algal products and applications (Diaz et al., 2023)

2 Bioactive species of marine microalgae

2.1 Fatty acid

The lipid content and fatty acid composition of microalgae change with variations in the

external environment and nutritional conditions. In most types of microalgae, the total lipid content exceeds 10% of their dry weight (Song et al., 2013; Huerlimann et al., 2010). Among them, unsaturated fatty acids with four to five double bonds, such as eicosaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (AA) (Figure 2), are not only components of animal and plant cell membrane structures but also play crucial physiological roles (Cheng et al., 2002; Banerjee et al., 2002; Koletzko et al., 1996). For example, EPA and DHA can lower blood lipids, reduce blood pressure, decrease cholesterol, prevent thrombosis, inhibit platelet aggregation, relax blood vessels, and are used in the prevention and treatment of inflammation, cancer, rheumatoid arthritis, and diabetes (Figueras et al., 2012; Machado et al., 2011; Taneda et al., 2010; Koller et al., 2014; Camacho et al., 2019). DHA is an important structural lipid found in the gray matter of the brain (Balakrishnan et al., 2021; Bradbury, 2011). It is also related to the development and formation of the retina (Bazan et al., 2005; Querques et al., 2011). It plays an important role in people with cardiovascular diseases (Breslow, 2006; Holub et al., 2009; Yamagata, 2017). It promotes the growth and development of brain cells, improves brain function, and can be used to prevent central nervous diseases and treatment (Camacho et al.,2019; Koller et al., 2014).

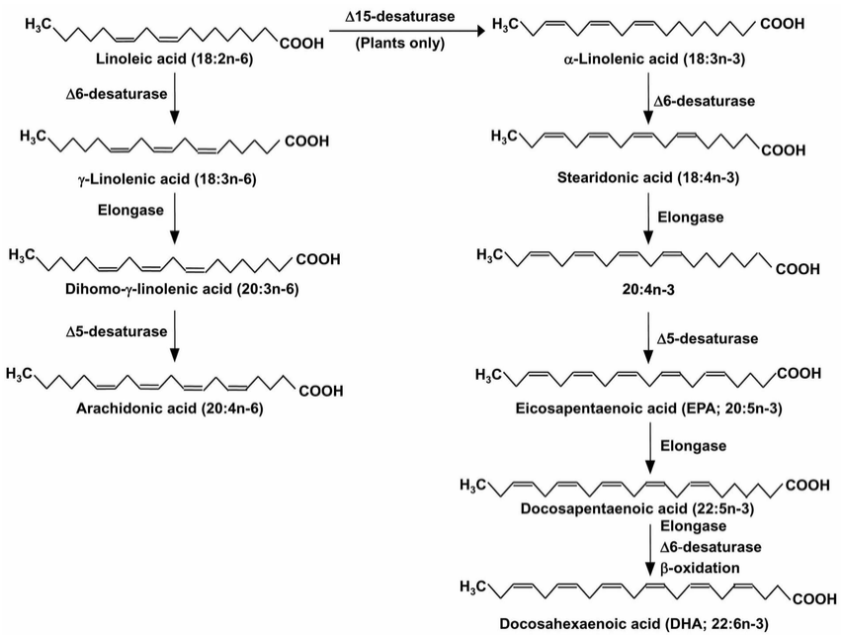

Figure 2 Polyunsaturated fatty acids (PUFAs) synthesis, including the production of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Calder, 2019).

Most higher plants and humans lack the necessary enzymes to synthesize polyunsaturated fatty acids (Nakamura et al., 2001). Humans must intake fatty acids from food. Currently, humans primarily obtain them from deep-sea fish. Using algae to produce fatty acids has several advantages (Behrens et al., 1996; Griffiths et al., 2011). 1: The PUFA content of some microalgae can reach 5 % to 6 % of dry weight, and its relative content is higher than that accumulated by fish through the food chain (Hempel et al., 2012). 2: PUFA produced from algae has no fishy smell and does not contain cholesterol (Remize et al., 2021). 3: The types of PUFA contained in some algae are relatively simple, making it easier to separate and purify single components (Guedes et al., 2011). 4: Algae grow quickly and can be cultivated using various incubators, and large-scale production can be achieved through precise control (Jayaraman et al., 2015; James et al., 2010). 5: Genetic modification can be carried out to increase its specific PUFA production (Ruiz-Lopez et al., 2015; Qi et al., 2020).

2.2 Polysaccharide

Polysaccharides are natural macromolecular substances widely present in microalgae and are an important component of all biological organisms. They exhibits unique biological activity, can enhance the body's immune function, and participates in various crucial life processes (Feng et al., 2023; Liu et al., 2020). Microalgae polysaccharides are a type of organic matter synthesized by microalgae through photosynthesis and metabolism, and the majority of it possesses biological activity. In recent years, microalgae polysaccharides have emerged as a research hotspot. These polysaccharides exhibit various physiological functions, including antiviral, anticancer, anti-radiation damage, anti-aging properties, as well as the ability to lower blood lipids and regulate the body's immune system (Mader et al., 2016; Mishima et al., 1998; de Jesus Raposo et al., 2013). For example, Yim et al. extracted the sulfated exopolysaccharide p-KG03 from the red tide microalga *Spirulina*, which has anti-encephalomyocarditis virus (EMCV) activity. There is also a *Spirulina platensis* polysaccharide (PSP) that can interfere with the adsorption of herpes simplex virus HSV-1 to host cells (Hong et al., 2002; Liu et al., 2020).

The polysaccharides produced by these algae also have a wide range of applications in the food, cosmetic, and pharmaceutical industries (Majee et al., 2017; Muthukumar et al., 2021).

2.3 Microalgae pigment

Due to changes in environmental conditions, the types and content of pigments in algal cells may vary. In addition to chlorophyll, microalgae have a variety of accessory pigments

and secondary pigments, such as phycobilin, carotenoids, astaxanthin, and rock yellow pigments (Zhang et al., 2018; Gilbert-López et al., 2015).

These natural pigments can enhance the utilization efficiency of light energy by algae, and some can also protect them (Ramanna et al., 2017). Some pigments also have biological activity, for example, carotenoids can delay aging, slow down cardiovascular disease, and enhance the body's immune response (Maria et al., 2015; Tan et al., 2019; Riccioni et al., 2011; Alishahi et al., 2015; Gui et al., 2022). β-carotene, astaxanthin, etc. are all carotenoids (Figure 3). β-carotene can be converted into vitamin A in the human body (Lin et al., 2015).

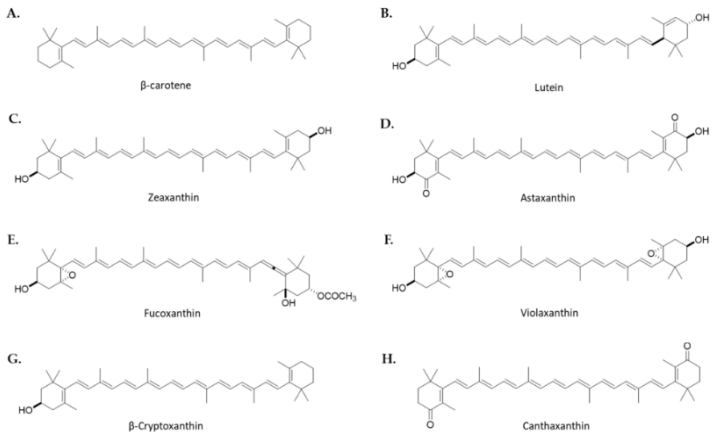

Figure 3 Structural representations of key functional carotenoids present in microalgae. Carotenes include β-Carotene (A), while xanthophylls comprise Lutein (B), Zeaxanthin (C), Astaxanthin (D), Fucoxanthin (E), Violaxanthin (F), β-Cryptoxanthin (G), and Canthaxanthin (H) (Ávila-Román et al., 2021).

Among known algae, the highest β-carotene content is 9 % and 0.17 % of the cell by volume in *Dunaliella salina* and *Spirulina platensis*, respectively (Sui et al., 2019; da Costa Ores et al., 2016). Astaxanthin is a natural red pigment with strong antioxidant and anti-tumor activity and can enhance the body's immune response (Raza et al., 2021; Faraone et al., 2020).

It has broad application prospects in the food, feed, and pharmaceutical industries (Olaniran et al., 2023; Oslan et al., 2021). Microalgae rich in astaxanthin include *Chlorella zofingiensis*, *Chlamydomonas nivalis*, and *Haematococcus pluvialis* (Liu et al., 2014; Remias et al., 2005; Dong et al., 2014). Among them, they can accumulate large amounts of carotenoids under specific conditions, of which more than 75 % is astaxanthin, with a content exceeding 2 % of the cell dry weight (Shah et al., 2016; Orosa et al., 2001). Rock yellow pigments refer to a type of yellow-brown pigment in some algae that can be used as a pigment or a source of xanthophylls for animals and humans (Prasanna et al., 2007). Fucoxanthin is widely present in the algae, and includes various carotenoids and xanthophylls that have yellow-orange colorations (Maoka et al., 2020; Shahidi et al., 1998). Fucoxanthin has a wide range of antioxidant, anti-obesity, anti-diabetes, anti-inflammatory, and also has therapeutic and preventive effects on various cancers and tumors (Méresse et al., 2020; Bae et al., 2020). The current market price is relatively high, reaching US $ 40,000-80,000 /kg, and the price is still increasing (Abu-Ghosh et al., 2021; Joel, 2016).

2.4 Phycobiliprotein

Zeaxanthin is a water-soluble pigment protein primarily found in blue, red, cryptic, and a few dinoflagellates (Roy et al., 2011; Larkum et al., 2016). It serves as a collective term for phycocyanin (PC), phycoerythrin (PE), allophycocyanin (AP), and phycoerythrocyanin. In red and blue algae, diverse phycobiliproteins come together to form highly organized supramolecular complexes known as phycobilisomes, connected by linkage peptides, as seen on Figure 4 (Manirafasha et al., 2016; Parjikolaei et al., 2013). These complexes are anchored

to the surface of the light-harvesting membrane, functioning as the primary light-harvesting and supplementary light-harvesting pigment systems (Lokstein et al., 2021; Simkin et al., 2022).

Cryptic and certain dinoflagellate species possess soluble phycobiliproteins that bind to photosynthetic pigments. These proteins then form complexes with the chlorophyll protein complex, creating a supplementary light-harvesting pigment system (Larkum et al., 2016; Satpati et al., 2020). The types and content of phycobiliproteins vary in different algae (Osório et al., 2020). Phycocyanin and allophycocyanin are prevalent in both blue and red algae (Chandra et al., 2017). Phycoerythrin only appears in red algae and some blue algae (Sfriso er al., 2018; Tan et al., 2022).

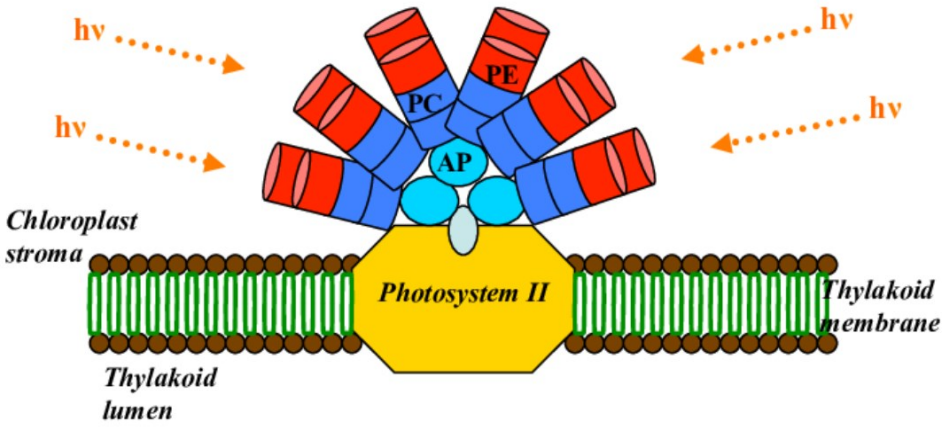

Figure 4 Schematic diagram of phycobilisome structure (Vernès et al., 2015).

Zeaxanthin can be used as a natural pigment in food, cosmetics, dyes, and other industries, and can also be used to make fluorescent reagents for clinical medicine, immunology, biochemistry, and biotechnology research fields, with high development value (Imchen et al., 2023; Prasanna et al., 2007). It is mainly used in the following applications: 1. Natural pigments. Replace artificial dyes used in cosmetics and food additives. For example,

extracted from *Spirulina platensis* powder is a type of open-chain tetrapyrrole chromophore-containing phycobiliprotein (Ding et al., 2017). 2. Medicine. Phycobiliprotein can stimulate human B lymphocyte proliferation and improve the body's immune response (Chang et al., 2011). R-phycoerythrin can produce a specific immune response with insulin antibodies, which has a certain therapeutic effect on diabetes (Wang et al., 2020). 3. Fluorescent probe. After phycobiliprotein is covalently linked with other proteins, the fluorescence quantum yield and emission spectrum remain unchanged (Sun et al., 2003).

In addition, phycobiliprotein has stable properties, high fluorescence quantum yield, low background interference, and easy linkage with biotin, antibodies, and glycoproteins (Pereira et al., 2020; Grabowski et al., 1978; Manirafasha et al., 2016; Glazer et al., 1990; Fairchild et al., 1991; Vinothkanna et al., 2020). It can be used as a new generation of fluorescent probes in clinical diagnosis, immunology, cell biology, histology, molecular biology, etc., replacing isotopes and enzymes as markers (Qiang et al., 2021; Sekar et al., 2008).

2.5 Toxin

Human beings have learned about shellfish toxins from poisoning incidents caused by eating toxic shellfish (Kao, 1993). However, research has found that the toxins are formed after the shellfish filter-feed toxic microalgae, causing the accumulation of microalgal toxins in the body or undergoing some chemical transformations (Landsberg, 2002). Microalgae are the initial producers of toxins (Hégaret et al., 2009). Currently, approximately 70-90 different microalgae species have been identified as toxin producers, with dinoflagellates comprising the majority, contributing to about 75% of the total. Additionally, certain species of

cyanobacteria and diatoms are capable of toxin production (Caruana et al., 2018; Lassus et al., 2015). Based on the clinical symptoms of poisoning, microalgal toxins can be categorized into several types, including paralytic shellfish toxins, diarrhetic shellfish toxins, neurotoxic shellfish toxins, memory-loss shellfish toxins, and fish maw toxins (Caruana et al., 2018; Lassus et al., 2015; Caruso et al., 2015).

Microalgal toxins are harmful to humans, but they are only intermediate metabolites in the growth process of algae and have biological activity and high development potential (Caruana et al., 2018; Lagos, 1998; Plumley, 1997). For example, some microalgal toxins are effective detoxification drugs that have certain effects on analgesia and anesthesia, spasmolysis, cough relief, and anticancer (Ismail et al., 2017; Luozao et al., 2022; Ślusarczyk et al., 2021; Stein et al., 1984; Keshri, 2012; Viviani, 1992). Selenium toxin mainly acts on ion channels in the nervous system and has a good cardiotonic effect (Araie et al., 2016; Ding et al., 2023). Diarrhetic toxin and active ingredient soft sponge acid are strong carcinogenic factors with unique carcinogenic mechanisms (López-Rodas et al., 2006; Cordier et al., 2000; Park et al., 2000). Unlike other carcinogens, they inhibit phosphatase activity, which is of great significance in studying cancer mechanisms and developing new anticancer drugs (Windust et al., 1996; Luu et al., 1993). Paralytic shellfish toxin has specific binding ability to sodium ion channels and is an important tool for molecular biology research (Zhang et al., 2013; Durán-Riveroll et al., 2017; Cusick et al., 2013).

3 The application potential of the microalgae

3.1 Medical applications

Microalgae not only have rich nutritional value, but also are a very rich resource of high-value chemicals and medicines (Rahman, 2020; Barkia et al., 2019; Leu et al., 2014; Chew et al., 2017). Algal cells contain certain components that are difficult to obtain from other organisms, such as unsaturated fatty acids, astaxanthin, beta-carotene, and various bioactive substances, which have therapeutic and medicinal value (Leu et al., 2014; Chew et al., 2017). Currently, the use of microalgae to produce polyunsaturated fatty acids such as gamma-linolenic acid, EPA, and DHA can prevent and treat cardiovascular diseases, cancer, and regulate the function of the central nervous system and visual nervous system (Ma et al., 2020; Kumar et al., 2019; Ulmann et al., 2017). Among them, the unsaturated fatty acids in spiral algae are mainly linoleic acid and gamma-linolenic acid, which have the effect of reducing blood plasma cholesterol levels (Ulmann et al., 2017; Horrobin et al., 1987).

Currently, it is found that gamma-linolenic acid can also inhibit the reproduction of cancer cells (Jiang et al., 1997; Hrelia et al., 1996). Applying *Dunaliella salina* to obtain beta-carotene, clinical confirmation adds beta-carotene can successfully treat hereditary photosensitive diseases (Sui et al., 2019). It is a prerequisite for vitamin A, and in large amounts can prevent and cure vitamin A deficiency (Ismail et al., 2012; Marinoa et al., 2020). At the same time, it also has anti-cancer, prevention of cardiovascular glaucoma and immune function (Emtyazjoo et al., 2012; Badr et al., 2014). Beta-carotene has the antioxidant effect of eliminating oxygen free radicals, which can prevent aging, and can prevent and treat

tumors (Chen et al., 2021; Pryor et al., 2000).

Astaxanthin also belongs to carotenoids, which can be obtained from *Haematococcus pluvalis* (Figure 5). It has a strong antioxidant ability to eliminate free radicals produced by ultraviolet radiation in the body, regulate and reduce these damages caused by photochemistry, and has a good therapeutic effect on skin cancer caused by ultraviolet radiation (Shah et al., 2016). Spiral algae water-soluble polysaccharides and glycoproteins have the effects of anti-radiation, anti-oxidation, improving immunity, promoting lymphocyte transformation, and inhibiting cancer cell proliferation (Zheng et al., 2023; Sun et al., 2022; Sajadimajd et al., 2019). Among them, phycocyanin has been used as a tracer and immunofluorescent agent in clinical medicine and as an anti-tumor drug (Jiang et al., 2017; Braune et al., 2021). *Chlorella vulgaris* cell digestion efficiency is low, which can be used as a food fiber to treat gastrointestinal system diseases (Panahi et al., 2016; Lv et al., 2022).

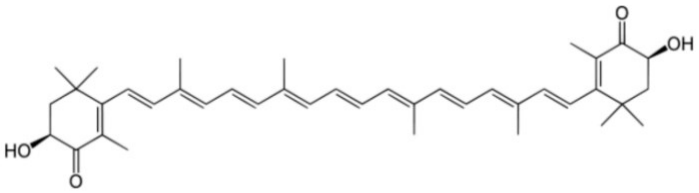

Figure 5 Astaxanthin molecular structure (Gammone et al., 2015).

3.2 Food applications

After the First World War, in order to solve the shortage of protein, European scientists were the first to develop and study microbial protein feed (Adedayo et al., 2011; Ugalde et al., 2002; Belasco, 1997). Their representatives are yeast and *Chlorella*. Now the practical microalgae that achieve mass production are only *Spirulina platensis*, *Spirulina maxima* and *Dunaliella tertiolecta (*Koller et al., 2014; EL-SAYED et al., 2018; Vonshak et al., 1988;

Borowitzka, 1990). Among them, *Spirulina sp.* contains more calcium, phosphorus, potassium, magnesium than milk, and is rich in trace elements, enzymes, and natural pigments, but the cholesterol content is very low (da Costa Ores et al., 2016). Therefore, *Spirulina sp.* has long been recommended by the Food and Agriculture Organization of the United Nations as the ideal health food for humans in the 21st century (Anvar et al., 2021). The annual output of global edible microalgae is only about 6000 tons (Faried et al., 2017). Microalgae are widely used in the food industry (Torres et al., 2020). Tableyogurt-shaped jujube powder can be used to produce food microalgae concentrated extraction liquid, which can be used as health food and food processing raw materials (Angiolillo et al., 2015). Microalgae contain a variety of nutrients and bioactive substances, which have the characteristics of health care and functional foods (Sun et al., 2018). Since the 1970s, Japan, the United States and Europe have successively developed health food products such as *Spirulina platensis*, *Spirulina maxima* and *Dunaliella tertiolecta* tablets or capsules, and have achieved mass production and sales (Lupatini et al., 2017; Wang et al., 2020).

3.3 Feed

Microalgae can be used as a potential protein source for various animals due to their rich nutritional value and ability to photosynthesize, release oxygen, and improve water quality (Yaakob et al., 2014). In addition, their moderate size allows them to be used directly as live feeds for aquatic animals such as mollusks, crustaceans, and sea cucumbers (Sun et al., 2018). They can also be used to cultivate zooplanktonic feeds such as rotifers, which serve as prey for larval stages of commercial aquatic animals such as shrimp, crab, and fish fry (Muller et

al., 2003; Zmora et al., 2013). Microalgae play an indirect role as feed in this process.

Currently, the following microalgae species are cultivated and used as feed: *Tetraselmis*, *Spirulina*, *Scenedesmus*, *Chlorella*, and *Isochrysis* (Guedes et al., 2012; Nagappan et al., 2021). Microalgae can also be used as feed additives due to their rich nutritional value, such as *Chlorella* being a potential protein source for various animals, and their high concentrations of valuable pigments such as β-carotene, astaxanthin, lutein, and fucoxanthin (Wang et al., 2018). These pigments are essential for the coloration of ornamental fish, shrimp, crab, salmon, and poultry yolks (Pasarin et al., 2018).

3.4 Pigment

Microalgae pigments play an important role in photosynthesis. Due to different species and environmental conditions, the types, and contents of pigments in microalgae cells may vary (Begum et al., 2016; Kuczynska et al., 2015). In addition to chlorophyll, diatoms also have a variety of accessory pigments and secondary pigments, such as phycobilin, carotenoids, etc. These natural pigments can not only promote the utilization efficiency of light energy by algae but resist the impact of sunlight rays and certain natural pigments also have biological activity and certain development and application value (Heydarizadeh et al., 2013; Patel et al., 2022). For example, carotenoids can scavenge free radicals, delay aging, slow down the deterioration of cardiovascular diseases, and enhance the immune system (Patel et al., 2010). Beta-carotene, astaxanthin, and zeaxanthin are all carotenoids. Beta-carotene can be converted into vitamin A in the human body, enhance immune function, and prevent diseases. It can inhibit the relaxation of alveoli in mammals and mice, prevent

the cancer of hamster ovarian cells, and also be used as a food colorant in the food industry (Prasanna er al., 2007; Lin et al., 2015; Jomova et al., 2013; Kennedy, 1991).

Astaxanthin is a natural red pigment with strong antioxidant and anti-tumor activity and can increase the body's immunity and has a wide application prospect in food, feed, and medicine (Zhang et al., 2015; Ambati et al., 2014; Faraone et al., 2020). Microalgae rich in astaxanthin mainly include *Chlorella vulgaris*, *Euglena gracilis*, and *Haematococcus pluvialis*, among which *Haematococcus pluvialis* cells can accumulate a large number of carotenoids under specific conditions (Vidhyavathi et al., 2008; Cuellar et al., 2015; Boussiba, 2000). Among them, more than 75 % is astaxanthin, with a content exceeding 2 % of the cell dry weight (Nobre et al., 2006). Zeaxanthin has positive effects on cardiovascular diseases protection and improving vision and immunity (Ribaya et al., 2004; Li et al., 2023; Bouyahya et al., 2021).

3.5 New energy

In the current world, the main fossil energy sources, such as oil, natural gas, and coal, are non-renewable resources, and their reserves have been decreasing and are expected to be exhausted in the near future (Arutyunov et al., 2017). In addition, the extraction and use of fossil energy also cause serious pollution to the environment (Omer, 2009). For example, in the oil and natural gas industry, there is a substantial risk of fuel leakage during extraction, transportation, and storage, especially in marine oil extraction and transportation. (Walker et al., 2019; Govorushko, 2013). In addition, in the refining process of oil and natural gas, a large amount of waste gas, wastewater, and toxic organic matter will be generated (Fakhru et

al., 2009). The use of refined oil will also lead to the emission of pollutants such as nitrogen oxides, hydrogen sulfide, sulfur dioxide, and greenhouse gases such as carbon dioxide, which will harm the environment and climate (Lin et al., 2009; Szklo et al., 2007). In view of the limitations of fossil energy and environmental issues, exploring renewable and clean energy has become an urgent task.

Although wind energy, solar energy, and hydrogen energy have made some development and application, there are still many shortcomings and limitations to make up for the shortage of fossil energy and replace fossil energy (Turkenburg et al., 2000; Halkos et al., 2020). Photosynthesis is a process that higher plants, algae, and cyanobacteria on Earth have the ability to perform. Through photosynthesis, plants can use natural light energy to take carbon dioxide in the air as the main raw material, and synthesize various organic compounds, carbohydrates, and lipids to meet their own growth needs. If these organic molecules are extracted, they can be made into various required bioenergy. Therefore, renewable biofuels produced based on green plants are highly regarded.

Currently, the main types of biofuels produced globally include bioethanol, biodiesel, biohydrogen, and biogas (Demirbas, 2017). The sources of traditional biofuels are all food crops, and there are two main production methods: one is to produce bioethanol using corn or sugar cane as raw materials; the other is to produce biodiesel using soybean, palm oil and other oil crops as raw materials (Somma et al., 2010; Braide et al., 2016; Bušić et al., 2018; Pimentel et al., 2005). However, these crops are traditionally seen as food sources. If used in large quantities for biofuel production, it may have an impact on food supply (Rosillo et al.,

2009; Meira et al., 2015; Pimentel et al., 2008).

In addition, lignocellulose, which has a wide range of sources, can also be used as a raw material for bioethanol production (Balat, 2011). When using these raw materials to produce bioethanol, the proportion of cellulose, hemicellulose, and lignin in the crop has a certain impact on the production efficiency of bioethanol (Limayem et al., 2012; Balat, 2011; Mood et al., 2013). At the same time, to release fermentable components, complex pretreatment of raw materials is required, which has high costs and requires a large amount of enzymes during fermentation (Mood et al., 2013; Kong et al., 2011; Kucharska et al., 2018). These issues still need further research and solutions. At present, the production cost of biodiesel is relatively high, and large-scale application is still in its infancy. According to statistics, the main component of biodiesel preparation cost is raw material cost (Moser, 2009). Therefore, seeking low-cost oil and fat raw materials has become a key link in the development of biodiesel research and application.

Currently, there is a contradiction and competition between many plant resources that can be used for the production of biofuels and food supply. Therefore, algae with photosynthetic systems and high oil content have great potential in the production of biofuels. Many algae have extremely high lipid content, and this special chemical property of lipids makes it possible for algae to produce biodiesel (Fon et al., 2013; Huang et al., 2018; Li et al., 2008; Gouveia et al., 2009). The oil content in some algae can reach 80 %, and it can also reach 20 % to 50 % in common varieties (Song et al., 2008; Singh et al., 2011). As a sustainable energy source, biodiesel can be added to diesel at any ratio and applied to diesel

engines (Hoseini et al., 2017). Compared with wood, algae grow in a water environment and can grow vertically without lignin crosslinking (Jones et al., 2012). Therefore, the lignin content in algae is low, and the ratio of lignin to hemicellulose in algae is also low. In addition, the sugar content in algae is high, which can be used for fermentation production of bioethanol (Li et al., 2014).

The cost of algal biodiesel is directly proportional to the efficiency of carbon sequestration by algae. Algae have the following advantages in producing bioenergy (Dalrymple et al., 2013; Kumar et al., 2020; Fortier et al., 2017; Smith et al., 2010; Moreira et al., 2016): 1. It can grow in wastewater, reclaimed water, and salt water, saving freshwater resources. 2. It has high biological yield and does not conflict with agricultural production. 3. While producing biofuels, high value-added products can be provided through other means. 4. Algae has a low demand for land. 5. Recover carbon dioxide in the air sufficiently to achieve green and renewable.

Biodiesel produced from algae has several advantages: 1. Biodiesel is easily decomposed by bacteria in the environment. Regular fossil fuels, especially diesel with complex components, and are not easily biodegradable (Griffiths et al., 2011; Datta et al., 2019; Zhang et al., 1998; Marchal et al., 2003). 2. Typically, biodiesel does not contain sulfur, aromatic hydrocarbons, or heavy metals (Tomic et al., 2014). It has high flash point, low toxicity, and low VOC content (Kralova et al., 2010). Biodiesel is less toxic to aquatic organisms than regular diesel (Khan et al., 2007). 3. It has good engine cold start performance. It can be used directly in existing diesel engines without any modifications

(Jiaqiang et al., 2019; Wang et al., 2000). Due to its ignition point of about 150 °C, which is higher than the ignition points of regular fossil fuels by 50 °C, it has good safety performance, making it easy for safe transportation and storage (Dharma et al., 2016). 4. Biodiesel is also an excellent lubricant that can effectively reduce wear on injectors, engines, and connecting rods, prolonging their service life (Gupta et al., 2021). In addition, studies on exhaust emissions from biodiesel have shown that the emissions of carbon monoxide, HCW, aromatic hydrocarbons, sulfur oxides, and carbon particles decrease as the biodiesel content in the fuel increases (Correa et al., 2006; Dincer, 2008). As a substitute fuel for diesel vehicles, biodiesel has lower emissions of toxic organic compounds, particulate matter, carbon dioxide, and nitrous oxides compared to regular diesel (Agarwal et al., 2011; Xing et al., 2002).

Microalgae can convert the energy of photosynthetically active radiation into biomass through photosynthesis. They can also carry out heterotrophic metabolism in dark conditions and synthesize lipids (Chisti, 2007). However, the essence of bioenergy is an energy conversion process. Therefore, using artificial energy to cultivate microalgae is meaningless in the commercial production of bioenergy. The only way to achieve the commercial production of microalgae biodiesel is to use natural light for photosynthetic growth and lipid accumulation (Chisti, 2007). Currently, the main purpose of most commercial microalgae cultivation is to obtain relatively high-value co-products such as beta-carotene, lutein, astaxanthin, etc. Few companies currently produce commercial microalgae biodiesel due to high costs. However, the potential of microalgae as a clean energy source for the future is widely recognized (Greenwell et al., 2010; Chisti, 2010; Chisti, 2008). Whether microalgae

biodiesel can be commercialized in the near future depends on the resolution of key issues. These include finding a high-yielding oil-producing species suitable for large-scale cultivation and reducing the cost of such cultivation. The cost of microalgae biodiesel is mainly influenced by factors such as the oil production capacity of the algae, the performance of the cultivation system, and the cost of separation and extraction (Chisti, 2007). Currently, the academic community is conducting extensive research on these aspects, and the core issue lies in how to improve the oil production capacity of microalgae. This can be divided into two aspects: how to increase the growth rate of microalgae and how to increase the oil content of cells. Factors that affect the growth rate of microalgae include light intensity, temperature, PH, salt concentration, nutritional factors, and carbon dioxide concentration. Factors that affect lipid accumulation in microalgae include nutritional factors, temperature, light, salt concentration, and trace elements.

4 Culture mode of the microalgae

The foundation of the development of the microalgae industry lies in ensuring an adequate supply of biomass fuel, and the choice of microalgae cultivation model has a crucial impact on increasing the biomass yield of microalgae. Therefore, the cultivation model of microalgae has always been the core focus of research. At present, the mainstream cultivation models of microalgae include batch culture (i.e. one-time culture), fed-batch culture, semi-continuous culture, and continuous culture (Lee et al., 2015; Zhu et al., 2015; Peter et al., 2022; Zheng et al., 2013).

4.1 Batch culture mode

Batch cultivation of microalgae has advantages such as simple operation and low cost, and is widely used in laboratories (Dębowski et al., 2020; Rawat et al., 2013). Under different conditions, such as light intensity, culture medium composition, pH value, and harvest time, the accumulation law of specific components in microalgae cells can be studied and the optimal culture conditions can be optimized. This provides a practical research method for artificial regulation of microalgae growth and improvement of specific product synthesis (Hiavova et al., 2015). The synthesis of some components in microalgae cells is closely related to the growth stage. In different growth stages, the content of each component in cells varies greatly (Fidalgo et al., 1998). For example, the total lipid content of some microalgae in the stationary phase is significantly higher than that in other stages. It is reported that the total lipid content at the end of the stationary phase is higher than that at the end of the logarithmic growth phase (Mansour et al., 2003). The lipid yield of Robaina at the stationary phase is 30 times that at the end of the logarithmic growth phase, while the total lipid content of Derxiat at the stationary phase is 1.3 times that at the logarithmic growth phase (Dunstan et al., 1993; Miller et al., 2014). Most microalgae can achieve massive accumulation of specific components under induced conditions, that is, in nutrient-limited or deficient conditions, they can synthesize large amounts of specific components (Yaakob et al., 2021; Rodolfi et al., 2009). Batch cultivation is conducive to strictly controlling induction conditions and is helpful to obtain target products.

In recent years, using microalgae to treat wastewater has become a research hotspot,

aimed at removing pollutants such as nitrogen, phosphorus, or heavy metals from wastewater to reduce pollutant concentrations to meet discharge standards. Batch cultivation has advantages in this regard and can effectively remove pollutants, especially divalent metal ions, whose removal ability is better than that of other cultivation modes (Hmmouda et al., 1995; Smori et al., 2013).

In summary, batch cultivation has simple operation and low cost and has wide applications in studying the growth and specific substance accumulation of microalgae. However, in batch cultivation, microalgae quickly consume nitrogen and phosphorus nutrients, which may lead to insufficient nutrition in the culture solution and limit algal cell growth and proliferation (Droop, 1975). Under these conditions, algal cells can accumulate specific substances such as oil and carotenoids. In recent years, batch cultivation has been widely used in two-step large-scale cultivation of cells for specific products. The first step is to provide sufficient nutrients for batch cultivation to accumulate high biomass; the second step is to achieve nutrient deficiency or limitation to increase the content of specific target products in microalgae cells. Therefore, batch cultivation has unique applications in large-scale cultivation for inducing oil production, astaxanthin production, carotenoid production, etc (Wehrs et al., 2019; Quinn et al., 2012).

4.2 Fed-batch culture mode

Fed-batch cultivation, also known as batch feeding cultivation, is a cultivation method in which one or more nutrients are added to the culture medium during the cultivation process (Minihane et al., 1986; Zheng et al., 2013). This technique is widely used in the cultivation of

microorganisms, animal, and plant cells, and has also been widely applied in the cultivation of microalgae (Yamanè et al., 2005; Coelho et al., 2014).

In the cultivation of microalgae, nitrogen and phosphorus are the most important nutrients for the growth of microalgae, and they are also the nutrients that are consumed the fastest and most prone to deficiency during the growth process of microalgae. Adding too much nitrogen and phosphorus at one time may have inhibitory and toxic effects on the growth of microalgae (Yaakob et al., 2021). Therefore, using a fed-batch cultivation model can effectively avoid substrate volition and toxic effects, promote cell growth and metabolism, and obtain higher biomass and metabolites (Chen, 1996; Scherholz et al., 2013).

Compared to batch culture, fed-batch culture can significantly increase the cell density and final biomass of microalgae (Shi et al., 2002). In addition, fed-batch culture plays an important role in enhancing the synthesis of specific metabolites in algal cells. Limiting fed-batch is a commonly used fed-batch culture method, which limits the concentration of nutrients in the culture solution by adding nutrients in low amounts. Limiting fed-batch of specific nutrients can promote the accumulation of specific substances in algal cells (Jeffryes et al., 2013; Wang et al., 2017; Dębowski et al., 2020).

Fed-batch cultivation is divided into two methods: constant-rate feeding and variable-rate feeding. Constant-rate feeding is a method of gradually adding nutrients to the culture solution at a uniform flow acceleration, while variable-rate feeding is a method of nonlinear feeding based on the growth characteristics of the cultured organisms. Because variable-rate feeding can adjust the nutrient concentration in the microalgae culture solution

in real time based on the growth status of the algal cells, creating a more suitable environment for the growth of microalgae or the synthesis of certain metabolites, it is more suitable for high-density cultivation than constant-rate feeding, and is more conducive to biomass accumulation (Yamanè et al., 2005; Geada et al., 2017).

Through fed-batch cultivation, especially variable-rate fed-batch cultivation, the concentration of nutrients in the algal cell growth process can be effectively regulated, maintaining the concentration of nutrients in the nutrient solution at an appropriate level (Geada et al., 2017). This can not only alleviate the inhibitory and toxic effects caused by high initial nutrient concentrations, greatly shorten the lag phase of algal growth, but also effectively solve the problem of nutrient limitation in batch cultivation, ensuring the continuous supply of nutrients (Geada et al., 2017; Panikov, 1995). Moreover, the addition of nutrients in the fed-batch cultivation mode is simple and easy to operate, which can stimulate the high accumulation of secondary metabolites while increasing the biomass of algal cells (Cao et al., 2022; Sun et al., 2021). In the cultivation of microalgae, especially in high-density cultivation, fed-batch culture plays an important role (Zheng et al., 2013).

4.3 Semi-continuous culture pattern

Semi-continuous cultivatiòn is a cultivation method based on one-time cultivation. When the algal cells reach a certain concentration, a certain amount of algal solution is harvested and supplemented with an equal amount of culture medium to continue cultivation. Semi-continuous cultivation is not only widely used in large-scale cultivation, but also a common cultivation mode in microalgal laboratory research (Solís et al., 2021; Benvenuti et

al., 2016; Gojkovic et al., 2021; Hewes, 2016). During semi-continuous cultivation, the use of fresh culture medium to replace equal amounts of original culture medium at regular intervals increases the nutrients in the culture medium, reduces the biological density, increases the light transmittance, and thus enhances the photosynthetic efficiency of the algal cells, resulting in faster growth rate and better growth condition of the algal cells (Hewes, 2016; Fu et al., 2012; Liu et al., 2019; Han et al., 2016).

The renewal rate, as one of the most important parameters in the semi-continuous cultivation mode, has a significant impact on the growth and intracellular biochemical components of microalgae. In many cases, the renewal rate of semi-continuous cultivation of microalgae is 20 % to 30 % (Fábregas et al., 1998). For example, *H. pluvialis* and freshwater microalgae. At a renewal rate of 20%, the cell yield reaches a maximum, significantly higher than other renewal rates (Gonçalves et al., 2022; Luo et al., 2016).

In recent years, there have been more and more studies on the application of semi-continuous cultivation in the accumulation of microalgae oil (Hsieh et al., 2009; Ho et al., 2014). Practice has proved that the semi-continuous cultivation mode is one of the best cultivation methods for the large-scale production of biodiesel from microalgae (Ashokkumar et al., 2014; Boonma et al., 2019; Dębowski et al., 2020). Under the semi-continuous cultivation mode, the optimal renewal rate of different microalgae varies, and the optimal renewal rate of microalgae in different cultivation systems also varies greatly (Imamoglu et al., 2010; Rosales et al., 2004; Luo et al., 2016). Therefore, in semi-continuous cultivation, it is necessary to select the appropriate renewal rate according to different seeding and

cultivation systems to improve the biomass production of large-scale cultivation of microalgae.

4.4 Continuous culture mode

Continuous culture is a cultivation method that maintains a stable growth environment for cells in the reactor by adding fresh culture medium to the cultivation system at a constant flow rate and removing culture medium at the same flow rate (Del Río et al., 2015; Bougaran et al., 2010; Palmer et al., 1975). This stable environment helps cells grow and metabolize in a relatively constant state, allowing for stable, high-speed cultivation of microalgae or the production of large amounts of metabolites (Palmer et al., 1975). The dilution rate is a key parameter in a continuous culture that has a significant impact on biomass production, cell yield, and metabolite accumulation of microalgae (San et al., 2013). Within an appropriate range of dilution rates, the biomass of microalgae increases as the dilution rate increases; however, if the dilution rate is too high, the cells cannot grow sufficiently and are washed away, resulting in a decrease in biomass. Research shows that each microalgae has an optimal dilution rate for continuous culture (Pereira et al., 2018; Sánchez et al., 2008).

During continuous cultivation, when microalgae cells are exposed to a specific dilution rate, their growth and metabolic activities are relatively stable. This environment is conducive to the efficient synthesis of certain important metabolites by microalgae cells. Compared to batch cultivation, continuous cultivation is more conducive to the accumulation of certain intracellular metabolites in microalgae cells. Therefore, continuous cultivation has unique advantages in stabilizing high-yield microalgae biomass and producing certain important

metabolites (Peter et al., 2022; Fernandes et al., 2015).

Different cultivation modes have significant effects on the accumulation of microalgal biomass and the synthesis of intracellular metabolites. Therefore, selecting an appropriate cultivation mode, especially based on the requirements of the target product, can improve the microalgae biomass and the yield of the target product (Dębowski et al., 2020). This helps to reduce the cultivation cost of energy microalgae, shorten the cultivation time, and provide strong support for the development of the energy microalgae industry. With the advancement of science and technology, the microalgae industry will receive more attention, and the cultivation mode will play an increasingly important role in the industrialization process of microalgae.

5 Culture equipment for the microalgae

5.1 Photobioreactor

Light bioreactors are devices specifically designed for the cultivation of photosynthetic microorganisms and tissues or cells with photosynthetic capabilities. Their structure is similar to that of general bioreactors, with regulation and control systems for culture conditions such as light, temperature, dissolved oxygen, carbon dioxide, pH, and nutrients. However, unlike bioreactors used for microbial fermentation, light bioreactors are primarily concerned with the efficiency of light energy absorption and do not require an aseptic operation, eliminating the need for equipment sterilization systems in the design (Posten et al., 2009; Yusoff et al., 2019; Xu et al., 2009). Ideal light bioreactors should have high light energy utilization efficiency, which can reach 18 % under ideal conditions, and allow continuous or

semi-continuous cultivation around the clock to achieve high density cultivation of microalgae and high biomass production yields per unit area or volume (Pulz et al., 2006).

Research on light bioreactors began in the 1940s, when the main goal was to cultivate a large number of microalgae and explore whether they could serve as a future food and fuel resource for humans. Since the 1950s, people have developed various closed light bioreactors, such as horizontal tubular, vertical tubular, and flexible belt types. However, these pioneering works were neglected for a long time and were only used as samples (Borowitzka et al., 2012; İhsan et al., 2020).

Open-pond cultivation systems have developed well due to their advantages of simple technology, low investment, easy construction, and simple operation (Dragone et al., 2010). They have been successfully used for large-scale cultivation of various microalgae such as *Spirulina*, *Chlorella*, achieving good results (Costa et al., 2019).

Despite the rapid progress in the research on light bioreactors, there is still a significant gap between land-based bioreactors and fermentation industries. Firstly, there is currently no mature light bioreactor product that can be produced in batches. Users can only make their own according to their understanding and needs, leading to a stagnant development status of the light bioreactor industry. Secondly, there is a significant difference between design concepts and practical applications. So far, there is a lack of truly industrialized products. Finally, due to the limited number of microalgae that can be cultivated on a large scale and the lack of in-depth research on the application of light bioreactors for cultivating microalgae, especially in the industrial cultivation of transgenic algae, it has limited the development of

light bioreactors (Yusoff et al., 2019).

To further promote the development of light bioreactors and applied research on high-density cultivation of microalgae, efforts need to be made in the following areas:

1. Strengthen the research on the design and manufacturing of light bioreactors to improve light energy utilization efficiency and cultivation efficiency.

2. Explore efficient cultivation methods and optimize cultivation conditions for different microalgae species.

3. Strengthen the research on microalgae transgenic technology and apply it to improve the production quantity and quality of microalgae.

4. Promote industrial production and application of light bioreactors to accelerate the rapid development of the microalgae industry.

5.1.1 Open mode photobioreactor

Open pond photobioreactors, also known as open pond culture systems, have significant advantages such as simple structure, low cost, and easy operation (Benemann, 2008).

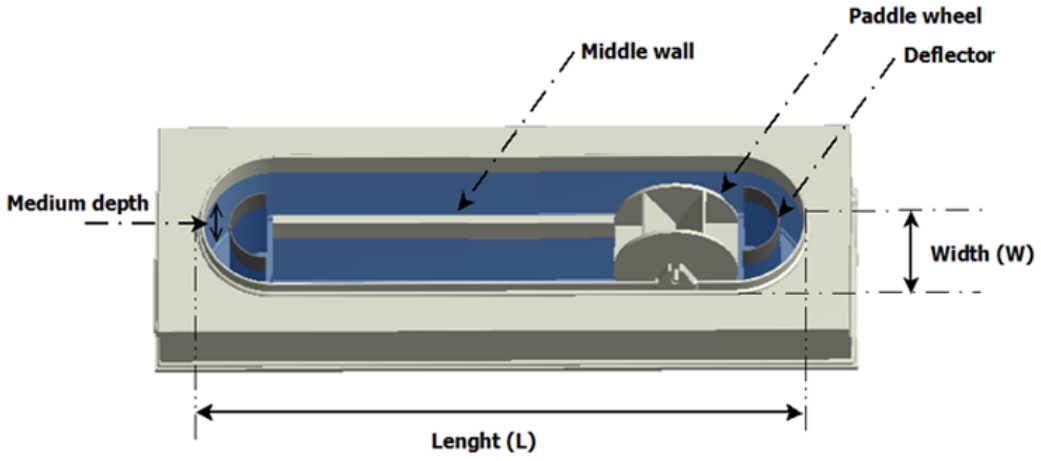

Figure 6 Race pound reactor (Rayen et al., 2019).

These reactors can be categorized into four main types: open pond reactors, raceway pond reactors, circulation pond reactors, and shallow pond reactors. Among them, the most typical and commonly used open pond culture system is the raceway pond reactor designed by Oswald in 1969 (Borowitzka et al., 2012; Chisti, 2013).

This system mainly consists of a closed elliptical ring water tank, and the culture solution inside the tank is driven by a set of paddles. Nutrients required for microalgae are added online, for example, in front of the rotating paddles (Figure 6). Simultaneously, mature biomass can be harvested behind the rotating paddles. The design of the rotating paddles facilitates the thorough mixing of microalgae and the necessary nutrients, carbon dioxide, and other gases, preventing the settling of microalgal cells.

Since the development of open pond culture systems, there have been some improvements in aspects such as mixing and online detection systems, but the overall structure has not changed significantly. It is the oldest type of algae cultivation reactor, more accurately described as a microalgae cultivation system. The application of such cultivation systems in production typically involves circular shallow ponds with an area of 1000 to 5000 square meters and a culture depth of 15 centimeters. The rotation of the impeller circulates the culture solution in the pond, preventing algal settling and improving the light energy utilization efficiency of algal cells (Borowitzka et al., 2012; Costa et al., 2019; Benemann et al., 1996; Sreekumar et al., 2016).

Despite various attempts by scholars to increase mixing effects and induce turbulence, such as using drag baffles, continuous flow, tidal aeration, liquid injection, helical propellers,

stirring pump circulation, and relying on gravity-driven flow, or even utilizing natural sources like wind and sunlight, as well as animals or humans, the system still has many weaknesses, including susceptibility to contamination and unstable cultivation conditions. Consequently, its photosynthetic efficiency remains low, resulting in low cell density of cultivated algae, typically ranging from 0.1 to 0.5 g/L (Kumar et al., 2015; Shen et al., 2009).

Although there have been numerous studies and improvements in the design and application of open pond systems since their introduction, they still have several shortcomings. Firstly, they are vulnerable to environmental influences, making it difficult to maintain suitable temperature and light conditions. Secondly, they may be prone to contamination by dust, insects, and miscellaneous microbes, making it challenging to maintain high-quality monoculture. Additionally, their light energy and carbon dioxide utilization rates are not high, preventing high-density cultivation. These factors contribute to low cell cultivation density, leading to higher harvesting costs (Lee, 2001; Borowitzka, 1999; Sreekumar et al., 2016).

Furthermore, microalgae suitable for large pond cultivation must be species capable of rapid growth in extreme environments, limiting the use to a few microalgae such as *Spirulina*, *Chlorella*, and Diatoms that can tolerate extreme conditions. For microalgae requiring mild cultivation conditions and having weaker population competition abilities, closed photobioreactors are the preferred method of cultivation (Borowitzka, et al., 2012). But now, more open-pond systems have been developed to meet various cultivation (Andersen, 2005; Sreekumar et al., 2016). However, the primary reason for the continued widespread use of

open pond culture systems is their low cost, which aligns better with the current stage of algae production and development. Although closed cultivation can ensure optimal growth conditions for algae, its drawbacks are evident, including the inability for large-scale production, increased costs, and hindrance to the widespread adoption of algae production at the current stage, making it less attractive to potential investors.

5.1.2 Closed mode photobioreactor

A closed cultivation system or photobioreactor is typically constructed using transparent materials, offering excellent light transmittance (Koller et al., 2015; Benemann, 2008; Grobbelaar et al. 2009). Apart from harnessing solar energy and possessing certain flexible characteristics, it shares many similarities with traditional bioreactors used in microbial fermentation. Despite having a history of 50 years, closed photobioreactors have garnered increased attention in the past decade, with numerous related patents emerging since the 1990 s (Masojídek et al., 2010).

In comparison, closed photobioreactors exhibit the following characteristics (Grobbelaar et al. 2009; Chacón et al., 2010; Yen et al., 2019):

Pollution-free, capable of achieving singular and purebred cultivation.

Cultivation conditions are easy to control.

High cultivation density, facilitating easy harvesting.

Well-suited for light exposure to all microalgae, especially conducive to the production of metabolic products of microalgae.

Highlights include a higher ratio of lighted area to cultivation volume and efficient

utilization of light energy and carbon dioxide.

In contrast to open cultivation systems, closed systems face greater limitations on cultivation scale (Fernández et al., 1999). In recent years, the development and utilization of closed photobioreactors have progressed rapidly, achieving high-density commercial cultivation (Dogaris et al., 2015). Common types of closed photobioreactors include pipeline-based, flat-panel, columnar acoustic, stirred, fermenter, and floating thin-film bag systems (Chang et al., 2017; Lehr et al., 2009; Koller et al., 2015; Pulz, 2001; Yen et al., 2019).

5.2 Major advantages and disadvantages of open and closed photobioreactors

According to structural characteristics, open photobioreactors typically adopt a raceway pond structure, which is relatively simple, easy to scale up, and cost-effective. However, due to their relatively small area and volume, they exhibit lower light utilization efficiency (Chang et al., 2017; Xu et al., 2009; Grobbelaar, 2009). In contrast, closed photobioreactors, represented by a closed tubular structure, have a more complex design, are challenging to scale up, and are associated with higher costs. However, they boast a larger area and volume, resulting in higher light utilization efficiency (Chang et al., 2017; Xu et al., 2009; Grobbelaar, 2009).

Regarding cultivation characteristics, open photobioreactors are simple to operate but challenging to control in terms of cultivation conditions and growth parameters. The cultivation environment is unstable, prone to contamination, and achieving sterile cultivation is difficult. Additionally, they have lower carbon dioxide utilization efficiency, higher

evaporation of moisture from the culture medium, and lower productivity (Chang et al., 2017; Xu et al., 2009; Grobbelaar, 2009; Yen et al., 2019; Pulz, 2001).

Closed photobioreactors are similarly easy to operate, with easy control over cultivation conditions and growth parameters. They provide a stable cultivation environment, are more easily controlled for contamination, and facilitate sterile cultivation. They exhibit high carbon dioxide utilization efficiency, lower evaporation of moisture from the culture medium, and higher productivity (Chang et al., 2017; Xu et al., 2009; Grobbelaar, 2009; Yen et al., 2019; Pulz, 2001).

In terms of production costs, open photobioreactors have lower costs but produce products of relatively lower quality. In contrast, closed photobioreactors have higher production costs but correspondingly higher product quality.

In summary, open and closed photobioreactors each have their advantages and disadvantages (Chang et al., 2017; Xu et al., 2009; Grobbelaar, 2009; Yen et al., 2019; Pulz, 2001). Open photobioreactors offer the advantages of simple structure, ease of scaling up, and lower costs, but with relatively lower light utilization efficiency. Closed photobioreactors, on the other hand, have the advantages of a larger area and volume, higher light utilization efficiency, but with a more complex structure, challenging scaling-up processes, and higher costs. In terms of cultivation characteristics, closed photobioreactors are more easily controlled for cultivation conditions and growth parameters, enabling sterile cultivation and improved carbon dioxide utilization efficiency. In production costs, open photobioreactors are more cost-effective but produce products of relatively lower quality, while closed

photobioreactors have higher production costs but correspondingly higher product quality.

Summary

Algae, as a class of organisms with tremendous potential, are poised to play a significant role in future development, spanning a wide range of fields such as food, energy, medicine, and environmental protection. In the realm of food, algae, rich in proteins, vitamins, and minerals, are considered a crucial component of the future food industry. It is anticipated to become a primary raw material for a variety of food products, including algae proteins and algae oils, meeting the growing demand for healthy and nutritionally rich food. Simultaneously, algae are set to experience opportunities in the energy sector. With its fast growth, high biomass, and oil-rich characteristics, algae are well-suited for bioenergy production. The renewable energy derived from algae, such as biodiesel and bio-natural gas, boasts lower carbon emissions and environmental impact, heralding a revolutionary shift in traditional energy industries. In the field of medicine, ongoing research into algae is uncovering its richness in bioactive substances with antioxidant, anti-inflammatory, and anti-tumor properties. Widely applied in pharmaceutical research and production, it is anticipated that more algae-based formulations will enter the market, contributing to the prevention and treatment of various diseases. The environmental role of algae is expected to intensify, given its capacity to absorb significant amounts of carbon dioxide while releasing oxygen. The large-scale cultivation and application of algae are envisioned as crucial measures for mitigating climate change and improving ecological conditions.

The future cultivation methods of algae are poised to make progress in multiple facets.

Optimization of light exposure and engineering design will focus on enhancing photosynthetic efficiency, ensuring appropriate light conditions at different growth stages. Biotechnology and genetic improvement techniques will be employed to enhance traits, such as stress resistance, growth rate, and yield. Emphasizing the principles of a circular economy, efforts will be directed toward comprehensive nutrient utilization from waste and sewage, lowering cultivation costs. The introduction of large-scale production and automation systems will enhance efficiency, reduce labor costs, and ensure consistency in product quality. The development of versatile algae cultivation systems will cater to diverse application needs, including symbiotic cultivation with other organisms for resource complementarity and efficient utilization.

In conclusion, through scientific research and technological innovation, harnessing algae resources is crucial for driving sustainable development across various industries. The future algae industry should resonate with diversification, efficiency, and sustainability, emerging as a vital force in addressing challenges related to food, energy, medicine, and the environment, contributing significantly to the progress of human society.

Knowledge gap

Currently there are several knowledge gaps: 1. Large-scale algal cultivation is mostly set up in limited area (coastal areas). 2. There are various photobioreactors for different situations, and the production cost is high. 3. Algae cultivation price still high (land occupation, hydro costs etc.). 4. There is a lack of real-time detection methods during the algae growth process.

Objectives of the work

As marine pollution continues to intensify, the breeding of marine microalgae and cultivation in inland areas has become a new direction. The experiment selected two types of marine microalgae, *Chrysotila dentata* and *Nannochloropsis oceanica* as research subjects. *Chrysotila dentata* is a marine microalga that is rich in oil and can absorb carbon dioxide to synthesize $CaCO_3$ shells (coccoliths) and has various application potentials; *Nannochloropsis oceanica* is widely used in aquacultural feed. The cultivation of both algae faces different problems: *Chrysotila dentata* is more fragile and requires harsher growth conditions. As for the outdoor cultivation of *Nannochloropsis oceanica*, the selection of photobioreactor and cultivation sites has always been an important part of its production process. So, this experiment has the following goals:

- Explore the optimal product accumulation conditions and growth conditions of *Chrysotila dentata*, and the effect of light on its calcium carbonate and lipid accumulation.
- Explore the growth and accumulation of valuable products of *Chrysotila dentata* in the presence of bacteria.
- Explore low-cost nitrogen sources in the process of *Nannochloropsis oceanica* and the feasibility of outdoor small-scale breeding.
- Explore the advantages and disadvantages of new photobioreactor and low costs photobioreactor, as well as the growth changes and oil accumulation of Nannochloropsis oceanica in outdoor long-term cultivation.

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Chapter 2 Optimization of *Chrysotila dentata* Lipid and Calcium Carbonate Production: Influences from Polyethylene Terephthalate and Algae-Bacterial Symbiotic System

Abstract

The marine microalgae *Chrysotila dentata* shows potential for producing oil and fixing carbon dioxide to calcium carbonate, making it a promising candidate for carbon neutralization applications. However, cultivating this species both indoors and outdoors presents many challenges. Our study illustrated the optimal photoperiod for *C. dentata* growth is 18L/6D, with a culture pH of 8.5 and a salt concentration of 2.5 %. The optimal algal-bacterial ratio for the Algal-Bacterial Symbiotic System (ABSS) with *Chrysotila (Pleurochrysis) dentata* and *Nitratireductor aquibiodomus* was determined to be 8:2. We also examined the impact of microplastic PET on the ABSS. A suitable amount of micro-PET (5-15 mg/L) promotes rapid algae growth, but high concentrations of microplastic (20-50 mg/L) slow the algal growth.

Keywords: *Chrysotila dentata, Nitratireductor aquibiodomus,* lipids, calcium carbonate, Algal-Bacterial Symbiotic System (ABSS), carbon neutralization

1 Introduction

Due to energy crises, food shortages, and carbon dioxide emissions, microalgal studies have become more and more popular. As essential primary producers, microalgae exhibit rapid growth, generating rich organic matter through photosynthesis. Algae also remove carbon dioxide and nutrients from water, releasing oxygen into the atmosphere and accumulating various lipids, proteins, and carbohydrates (Rai et al., 2000; Judd et al., 2015; Singh et al., 2013; Li et al., 2008; Rahman, 2020; Barkia et al., 2019; Leu et al., 2014; Chew et al., 2017). Almost 70 % of the oxygen in the atmosphere is contributed by algae, surpassing the combined contribution of all forests (Rai et al., 2000; Proksch, 2013).

Algae can survive in wide array of different environments, including forests, under polar ice sheets, in subglacial soil, in the hottest and driest deserts, in swimming pools, aquariums, and waterways (Williamson et al., 2020; Andersen, 1992; Chapman et al., 2006). Their structure, it enables algae not only to survive in extremely harsh environments but also to produce high-value biomass (Seckbach, 2015).

The marine microalgae *Pleurochrysis dentata*, also known as *Chrysotila dentata* (Andersen et al., 2015), can utilize bicarbonate in the environment to synthesize $CaCO_3$ shells (10 % of algal dry weight). Additionally, its lipid content can reach 33.61 % of the algal dry weight (Marsh, 1999; Marsh, 2003; Chen et al., 2019). Due to its excellent carbon dioxide fixation ability and high oil production rate, *C. dentata* holds potential applications in carbon neutralization and biofuel production (Moheimani, 2005; Nimer & Merrett, 1993; Sikes et al., 1980).

Many photoautotroph organisms in the dim sea are not green because surface water absorbs red and violet light, which chlorophyll cannot efficiently absorb. In the deep ocean, algae and phytoplankton rely on accessory pigments, also called 'mask pigments' to harvest blue and green light and then transfer that energy to chlorophyll molecules for photosynthesis (Rowan, 1989; Haxo, 1960). Accessory pigments can mask the green appearance of chlorophyll. As a result, the algae have a yellow-brown or red appearance (Calkins, 2011; Haxo, 1960). Light has been shown to be linked with the occurrence of algal bloom and algal growth. (Zhou et al., 2008; Zhou et al., 2018; Riebesell et al., 2007). *C. dentata* presents a brown color, and the optimal photoperiod for its indoor growth is unclear.

Since the industrial revolution, burning of fossil fuels has affected marine pH, impacting the marine ecosystem (Fabry et al., 2008; Riebesell et al., 2007). Previous studies have investigated the $CaCO_3$ accumulation of *C. dentata* in different pH environments (Song et al., 2014). However, during algal cultivation, medium evaporation can lead to an increase in salt concentration (The typical ocean salinity is 3.5%). Therefore, identifying algae with wide salinity tolerance poses a challenge in both indoor and outdoor microalgae cultivation (Belay, 1997; Packer, 2009). Microalgae with broad adaptability to salt concentration can be cultivated over a wider range and in different regions, exhibiting good adaptability to variations in salt concentration caused by evaporation and precipitation. Furthermore，after several generations of indoor cultivation, algae may show a weakened state, possibly leading to death (de Oliveira, 2020). The hypothesis suggests that the presence of bacterial pollution may lead to the algal death. Previous studies reported that bacteria are present in

open-raceway ponds and all types of open cultivation systems (Benemann, 1996; Huntley et al., 2007; Packer, 2009). Nitrifying bacteria oxidize ammonia nitrogen into nitrite and nitrate ions, which are widely used in aquaculture (Urakawa et al., 2020).

Nevertheless, marine microalgae cultivated in inland and small-scale indoor conditions still have challenges. Therefore, this experiment focuses on investigating suitable conditions for the indoor cultivation of *C. dentata* and algae-bacterial symbiotic system.

The optimal photoperiod for *C. dentata* growth was determined, aiming to accumulate more valuable bioproducts. This experiment further explores the accumulation of lipids and $CaCO_3$ under different salt concentrations. This research also investigates whether *Nitratireductor aquibiodomus* promotes or inhibits the growth of *C. dentata* and the changes in algal biomass under symbiosis.

2 Methods and materials

2.1 Algal and bacteria strain

Chrysotila (Pleurochrysis) dentata was kindly provided by Dr. Fan Lu, Hubei University of Technology, China. Whereas bacterial strain *Nitratireductor aquibiodomus* was isolated from the previous *C. dentata* culture medium.

2.2 Lipid measurement

The chloroform-methanol extraction method was used for algal lipid content measurement (Bligh & Dyer, 1959). The procedure is as follows:

Dried algal powder (0.01 g) was transferred to a 1.5 mL centrifuge tube (T0). Then chloroform, methanol, and water were added to make the volume ratio of 1:2:0.8. Following

thism the solution was mixed by a sonicator at 30 KHz for 30 seconds. After that, the solution was vortexed for 3-5 minutes. Then, centrifugation was done at 13,800 g for 3 minutes and the supernatant was transferred to a 15 mL tube. The above process was repeated three times. All supernatants were combined in the 15 mL tube, then water and chloroform were added, to make the final chloroform: methanol: water volume ratio was 1:1:0.9, mixed well and stood the 15 mL tube until the liquid layers separated. The bottom layer was then transferred to a pre-weighed tube (M1) and placed it in a fume hood overnight. Dried the tube (M1) in oven at 105 ℃ until the weight stabilized. The tube was weighed and recorded as M2 (g). To calculate the total lipid content, use the following equation:

$$\text{Lipids content (\%)} = \frac{\text{M2(g)} - \text{M1(g)}}{0.01\text{g}} \times 100\%$$

2.2 Optimization of cultural conditions

2.2.1 Algal culturing and sample collection under different photoperiods, pH, and salinity

C. dentata was cultured in f/2 medium (Chen et al., 2019) under six different photoperiods: 10L/14D, 12L/12D, 14L/10D, 16L/8D, 18L/6D, and 20L/4D, each with three repetitions. Similarly, the cultural conditions for *C. dentata* were optimized under five different pH values of the f/2 medium were set: 6, 7, 8, 8.5, and 9, and six different salinity concentrations (Instant Ocean® sea salt): 1.5 %, 2.5 %, 3.5 %, 4.5 %, 5.5 %. The initial cell number of algae was 1×10^6 per liter. Algal samples were collected on Day 0, Day 10, Day 15, Day 20, Day 25, and Day 30. For the determination of algal weight, 200 mL of algal medium was collected by centrifugation at 2400 g for 10 minutes and then dried in a 105 ℃ oven for

24 hours.

2.2.2 Algal culturing and sample collection under different algae bacteria ratio

The 11 algal-to-bacterial inoculation ratios (Table 1) were created to investigate the influence of bacteria on algal growth. The algae and bacteria were cultivated in 2 L of f/2 medium with an 18-hour light/6-hour dark cycle, pH of 8.5, and a salt concentration of 2.5 %. Algae and bacteria in the logarithmic growth stage were selected, and both were present at a cell number of 1×10^6 per liter.

Table 1 The ratio of *Chrysotila dentata* and *Nitratireductor aquibiodomus*

Chrysotila dentata: Nitratireductor aquibiodomus volume ratio										
10:0	9:1	8:2	7:3	6:4	5:5	4:6	3:7	2:8	1:9	0:10

2.3 Algal culturing and sample collection under different concentrations of PET

In this experiment, polyethylene terephthalate (PET) powder (Goodfellow™, England) with a maximum particle size of 300 microns and white color was used. Different concentrations of microplastic PET (0 mg/L, 5 mg/L, 10 mg/L, 15 mg/L, 20 mg/L, 25 mg/L, 30 mg/L, 35 mg/L, 40 mg/L, 45 mg/L, 50 mg/L) were added to the culture medium to explore the effects of microplastics on algae-bacteria symbiosis. Based on the results from optimization of cultural conditions, a 2.5 L glass photobioreactor was used for the experiment with 2 L of f/2 medium, 18-hour light/6-hour dark cycle, pH of 8.5, 2.5 % salt concentration, and an algae-to-bacteria volume ratio of 8:2.

2.4 Calcium carbonate measurement

The ethylenediaminetetraacetic acid (EDTA) complexometric titration method was used for calcium content measurements (Chen et al., 2019). The procedure is as follows:

In a 1.5 mL centrifugation tube (T1), 1 mL of 75 % ethanol was mixed with 0.01 g of dry *C. dentata* powder to start the decolorization steps. T1 was then transferred to an 80 ℃ water bath for 30 minutes followed by centrifugation at 13,800 g for 3 minutes with the resulting green supernatant being discarded. This step was repeated until the solution became colorless. 1.5 mL of HCl (2 N) was added into the decolorized sample in T1 and then incubated in an 80 ℃ water bath for 30 minutes to remove CO_2. After that, the supernatant was collected by centrifugation at 13,800 g for 3 minutes. Then, the EDTA complexometric titration method was used to measure calcium ions in the supernatant [35]. Supernatant (1 mL) was mixed with 800 μL of dd-water, 4.5 mL of calcium indicator ammonium triacetate (20 %), and 1 mL of $NaNO_3$ (20 %). Then the pH was adjusted between 12.5 and 13. After that, 200 μL of calconcarboxylic acid (5 mg/mL) was added followed by 0.02 M of EDTA · 2Na added drop by drop until the color changes red to blue (the color was maintained for at least 30 seconds). Using the following equation calcium ion content (mg/L) was calculated:

$$\text{Calcium ion content} = \frac{\text{M} \times \text{a} \times 40.08}{\text{V}} \times 1000$$

M: standard solution molar concentration of EDTA.

a: The volume (mL) of used EDTA during the titration.

V: Sample volume (mL).

The atomic weight of calcium: 40.08.

2.5 Scanning electron microscope (SEM)

The Scanning Electron Microscope (SEM) was used to observe the $CaCO_3$ shell of *C.*

dentata (Calvert et al., 1976). The steps used are as follows: (1) Fixed *C. dentata* cells using a 2.5 % to 3 % concentration of glutaraldehyde with a pH ranging between 6.8 and 7.4. *C. dentata* cells were placed in the glutaraldehyde fixation solution for 30 minutes (Chen et al., 2019). (2) The fixed *C. dentata* cells were then dehydrated using gradient concentrations of ethanol (50 %, 60 %, 70 %, 80 %, 90 % and 100 %), with a soak time of 15 minutes for each concentration. (3) These samples were kept in -80 °C freezers until use. (4) Before SEM analysis, the samples were transferred from -80 °C freezers to a freeze dryer (LABCONCO freezone 12, Kansas City, MO, USA) for 24 hours.

2.6 Chlorophyll α measurement

The chlorophyll α content of *C. dentata* was measured using the methanol extraction method (Chen et al., 2008). In this experiment, a quicker chlorophyll α measurement method was used. The steps are as follows:

C. dentata (1 g) was collected by centrifugation at 13,800 g for 3 minutes and discarded the supernatant. The collected algae were mixed with 1 mL of methanol and placed in a 50 ℃ water bath for 5 minutes, vortexing occurred 2-3 times during the water bath. Then, the sample was immediately cooled to room temperature, and centrifuged at 13,800 g for 3 minutes. The supernatant was transferred into a 1.5 mL centrifugation tube, and then methanol was added to make the final volume 1 mL. The above steps were repeated until the samples were not green. The optical density of the methanol extract was measured against a methanol blank at 665 nm and 750 nm using an Epoch™ Microplate Spectrophotometer BioTek®. The chlorophyll α concentration was determined by using the following equation:

$$\text{Chlorophyll } \alpha \ = 13.9(OD665 - OD750) \times \frac{U}{V}$$

U = the final methanol volume

V = the sample volume.

2.7 Box-Behnken design (BBD) for optimizing the bioproduct production.

The optimization of growth conditions for algal dry weight, lipid content, calcium content, and chlorophyll α in the culture medium was conducted using the Response Surface Methodology (RSM). Based on the results of single-factor experiments, the Box-Behnken design (BBD) was employed, selecting three factors with three levels each. The RSM model was constructed using Design-Expert 10 software (Stat-Ease, Inc., USA), incorporating the BBD table. To assess the goodness of fit of the second-order polynomial model equation, an F-test was conducted with the R^2 coefficient and lack of fit set at a significance level of 5 %. Additionally, the highest production of algal dry weight, lipid, calcium, and chlorophyll α, along with the corresponding culture conditions, was calculated based on the RSM model.

2.8 Statistical Analysis

All experiments were conducted in triplicate. The results obtained from these triplicates underwent statistical analysis using the one-way ANOVA function in IBM SPSS® software. For comparison, we employed Duncan' s multiple-range test, with a significance level (P-value) set at 0.05. The statistical significance results for $CaCO_3$, lipid, and chlorophyll α contents are individually reported in the supplementary information file.

3 Results and discussion

3.1 The results of optimum photoperiod for *C. dentata* growth

The algal samples were collected on day 0, day 10, day 15, day 20, day 25, and day 30. After 30 days of cultivation, the highest algal dry weight (0.24 g/ 200 mL) was observed in the 20L/4D photoperiod, higher than the other photoperiod groups.

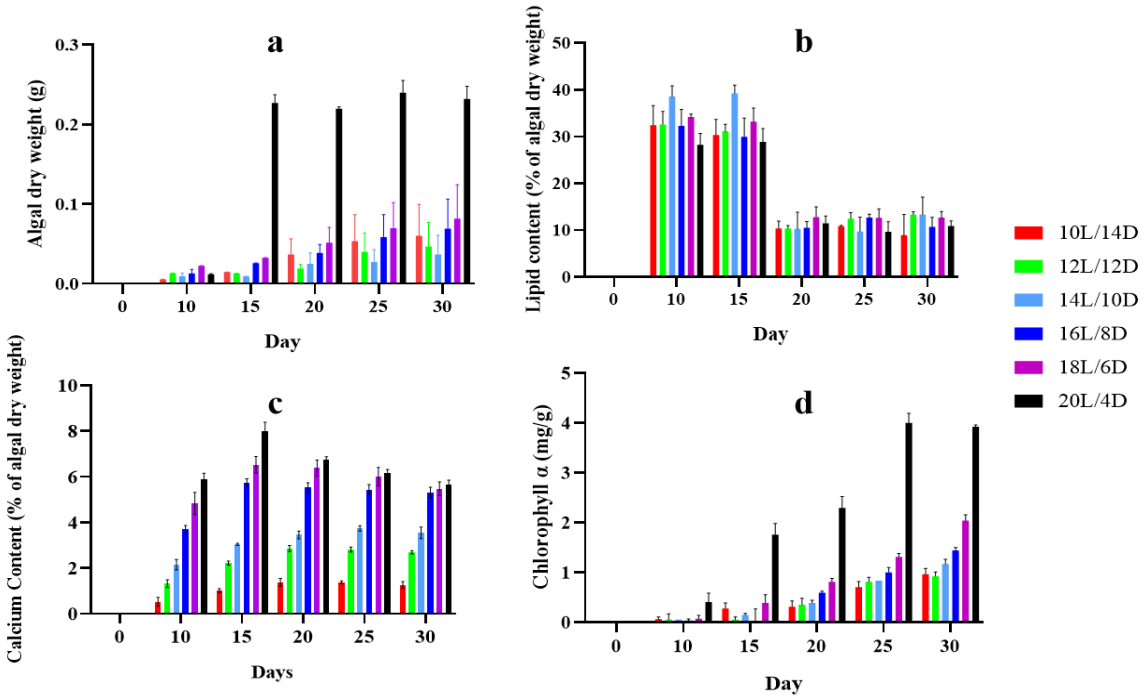


Figure 1 Bioproducts of *C. dentata* under different photoperiods. **a:** Algal dry weight. **b:** Lipid content. **c:** Calcium carbonate content. **d:** Chlorophyll α

However, rapid growth does not necessarily translate to better accumulation of other bioproducts. Algae tend to consume energy rather than accumulate substances during rapid growth (Packer, 2009). Our results also support this perspective; the lowest lipid accumulation in *C. dentata* occurs under the 20L/4D photoperiod (Figure 1-b). When the photoperiod is 14L/10D, *C. dentata* exhibits the highest lipid content on the 15th day,

constituting 39.22 % of algal dry weight, which is higher than the known 33 % lipid content of *C. dentata* (Moheimani et al., 2006). The second-highest lipid content was observed under 18L/6D photoperiod, at 34.18 %.

From figure 1-c, *C. dentata* accumulated more $CaCO_3$ when the light period was long. Under the 20L/4D photoperiod, *C. dentata* exhibits the highest calcium content on day 15, reaching 7.97 % of the algal dry weight. The second-highest calcium content was observed in 18L/6D photoperiod, which took 6.55 % of the algal dry weight.

The hypothesized function of *C. dentata*' s $CaCO_3$ shell was to shield it from external mechanical forces (Chen et al., 2019). However, the results of this experiment showed another potential function of the *C. dentata* shell. It may serve to protect *C. dentata* from light damage by blocking strong light and reducing the impact on coccolith.

Thus, scanning electron microscopy (SEM) was used on *C. dentata* cultivated under different photoperiods to assess potential alterations in the thickness of the $CaCO_3$ shell. Algae from Day 15 were selected for SEM analysis. Based on the SEM results (Figure 2), we utilized the thickness of the outer tube of the V unit coccolith as the criterion.

The coccolith thickness was found to increase with prolonged illumination duration. Specifically, under a 20L/4D light cycle, the outer tube of the V unit measures 132 nm (Figure 2-f). In contrast, the thickness is reduced to 70.7 nm under a 10L/14D light cycle (Figure 2-a), and the second thickest outer tube is observed under an 18L/6D photoperiod, measuring 98.5 nm. The result indicates that the most suitable light intensity for *C. dentata* $CaCO_3$ shell accumulation is under a 20L/4D photoperiod.

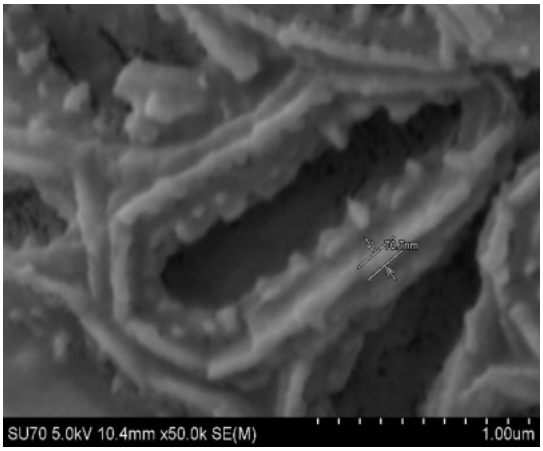
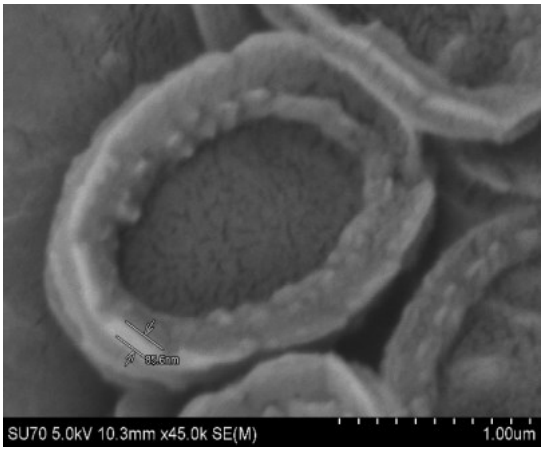
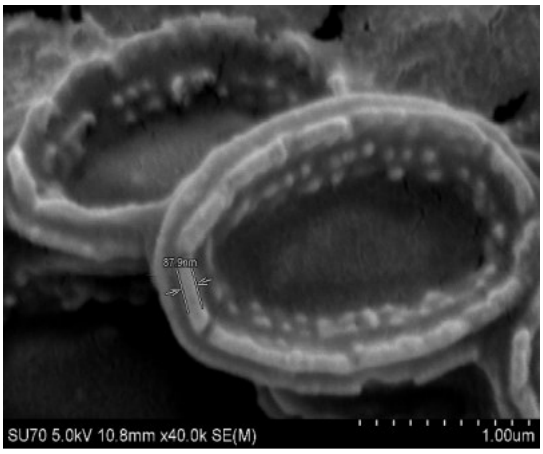
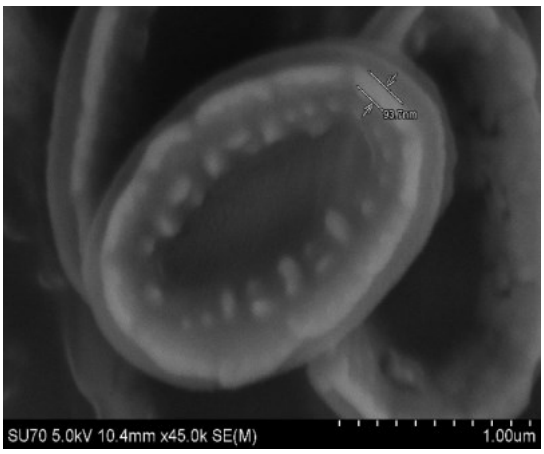
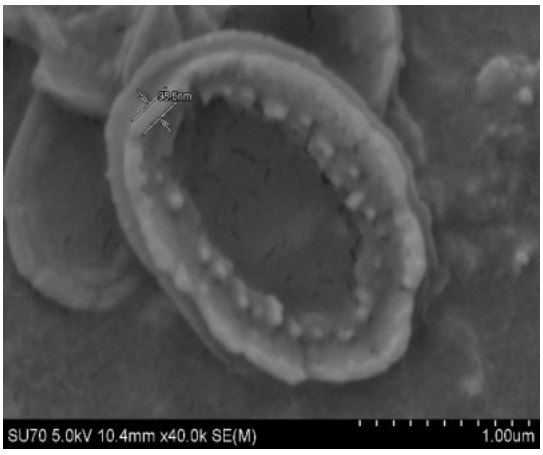
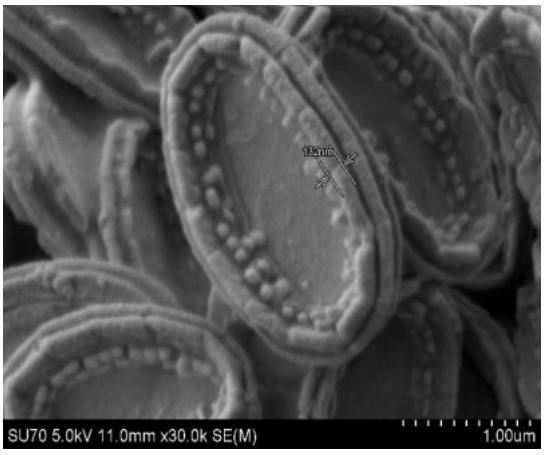
a: 10L/14D	**b:** 12L/12D
	
c: 14L/10D	**d:** 16L/8D
	
e: 18L/6D	**f:** 20L/4D
	

Figure 2 Coccolith thickness of *C. dentata* grown under different photoperiods. a: 10L/14D; b: 12L/12D; c: 14L/10D; d: 16L/8D; e: 18L/6D; f: 20L/4D.

Our findings confirm that the photoperiod influenced the shell thickness of *C. dentata*.

This also presented new ideas for improving the carbon fixation capacity of *C. dentata*, which was to extend the photoperiod appropriately or place the cultivation photobioreactors in an open area with more light. Further research is needed to determine whether *C. dentata* can mitigate the damage caused by excessive light by increasing the thickness of its shell.

The results for chlorophyll α exhibited a similar trend to that of the algal dry weight. Under the 20L/4D photoperiod, *C. dentata* showed the highest chlorophyll α content, at 4.00mg/g (Figure 1-d). The second-highest chlorophyll α content, at 2.04 (mg/g), was observed under the 18L/6D photoperiod on day 30. The experimental results showed positive correlation between light intensity and chlorophyll α content.

3.2 Optimization of cultural conditions

3.2.1 The results of optimum pH values for *C. dentata* growth

At a pH of 9, *C. dentata* exhibited the highest dry weight, with the second-highest dry weight observed in *C. dentata* grown in a medium at pH 8.5 (Figure 3-a). One fascinating observation from this experiment was that *C. dentata* autonomously regulated the pH of its surrounding environment (Figure 4). Previous studies have highlighted that *Emiliania huxleyi* produces dimethylsulfur (DMS) during its growth (Van Rijssel et al., 2002). Dimethyl sulfoniopropionate (DMSP) is commonly found in marine phytoplankton cells. After decomposition, it can be converted to DMS and enters the atmosphere which forms acid rain (Charlson et al., 1987; Kirst et al., 1991; Cuhel et al., 1987; Van et al., 2002; Stefels, 2000).

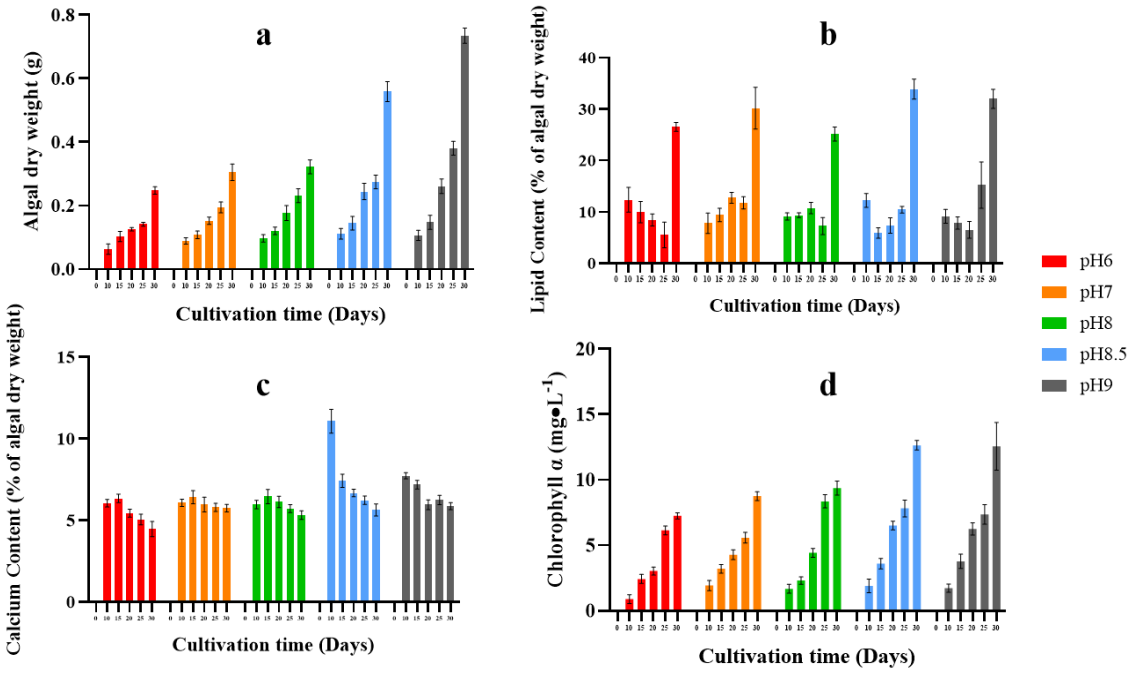


Figure 3 Bioproducts of *C. dentata* under different pH values. **a:** Algal dry weight. **b:** Lipid content. **c:** Calcium carbonate content. **d:** Chlorophyll α

From Figure 4, the pH value in the pH 9 group consistently decreased. The other four experimental groups' pH values were increased from day 0 to day 5. After day 5, the pH values gradually decreased, and stabilized around 7.5 by day 30. We hypothesized that after day 5, *C. dentata* begins to secrete dimethylsulfur (DMS) during its growth (Need to be verified in future experiments).

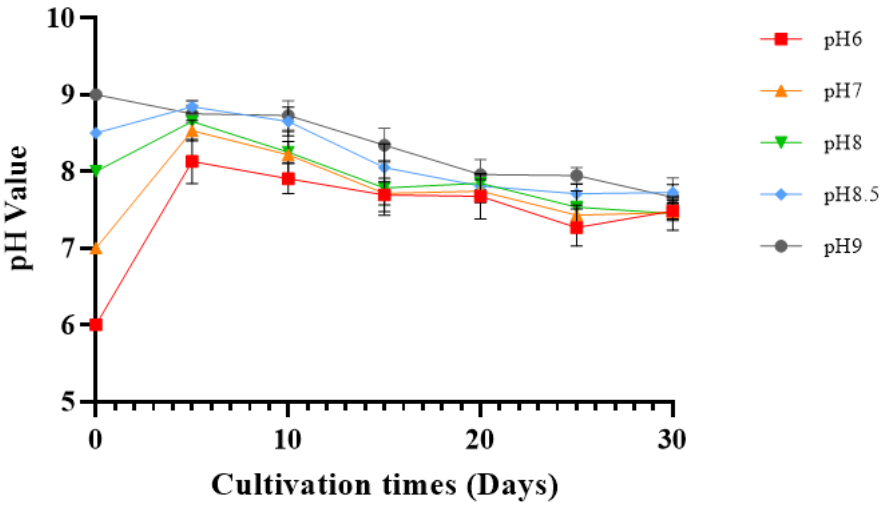


Figure 4 The changes of medium pH value when *C. dentata* growth.

In Figures 3-b and 3-c, When the medium pH value was 8.5, it showed the highest lipid and $CaCO_3$ content, reaching 33.88 ± 1.95 % and 11.08 ± 0.73 % of algal dry weight, respectively. Conversely, when the medium was adjusted to a pH of 6, the content of both lipid and $CaCO_3$ was relatively low.

The results of pH on chlorophyll α content differed from the results of pH on lipid and $CaCO_3$ content. The optimum pH environment for *C. dentata* chlorophyll α accumulation was at pH of 8.5 (12.64 mg/mL). The lowest amount of chlorophyll α appeared at pH 6 on day 30.

3.2.2 The results of optimum salinity for *C. dentata* growth

The salt concentration was not constant, whether in a laboratory culture, an outdoor open culture, or the natural marine environment, factors such as natural evaporation, river estuary discharge, and rainfall contribute to continuous salinity fluctuations (Summer et al., 2005). The experiment cultured *C. dentata* at different salt concentrations to observe the effects of salt concentration on algal growth. According to the results of figures 5, *C. dentata* salinity tolerance range was 1.5 % to 3.5 %. High salinity levels, such as 4.5 % and 5.5 %,

have an inhibitory effect on the growth of *C. dentata*.

As shown in Figure 5-a, the optimal salinity for achieving the highest *C. dentata* dry weight was 2.5 %, reaching 0.1696 g on day 25. Interestingly, when salinity was 4.5 % and 5.5 %, the algal dry weight remained almost unchanged.

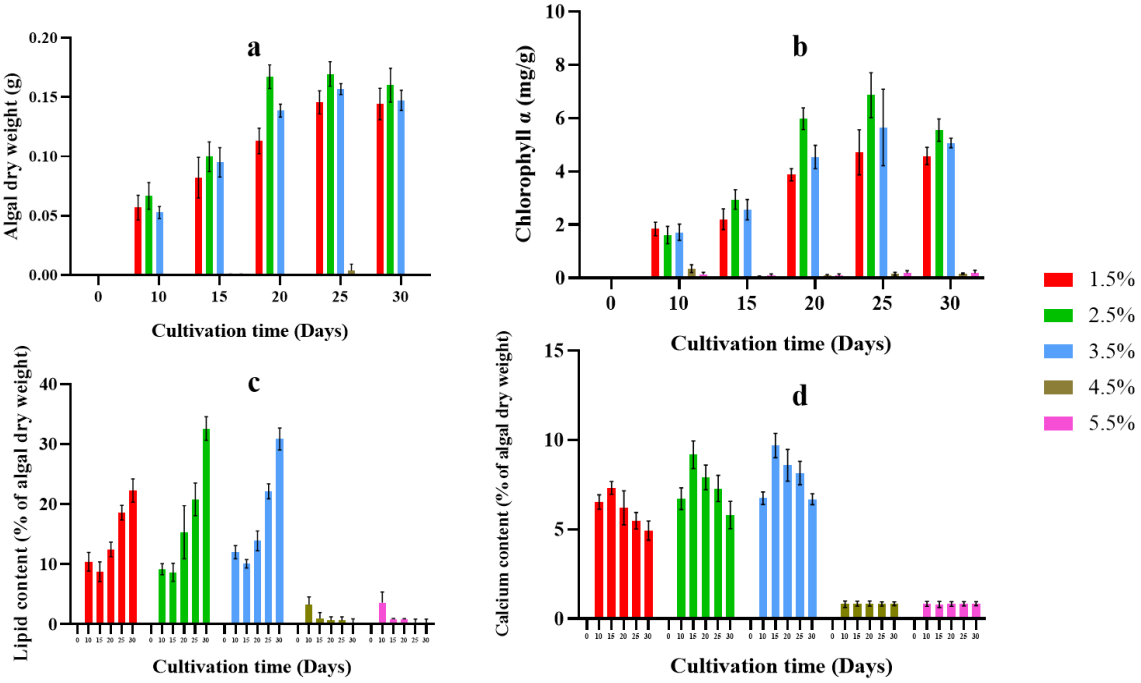


Figure 5 Bioproducts of *C. dentata* under different salt concentrations. **a:** Algal dry weight. **b:** Chlorophyll α. **c:** Lipid content. **d:** Calcium carbonate content.

The *C. dentata* cultures exhibited the highest chlorophyll α content (6.86 mg/g) when cultivated with 2.5 % salinity on day 25. In contrast, when the salinity was set at 3.5 %, the chlorophyll α content only reached 5.65 mg/g on day 25. Notably, elevated salinity levels, such as 4.5 % and 5.5 %, were found to inhibit the accumulation of algal chlorophyll α.

The lipid content of *C. dentata* increased from day 0 to day 10 across all five salinity levels (Figure 5-c). However, a notable difference emerged after day 10: the lipid content in

the 3.5 %, 4.5 %, and 5.5 % salinity groups began to decrease, while the lipid content in the 1.5 % and 2.5 % salinity groups continued to increase. From Figure 5-c, the optimal salt concentration for lipid accumulation was 2.5 %, at 32.57 % of algal dry weight.

Under five different salinity conditions, $CaCO_3$ content exhibited an increasing trend from day 0 to day 10 in all five groups (Figure 5-d). However, after day 10, the $CaCO_3$ content showed a decreasing trend in the 1.5 %, 2.5 % and 3.5 % salinity groups. However, in the 4.5 % and 5.5 % salinity groups, the $CaCO_3$ content remained stable after day 10. When salinity was 3.5 %, the highest $CaCO_3$ content reached was 9.68 % of *C. dentata* dry weight on day 15, then the $CaCO_3$ content began to decrease.

In summary, the optimal salt concentrations for algal dry weight, chlorophyll α and lipid accumulation were 2.5 %, for calcium accumulation a salinity of 3.5 %. Although it is generally recommended to use a salinity of 3.5 % for the f/2 medium, our results indicated that the standard f/2 medium salinity was not suitable for *C. dentata*.

3.4 Optimization of algal dry weight, lipid content, calcium content, and chlorophyll α content using response surface methodology

Response surface methodology (RSM) is a commonly used statistical approach for optimizing the production of bioproducts. It not only provides guidance during the optimization process but also explains the interactions among several factors. Response surface methodology plays an important role in the algae-related industries, enabling large-scale production of specific products.

Table 2 Box-Behnken design for optimizing bioproducts from *C. dentata*.

Run	A: Photoperiod (h)	B: pH Value	C: Salinity (%)	Algal dry weight (g)	Lipid content %	Calcium content %	Chlorophyll α content
1	16	8	2.5	0.5573	31.9	8.81	1.57
2	20	8	2.5	0.6456	32.22	9.43	2.58
3	16	9	2.5	0.6499	32.36	9.51	2.62
4	20	9	2.5	0.67	32.59	9.63	2.67
5	16	8.5	1.5	0.4866	31.76	8.6	1.23
6	20	8.5	1.5	0.5833	32.02	9.11	1.79
7	16	8.5	3.5	0.5891	32.12	9.29	1.99
8	20	8.5	3.5	0.6899	32.97	9.93	2.99
9	18	8	1.5	0.4915	31.85	8.72	1.39
10	18	9	1.5	0.5667	31.97	8.96	1.66
11	18	8	3.5	0.6709	32.71	9.73	2.7
12	18	9	3.5	0.6723	32.86	9.86	2.87
13	18	8.5	2.5	0.7499	33.89	10.71	3.89
14	18	8.5	2.5	0.7293	33.51	10.41	3.71
15	18	8.5	2.5	0.7351	33.62	10.52	3.75
16	18	8.5	2.5	0.7598	33.97	10.89	3.98
17	18	8.5	2.5	0.7403	33.72	10.65	3.79

For instance, optimizing the conditions for algae lipid accumulation can facilitate the production of biodiesel, and further optimization for algae metal adsorption enhances its application value (Hallenbeck et al., 2015；Hasnain et al., 2023；Jaafari et al., 2019). In other fields, such as optimizing the production conditions of enzymes, RSM has also found extensive application (Bandal et al., 2021). After further optimization through BBD and design matrix, the production results of algal dry weight, lipid content, calcium content, and chlorophyll α content are detailed in Table 2.

The photoperiod (A: 16 hours, 18 hours, 20 hours), initial medium pH (B: 8, 8.5, 9), and salinity (C: 1.5 %, 2.5 %, 3.5 %) were selected as independent factors, while algal dry weight, lipid content, calcium content, and chlorophyll α content were set as response values.

Following statistical analysis using response surface methodology, the relationship between algal dry weight, lipid content, calcium content, and chlorophyll α content and individual factors can be expressed by the following formulas:

$$\textbf{Alage dry weigh} = 0.74 + 0.038A + 0.024B + 0.062C - 0.017AB + 0.00125AC - 0.018BC - 0.0063A^2 - 0.05B^2 - 0.093C^2$$

$$\textbf{Lipid content} = 33.74 + 0.21A + 0.14B + 0.38C - 0.023AB + 0.15AC + 0.0075BC - 0.8A^2 - 0.67B^2 - 0.72C^2$$

$$\textbf{Calcium content} = 10.64 + 0.24A + 0.16B + 0.43C - 0.13AB + 0.033AC - 0.028BC - 0.69A^2 - 0.6B^2 - 0.72C^2$$

$$\textbf{Chlorophyll α content} = 10.64 + 0.24A + 0.16B + 0.43C - 0.13AB + 0.033AC - 0.028BC - 0.69A^2 - 0.6B^2 - 0.72C^2$$

The results of the analysis of variance (ANOVA) for the model are presented in supplementary material Table S1 to 4. The R-squared values for algal dry weight (0.9795), lipid content (0.9744), calcium content (0.9759), and chlorophyll α content (0.9872) indicate that many of the response values (dependent variables) can be explained by the independent variables and the equation.

The p-values for the regression and lack-of-fit tests for the four bioproducts were all greater than 0.001. Based on the results of lack of fit, the results for algal dry weight (0.0947), lipid content (0.4944), calcium content (0.5751), and chlorophyll α content (0.1223) suggested that the established quadratic equation was relatively reliable in explaining the impact of these independent factors on bioproduct production, and there was no lack of fitness issues.

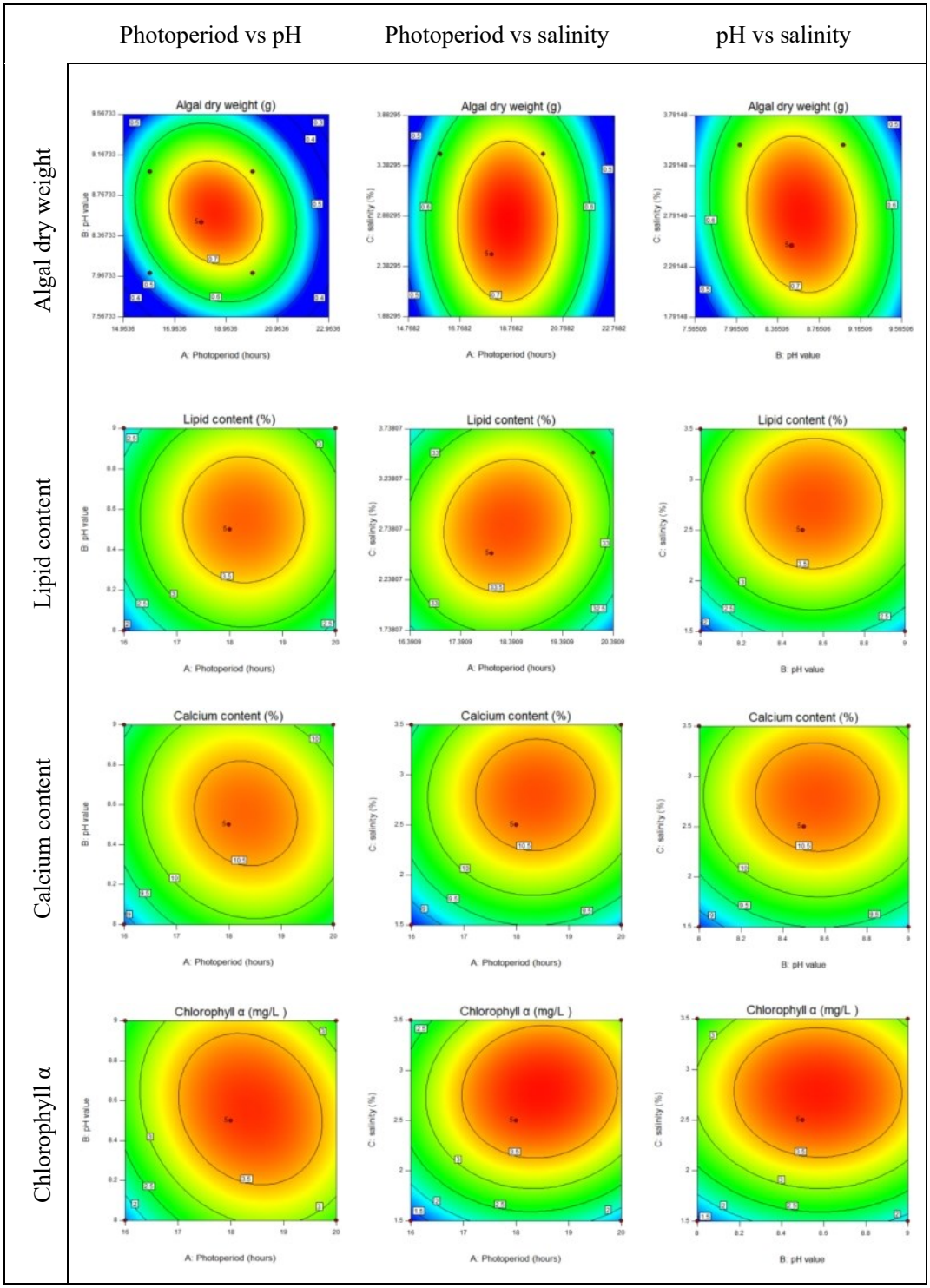


Figure 6 The 2-D counter plots of the established RSM model indicate the interaction between different individual factors.

The 2D contour plots of the equation in Figure 6 illustrated that optimal values for algal dry weight (0.755 g), lipid content (33.787 %), calcium content (10.697 %), and chlorophyll

α content (3.913 mg/g) can be achieved under the tested conditions (Photoperiods: 18.485 L; pH value: 8.549; Salinity: 2.604). Additionally, the elliptical shape of all contour plots suggested that these variables were influenced by the interaction between independent factors. The equation and contour plots illustrated that the duration of light exposure had the most significant impact on dry weight, lipid content, calcium content, and chlorophyll α content. Next in significance were the pH value and salinity. However, this did not imply that salinity and pH had an insignificant impact on the products; on the contrary, all three factors played a crucial role in the accumulation of products, as indicated by the results.

Based on the equation within the range of the BBD design, it was predicted that under the conditions of 18 hours of light, medium pH 8.5, and 2.5 % salinity, the highest dry weight of *C. dentata* could reach 0.7595 ± 0.09 g, the highest lipid content of dry weight could reach 33.97 ± 0.77 %, the highest calcium content could reach 10.89 ± 0.74 % of algal dry weight, and the highest chlorophyll α content possible is 3.98 ± 0.94 mg/g. It was anticipated that the impact of cultivation conditions on the production of these various components might be slightly flexible, depending on the target product.

This finding held significant implications for algal cultivation in controlled indoor small-scale environments. However, if we contemplate expanding the cultivation setting to the outdoors, a more comprehensive consideration of factors such as natural patterns and economic costs is required for a better optimization of cultivation conditions to adapt to diverse environments. This systematic optimization approach is poised to enhance the efficiency and sustainability of algal production, offering valuable guidance for future

research and industrial applications.

3.5 The results of optimum algae and bacteria ratio for *C. dentata* growth

During the algal culturing process, algae appeared to being poor state after several generations of cultivation, and in some cases, they even died, blocking the culture continuation. It was speculated that the algal mediums were contaminated by bacteria, leading to weakened growth and eventual death (Ji et al., 2018; Li et al., 2023; Saravanan et al., 2021). A bacterial strain, *Nitratireductor aquibiodomus*, was subsequently isolated from the suboptimal algal culture medium, confirming the speculation.

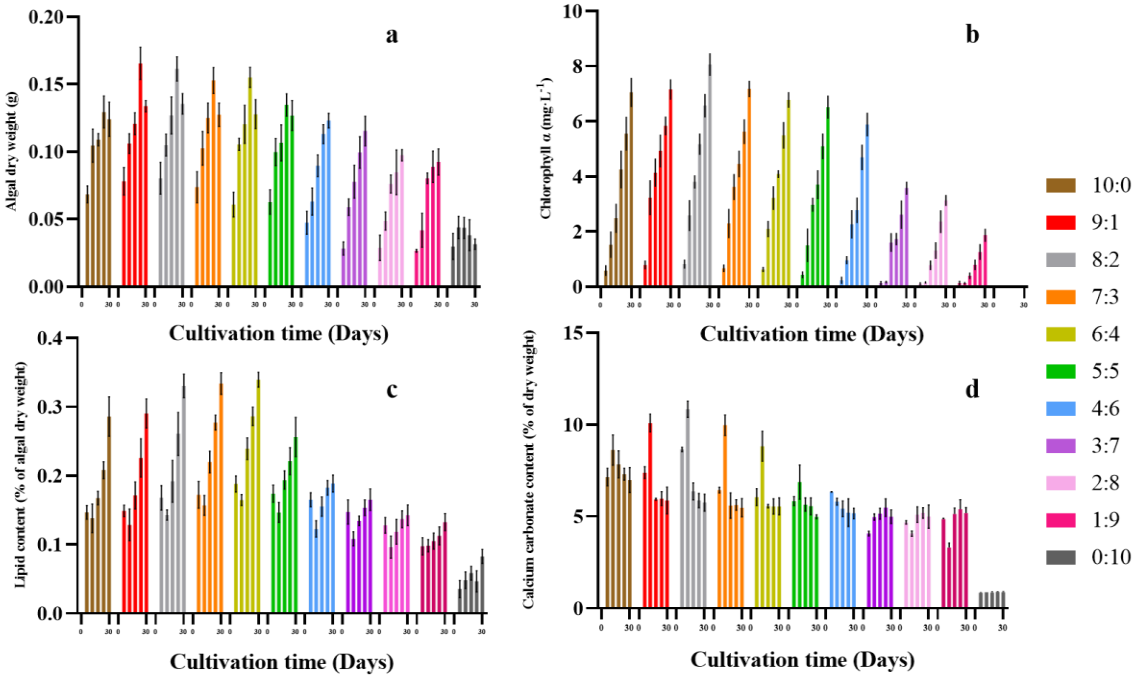


Figure 7 Bioproducts of *C. dentata* under different algal- bacterial ratios. **a:** Algal dry weight. **b:** Chlorophyll α. **c:** Lipid content. **d:** Calcium carbonate content.

The challenge in algae application production is the presence of bacteria, and whether the relationship is symbiotic or competitive needs exploration. In this experiment, symbiosis

tests were conducted using marine microalgae *C. dentata* and bacteria *Nitratireductor aquibiodomus* to examine the changes in biological products under different algal-bacterial inoculation ratios and the growth status of algae.

According to the results in Figure 7-a, except for the algal-bacterial 0:10 group, all other groups exhibited increasing dry weight from day 0 to day 25. The group with algal-bacterial ratio of 9:1 showed the highest dry weight on day 25 (0.17 g). The dry weight ranking from high to low on day 25 was as follows: 9:1 > 8:2 > 7:3 > 6:4 > 5:5 > 10:0 > 4:6 > 3:7 > 2:8 > 1:9 > 0:10.

Figure 8 The growth situation of different algal- bacterial ratio on Day 10 (From left to right: 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10).

Photos taken on day 10 (Figure 8) revealed interesting phenomena. When algal-bacterial ratio was 10:0, the algal showed light yellow color. The population of *C. dentata* was higher from an algal-bacterial ratio 9:1 to 5:5 than 4:6 to 0:10.

The results from chlorophyll α analysis indicated that the combination of *C. dentata* and *N. aquibiodomus* could influence chlorophyll α accumulation (Figure 7-b). The highest chlorophyll α content was 8.06 mg/L and observed when the algal-bacterial ratio was 8:2 on day 30, followed by 7.17 mg/L with 7:3 algal-bacterial ratio. From Figure 7-c, the 6:4 group exhibited the highest lipid content on day 10, accounted for 33.95 % of the dry weight. The

second highest lipid content on day 10 was observed in the 7:3 group at 33.38 %, followed by the 8:2 group at 33.05 %.

These findings suggest that the growth of *C. dentata* was not entirely independent of *N. aquibiodomus*. Compared to the group without bacteria, when *C. dentata* inoculation was high, *N. aquibiodomus* could coexist and even promote the growth of *C. dentata*. However, excessive inoculation of *N. aquibiodomus* could inhibit the growth of *C. dentata*. The results indicated that *C. dentata*' s lipid accumulation was influenced by *N. aquibiodomus*, especially from 0 to 10 days, when *N. aquibiodomus* enhanced lipid accumulation. Excessive bacteria could induce stress on *C. dentata* growth, leading to nutrient accumulation in algae to survive. This explained why, after 10 days, the group with a larger bacterial content showed a downward trend in lipid content.

In the figure 7-d, a peak was observed on day 10. Among these, the highest accumulation of $CaCO_3$ occurred when the algal-bacterial ratio was 8:2, accounted for 10.83 % of the dry weight. Followed by ratio 9:1, where $CaCO_3$ accounted for 10.09 % of the dry weight. The 10:0 group contained 8.62 % of dry weight in $CaCO_3$. After day 10, the accumulation of $CaCO_3$ continued to increase in most groups where bacteria dominated. However, in groups where algae were predominant, the accumulation of $CaCO_3$ gradually decreased. All groups began to level off at day 15. Also, it was evident from Figure 7-d that the optimal algal-bacterial ratio for $CaCO_3$ content accumulation is 8:2 on day 10.

3.6 Effects of polyethylene terephthalate on algal-bacterial symbiotic system.

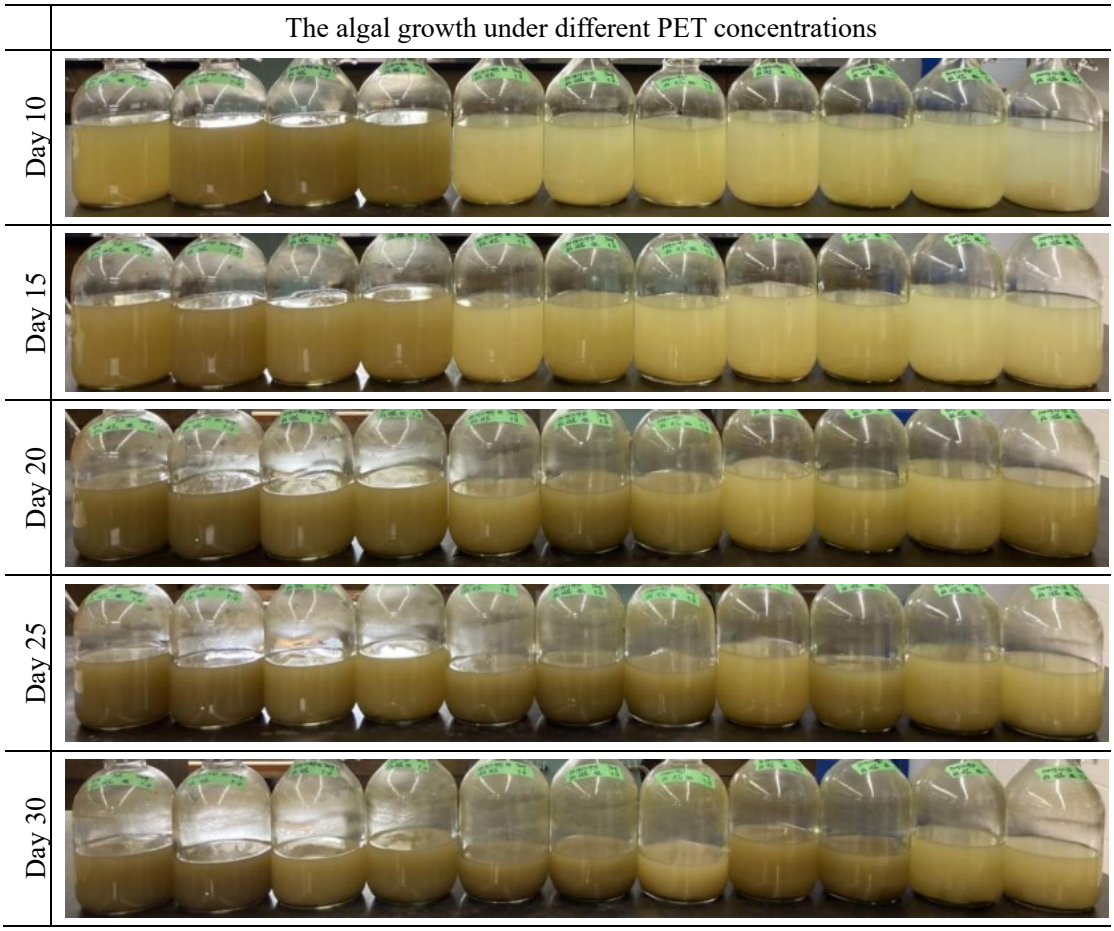


Figure 9 The algal growth under different PET concentrations on day 10, 15, 20, 25, 30 (from left to right the PET concentration is 0 mg/L, 5 mg/L, 10 mg/L, 15 mg/L, 20 mg/L, 25 mg/L, 30 mg/L, 35 mg/L, 40 mg/L, 45 mg/L, 50 mg/L).

Through the four tests conducted under the conditions of a photoperiod of 18L/6D, pH of 8.5, 2.5 % salinity, and an algal-bacterial ratio of 8:2, the impact of microplastic PET on the growth state of the algal-bacterial symbiotic system was observed. Photobioreactor images from the experiment are shown in Figure 9 which illustrates PET concentrations from 20-50 mg/L slow the growth of *C. dentata*, primarily by blocking light. Interestingly, concentrations around 5 mg/L to 15 mg/L promoted *C. dentata* growth (Figure 9).

The algal-bacterial symbiotic system containing a high concentration of PET showed

significant growth starting from day 20. This may have been due to PET providing an attachment for algae. The size of microplastic PET used in this experiment was 300 microns, significantly larger than *C. dentata* (around 5 microns) cell size. The microplastic' s larger size offered attachment points for *C. dentata*, potentially reducing damage caused by shear forces (Packer, 2009). This phenomenon also provides insight into how microplastics enter the food chain. Algae predators consume the mixture of algae and microplastics, with the microplastic eventually entering the human body through the food chain (Leslie et al., 2022).

This finding suggests a novel approach to microplastic collection, using algae to capture microplastics and subsequently recovering the algae-microplastic mixture.

Alternatively, the ABSS may have degraded PET as a carbon source which merits further exploration in future research. Nevertheless, when micro-PET concentration was 50 mg/L, it could inhibit the growth of *C. dentata* by obstructing light and diminishing photosynthetic efficiency. In contrast, *C. dentata* growth slowed down due to microplastic PET blocking light, but bacteria *N. aquibiodomus* remained unaffected. When the population of *N. aquibiodomus* exceeded that of *C. dentata*, as suggested by our Algal-Bacterial ratio experiment, *N. aquibiodomus* could inhibit the growth of *C. dentata*, presenting a challenging situation for the algae.

On day 10, algal dry weight revealed a small peak (Figure 10-a), with concentrations of 5 mg/L, 10 mg/L, and 15 mg/L of PET in the medium exhibiting high dry weights of 0.0649 g, 0.0726 g, and 0.0627 g, respectively. This phenomenon occurred because the low concentration of microplastic PET minimally blocks light, and concurrently provides an

attachment point for *C. dentata*, enabling faster growth. In contrast, when the microplastic PET concentration was 0 mg/L, the dry weight was 0.0439 g, indicating slower dry weight accumulation in the absence of micro-PET. This observation indicated that *C. dentata* experienced accelerated growth in the presence of attachment points, or that proper shading was beneficial for *C. dentata* growth. On day 30, the highest dry weight was observed when the microplastic PET concentration was 25 mg/L, at 0.1787 g (Figure 10-a).

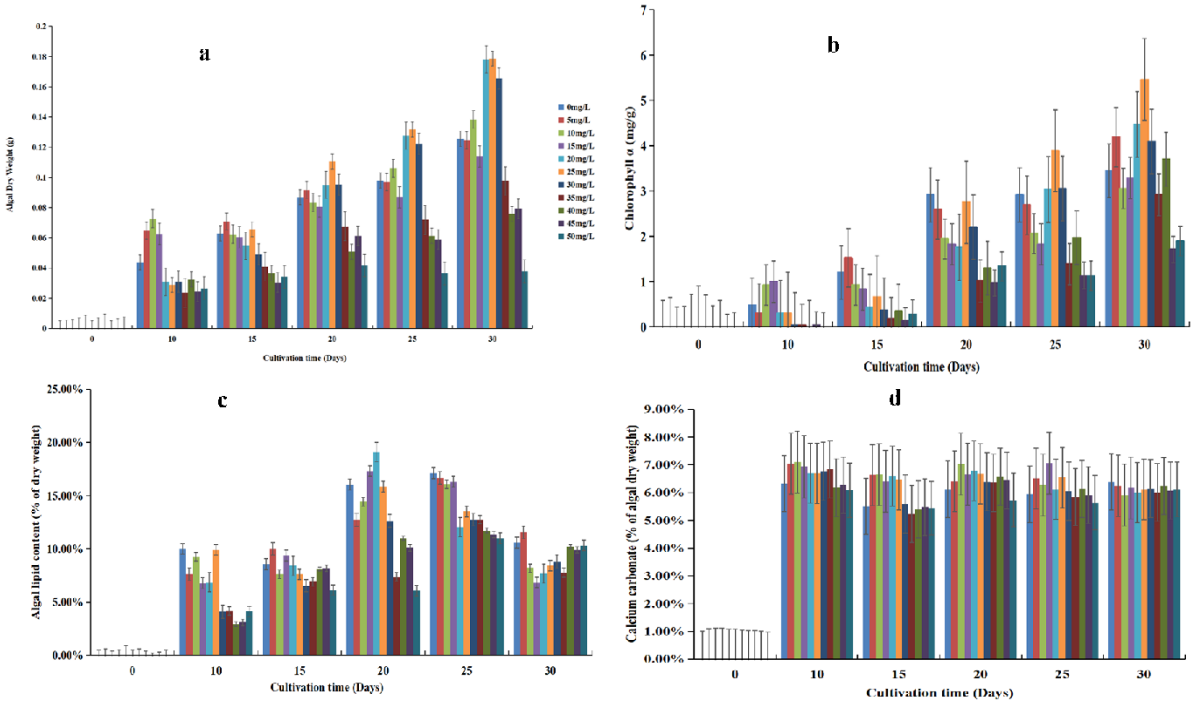


Figure 10 Bioproducts of *C*. dentata under different PET concentrations. **a:** Algal dry weight. **b:** Chlorophyll α. **c:** Lipid content. **d:** Calcium carbonate content.

From day 0 to day 10, the medium containing 15 mg/L micro-PET exhibited the highest chlorophyll α content (Figure 10-b), measuring 0.9962 mg/g. By day 30, the chlorophyll α content peaked at 5.4673 mg/g, which was observed in the medium with 25 mg/L micro-PET.

The second-highest chlorophyll α content was 4.4712 mg/g, occurring at 20 mg/L microplastic PET.

The lipid content revealed three peaks (Figure 10-c): the first on day 10, the second on day 20, and the third on day 25. Without microplastic PET in the medium, the highest lipid accumulation occurred on day 10, accounting for 10 % of algal dry weight. Across all groups, the medium with 20 mg/L PET exhibited the highest lipid content on day 20, reaching 18.98 %. On day 25, the medium without any micro-PET displayed the highest lipid content at 17.24 % of algal dry weight. On day 10, high concentrations of micro-PET (30-50 mg/L) inhibited lipid accumulation. Microplastics obstruct light, reducing photosynthesis efficiency (Liu et al., 2019).

On day 10, the maximum accumulation of $CaCO_3$ was 7.1 % when the PET concentration was 10 mg/L (Figure 10-d). On day 15, the content of $CaCO_3$ decreased when the medium contained 0 mg/L and 30 mg/L to 50 mg/L of micro-PET. However, in the medium with lower PET content (5 to 25 mg/L), the $CaCO_3$ accumulation slightly decreased. From day 10 to day 30, $CaCO_3$ levels remained between 5 % and 7 %.

Combined with previous results (Figures 1-c, 3-c, 5-d, 7-d), the general trend of $CaCO_3$ accumulation peaks from day 10 to 15 and then stabilizes. However, the amount of $CaCO_3$ fluctuates after day 15 In the culture medium containing PET. It was speculated that high PET concentrations caused collisions with each other, resulting in the detachment of algae attached to the microplastics.

Previous studies showed that the shell of *C. dentata* is formed by many coccoliths

crossing over each other (Chen et al., 2019; Kazuko et al., 2011). This structure implied that even if one or two coccolith fall off, it did not impact the whole, and the alga could synthesize the coccolith again. In other words, *C. dentata* undergoes a cycle of shading coccoliths, followed by a regrowth of the coccoliths, and then a shading of coccoliths again. This explains why the results show fluctuations up and down.

Conclusion

The optimal culture condition for *C. dentata* was photoperiod 18L/6D, pH 8.5, salinity 2.5%. And the optimum algal-bacterial ratio of *C. dentata* to *N. aquibiodomus* is 8:2.

Microplastics (micro-PET) had impact on algal-bacterial growth. Interestingly, 5–15 mg/L of microplastics promoted algal growth. However, high concentrations of microplastics (20-50 mg/L) slow the algal growth.

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Supplementary materials

Table S1. The Analysis of Variance table for the RSM model of algal dry weight.

Response 1 Algal dry weight

ANOVA for Response Surface Quadratic model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.12	9	0.013	37.11	< 0.0001	significant
A-Photoperic	*0.012*	*1*	*0.012*	*32.70*	*0.0007*	
B-pH value	*4.685E-003*	*1*	*4.685E-003*	*13.10*	*0.0085*	
C-salinity	*0.031*	*1*	*0.031*	*85.32*	*< 0.0001*	
AB	*1.163E-003*	*1*	*1.163E-003*	*3.25*	*0.1144*	
AC	*4.202E-006*	*1*	*4.202E-006*	*0.012*	*0.9167*	
BC	*1.362E-003*	*1*	*1.362E-003*	*3.81*	*0.0920*	
A^2	*0.017*	*1*	*0.017*	*46.21*	*0.0003*	
B^2	*0.010*	*1*	*0.010*	*28.88*	*0.0010*	
C^2	*0.036*	*1*	*0.036*	*101.82*	*< 0.0001*	
Residual	2.504E-003	7	3.577E-004			
Lack of Fit	*1.916E-003*	*3*	*6.388E-004*	*4.35*	*0.0947*	*not significant*
Pure Error	*5.872E-004*	*4*	*1.468E-004*			
Cor Total	0.12	16				

Table S2. The Analysis of Variance table for the RSM model of lipid content.

Response 2 Lipid content

ANOVA for Response Surface Quadratic model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	9.36	9	1.04	29.63	< 0.0001	significant
A-Photoperic	*0.34*	*1*	*0.34*	*9.81*	*0.0165*	
B-pH value	*0.15*	*1*	*0.15*	*4.31*	*0.0765*	
C-salinity	*1.17*	*1*	*1.17*	*33.35*	*0.0007*	
AB	*2.025E-003*	*1*	*2.025E-003*	*0.058*	*0.8171*	
AC	*0.087*	*1*	*0.087*	*2.48*	*0.1593*	
BC	*2.250E-004*	*1*	*2.250E-004*	*6.411E-003*	*0.9384*	
A^2	*2.71*	*1*	*2.71*	*77.21*	*< 0.0001*	
B^2	*1.90*	*1*	*1.90*	*54.22*	*0.0002*	
C^2	*2.20*	*1*	*2.20*	*62.58*	*< 0.0001*	
Residual	0.25	7	0.035			
Lack of Fit	*0.10*	*3*	*0.034*	*0.96*	*0.4944*	*not significant*
Pure Error	*0.14*	*4*	*0.036*			
Cor Total	9.61	16				

Table S3. The Analysis of Variance table for the RSM model of calcium content.

Response 3 Calcium content

ANOVA for Response Surface Quadratic model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	8.52	9	0.95	31.44	< 0.0001	significant
A-Photoperic	*0.45*	*1*	*0.45*	*14.82*	*0.0063*	
B-pH value	*0.20*	*1*	*0.20*	*6.69*	*0.0361*	
C-salinity	*1.46*	*1*	*1.46*	*48.54*	*0.0002*	
AB	*0.063*	*1*	*0.063*	*2.07*	*0.1929*	
AC	*4.225E-003*	*1*	*4.225E-003*	*0.14*	*0.7191*	
BC	*3.025E-003*	*1*	*3.025E-003*	*0.10*	*0.7606*	
A^2	*1.99*	*1*	*1.99*	*66.17*	*< 0.0001*	
B^2	*1.53*	*1*	*1.53*	*50.83*	*0.0002*	
C^2	*2.16*	*1*	*2.16*	*71.56*	*< 0.0001*	
Residual	0.21	7	0.030			
Lack of Fit	*0.076*	*3*	*0.025*	*0.75*	*0.5751*	*not significant*
Pure Error	*0.13*	*4*	*0.034*			
Cor Total	8.73	16				

Table S4. The Analysis of Variance table for the RSM model of chlorophyll α content.

Response 4 Chlorophyll α

ANOVA for Response Surface Quadratic model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	13.86	9	1.54	59.87	< 0.0001	significant
A-Photoperic	*0.86*	*1*	*0.86*	*33.36*	*0.0007*	
B-pH value	*0.31*	*1*	*0.31*	*12.13*	*0.0102*	
C-salinity	*2.51*	*1*	*2.51*	*97.55*	*< 0.0001*	
AB	*0.23*	*1*	*0.23*	*8.96*	*0.0201*	
AC	*0.048*	*1*	*0.048*	*1.88*	*0.2125*	
BC	*2.500E-003*	*1*	*2.500E-003*	*0.097*	*0.7643*	
A^2	*2.76*	*1*	*2.76*	*107.29*	*< 0.0001*	
B^2	*1.80*	*1*	*1.80*	*70.13*	*< 0.0001*	
C^2	*4.33*	*1*	*4.33*	*168.51*	*< 0.0001*	
Residual	0.18	7	0.026			
Lack of Fit	*0.13*	*3*	*0.044*	*3.63*	*0.1225*	*not significant*
Pure Error	*0.048*	*4*	*0.012*			
Cor Total	14.04	16				

Chapter 3. The Optimal Cultivation of *Nannochloropsis oceanica* and Outdoor Small-scale Breeding Throughout the Four Seasons

Abstract

Microalgae, due to their rapid growth, ability to absorb carbon dioxide, and high lipid accumulation, have the potential to address energy, climate, and food crises. We analyzed various culture conditions affecting a feed algae species, *Nannochloropsis oceanica*. The results indicated that 0.5 g/L of urea was a suitable nitrogen source for *N. oceanica* cultivation, while using ammonium chloride should be avoided. The salt concentration for cultivation should be maintained between 2.5 % and 3.5 %. Outdoor experiments revealed that *N. oceanica* is a cold-tolerant algae species; excessively high temperatures (over 30 ℃) are not conducive to its cultivation. It is recommended to provide adequate shading during the summer. Chlorophyll fluorescence provides real-time monitoring of growth inhibition factors, aiding in timely adjustments to cultivation conditions.

Key words: *Nannochloropsis oceanica*, urea, ammonium chloride, Chlorophyll fluorescence, Fv/Fm; outdoor

1 Introduction

Nannochloropsis oceanica is a type of microalgae belonging to the *Chlorophyta* phylum, widely found in marine and freshwater environments. *Nannochloropsis oceanica* is typically spherical or elliptical, with a small diameter (Heimann et al., 2015). It is widely distributed in various water bodies globally, especially in warm marine environments. *Nannochloropsis oceanica* exhibits strong adaptability to light and moderate salinity levels, allowing it to thrive and reproduce in diverse aquatic environments (Martínez et al., 2014; Bartley et al., 2013; Wahidin et al., 2013). *Nannochloropsis oceanica* has garnered attention in biotechnology due to its rapid growth and rich lipid content. The lipids, primarily triglycerides (TAGs), can be extracted to produce biofuels such as biodiesel or other valuable bioproducts (Ma et al., 2016). Additionally, biomass derived from *Nannochloropsis oceanica* can be utilized as bait, feed, and biofertilizer. It serves as an excellent source of omega-3 polyunsaturated fatty acids, particularly eicosapentaenoic acid (EPA), and is recognized as one of the most promising producers of EPA (Sukenik et al., 1989; Renaud et al., 1991).

Since the late 1980s, *N. oceanica* has emerged as the primary algae employed in numerous aquaculture hatcheries across Europe. This species has effectively supplanted commonly used algae such as *Isochrysis galbana* and *Monochrysis sp.*, significantly contributing to EPA levels in the food web (Sukenik et al., 1993; Lubzens et al., 1995, 1997). Cultivating *N. oceanica* serves two main purposes: (1) serving as the primary or partial feed for rotifer production; and (2) feed for aquatic farm and creating a "green water effect" in fish larval ponds (Lubzens et al., 1995).

Currently, several standard cultivation methods exist, including (1) using polyethylene bags of 100 or 150 liters, (2) using polyethylene bags or transparent fiberglass columns of 400-800 liters for batch or continuous cultivation, (3) utilizing large outdoor circular or rectangular ponds (e.g., from 8000 to 300,000 L), which can be outdoor ponds (Renaud et al., 1991) or outdoor racetrack ponds (Lubzens et al., 1995; Hoffman, 1999).

Chlorophyll fluorescence analysis is a technique for determining and diagnosing plant activity based on the principles of photosynthesis. By utilizing chlorophyll within plant cells as a natural probe, this method facilitates the study and detection of the photosynthetic physiological status of plants, as well as the influence of external factors on them (Murchie et al., 2013; Kalaji et al., 2017; Daley, 1994). Its advantages, such as rapidity, sensitivity, and non-invasiveness to cells, make it an exceptional tool for investigating plant photosynthesis (Ni et al., 2019). With the ongoing development of modulated fluorescence techniques, the application scope of chlorophyll fluorescence has progressively expanded into various fields, including plant ecology, agronomy, forestry, limnology, and environmental science (Murchie et al., 2013).

The chlorophyll fluorescence technique allows for real-time assessment of the photosynthetic efficiency in algae (Kumar et al., 2014; Oxborough et al., 2004). By measuring chlorophyll fluorescence parameters, it provides a comprehensive understanding of their utilization efficiency of light energy and the overall health of the photosynthetic process (Solovchenko et al., 2022). In algal cultivation, facing various environmental stressors such as high temperature and high salinity, the chlorophyll fluorescence technique

can monitor the impact of these stressors on algae, aiding in identifying their physiological status and adaptive capabilities (Bazzani et al., 2021).

Obtaining sodium nitrate in some countries and regions is difficult due to cost and safety concerns. Therefore, choosing more economical nitrogen sources for algal cultivation has become a significant issue. Thus, the objectives of this experiment are to find the optimal nitrogen sources and salinity for *N. oceanica* growth by using the chlorophyll fluorescence technique, and exploring the feasibility of outdoor small-scale breeding across all seasons.

2 Methods and materials

2.1 Algal strain

Marine algae *N. oceanica* was kindly provided by Dr. Lu Fan, Hubei University of Technology, China.

2.2 Algal cell optical density measurement

The 5 mL algae liquid was measured every day using a UV spectrophotometer to determine its optical density at 680 nm (OD_{680}).

2.3 Algal cultivation

This experiment was conducted indoors and outdoors, cultivating *N. oceanica* using an f/2 medium with 3.5 % salinity (Sea Salt), in a 700 mL columnar bioreactor (Guillard, 1975). Indoor cultivation involved 24-hour exposure to fluorescent light (3000 Lux) and aeration (0.6 L min^{-1}). The outdoor experimental part relied on natural light and air pump for cultivation.

2.4 Algal cell count

At the initial stage of cell counting, there was no need to dilute when the cell concentration was low. The mixed algae solution (10 μL) was taken onto a Hemocytometer, covered with a cover slip, and the algae were counted in 25 large grids. When the concentration was high, the algal concentration solution was diluted until the number of algae in 25 large grids was about 100. The dilution factor was recorded, and the cell number was obtained using the following formula:

$$\text{Cell/mL} = \text{Cell number} \times 10000 \times \text{Dilution factor}$$

2.5 Chlorophyll α measurement

The chlorophyll α determination was performed by methanol extraction method (Chen et al. 2008). Algae liquid (1 mL) was centrifuged (13,800 g, 1 min), and the supernatant was discarded. Methanol (1 mL) was added to pellets, then heated in the water bath (50 ℃, 5 min) and vortexed 1-2 times. The mixture was cooled at room temperature (25 ℃) and centrifuged again (13,800 g, 1 min). The green supernatant was measured at 665 and 750 nm using spectrophotometer. The chlorophyll α content was calculated according to the following formula：

$$\text{Chlorophyll } \alpha = 13.9\,(\text{OD665} - \text{OD750}) \times \frac{\text{U}}{\text{V}}$$

U：the final methanol volume.

V: the sample volume.

2.6 Algal dry weight measurement

The algae solution was dried in oven (80 ℃) for 24 h and cooled down at room

temperature in a drying dish with Jin Teng brand microporous filter membrane filter paper (0.45 μm pore size; diameter 50 mm) and pre-weighed (m1) for further uses. Then, the algal solution (5 mL) was pumped onto a filter paper and washed with 0.65 M ammonium bicarbonate solution to remove the salt. The filtrate was dried at 80 ºC overnight, cooled and weighed to obtain the constant weight (m2). Measurements were done in triplicate at each time interval, and the final dry weight of algae (DCM, g/L) was calculated using the following formula:

$$\text{Cell Weight} = \frac{m2 - m1}{5} \times 1000$$

2.7 Chlorophyll fluorescence assay

Utilizing the WATER-PAM system (WALZ Photosynthesis Instruments) for Pulse Amplitude Modulation (PAM) in the determination of algae chlorophyll fluorescence efficiency:

1. The 5 mL algal sample stood in darkness for 20 minutes.

2. After the dark adaptation, the algal sample was introduced into the fluorescence detector to commence the chlorophyll fluorescence measurement.

2.8 Effect of urea on *N. oceanica* growth

Three urea concentrations (0.1, 0.5, and 0.2 g/L) were selected. Sodium nitrate was used as the control group, and a blank control was also set. The culture medium was composed of an f/2 medium and corresponding modifications to the nitrogen source. PAM, OD_{680}, cell number and Chlorophyll α were estimated from day 0 to 10, and algal dry weight was determined on days 0, 3, 5, 7 and 9.

2.9 Effect of ammonium chloride on *N. oceanica* growth

Seven groups of nitrogen sources were established as follows: 0.1 g/L $NaNO_3$ (tube 1), 1 g/L $NaNO_3$ (tube 2), 2 g/L $NaNO_3$ (tube 3), 0.16 g/L NH_4Cl (tube 4), 1.56 g/L NH_4Cl (tube 5), 3.18 g/L NH_4Cl (tube 6), and 0 % nitrogen (tube 7).

PAM and OD_{680} measurements were conducted from days 0 to 7 and algal dry weight, cell number, and chlorophyll α were assessed on days 0, 1, 3, 5 and 7. Algae were cultivated in a 3.5 % salinity environment. The well-grown algal liquid (250 mL, grown indoors) was collected. After centrifugation (1200 g, 10 min), the supernatant was discarded, and f/2 medium with the appropriate nitrogen source concentrations (using None Nitrogen f/2 medium for one wash) was added. The mixture was transferred to a 700 mL glass photobioreactor with continuous air pumping.

2.10 Comparative effect study of sodium nitrate, urea, and ammonium chloride on optimal *N. oceanica* growth

As seen in results from 2.8 and 2.9 that optimal *N. oceanica* growth was observed at 1 g/L $NaNO_3$, 0.5 g/L Urea, and 0.16 g/L NH_4Cl. This study compared these nitrogen concentrations to identify the optimum nitrogen source.

The experiment was conducted under 3.5 % salinity f/2 medium conditions, 1.0 g/L $NaNO_3$, 0.5 g/L Urea, and 0.16 g/L NH_4Cl. Culture without nitrogen was used as the control (None N).

2.11 Effect of high ammonium chloride concentrations on algal chlorophyll fluorescence.

Table 1 Measurement timetable for chlorophyll fluorescence measurement.

	Day 0 (October 16）							Day 1	Day 2
Measurement time	8:00	8:30	9:00	10:00	12:00	16:00	20:00	8:00	8:00
1.0 g/L $NaNO_3$	0 h	1/2 h	1 h	2 h	4 h	8 h	12 h	24 h	48 h
	Day 0 (October 17）							Day 1	Day 2
Measurement time	8:00	8:30	9:00	10:00	12:00	16:00	20:00	8:00	8:00
0.16 g/L NH_4Cl	0 h	1/2 h	1 h	2 h	4 h	8 h	12 h	24 h	48 h
	Day 0 (October 18）							Day 1	Day 2
Measurement time	8:00	8:30	9:00	10:00	12:00	16:00	20:00	8:00	8:00
4.76 g/L NH_4Cl	0 h	1/2 h	1 h	2 h	4 h	8 h	12 h	24 h	48 h

The effect of the high concentrations of ammonium chloride on *N. oceanica* chlorophyll fluorescence was observed (results of 2.9). The effect of high ammonium chloride concentrations on photosynthetic efficiency was investigated in the present study.

Algae were cultured in 3.5 % salinity f/2 medium with different nitrogen sources ($NaNO_3$ 1.0 g/L, NH_4Cl 0.16 g/L, and NH_4Cl 4.76 g/L). The chlorophyll fluorescence efficiency was evaluated at 0, 1/2, 1, 2, 4, 8, 12 and 24 h, and days 2 and 3. Since the measurement of a single sample takes a long time, day 0 measurements of different concentrations are selected on different days. The timetable for measuring chlorophyll fluorescence efficiency is presented in Table 1.

2.12 Effect of salt on *N. oceanica* growth

N. oceanica was cultured indoors at different salt concentrations, and the differences in its chlorophyll fluorescence efficiency were monitored. Salt concentrations were selected (0, 0.5, 3.5 and 5.1 %). PAM measurements were taken at various time points (0, 1/6, 1/2, 1, 2, 3,

4, 8, 12, 24, 48, 72, 96, 120, 144, 168. 192, 216 and 240 h). Well-grown algal liquid (500 mL) was obtained through centrifugation (1200 g, 30 min). After removing the supernatant, f/2 medium with the appropriate salinity concentrations was added. The mixture was transferred to a 700 mL glass photobioreactor with continuous air pumping (0.6 L/min). PAM measurements at 0 h served as baseline. The nitrogen source was $NaNO_3$ at 1.0 g/L.

2.13 Variations in *N. oceanica* growth with various initial inoculation concentrations throughout four seasons in outdoor conditions

Four algal concentrations were selected and cultivated in 700 mL bioreactor with continuous aeration. The cultivation conditions were as follows: The light exposure was 9 am - 3 pm, 7 am - 6 pm, 8 am - 3 pm and 10 am - 2 pm, respectively, in spring, summer, autumn and winter. Daily weather conditions, outdoor and water temperatures, light intensity, and overall weather conditions were recorded.

2.13.1 Spring experiment (March 27-April 3)

Four initial OD_{680} values of *N. oceanica* were selected (0.2606, 0.5886, 1.1144, and 2.1341), and chlorophyll fluorescence efficiency measurements were conducted daily at 9 am, 2 pm, and 6 pm. OD_{680}, cell numbers, algal dry weight, and chlorophyll α were also evaluated.

2.13.2 Summer experiment (June 26- July 3)

Four different initial OD_{680} values (0.2621, 0.5962, 1.0252, and 2.0474) were selected, and chlorophyll fluorescence efficiency measurements were performed daily at 9 am, 2 pm, and 6 pm. OD_{680}, cell numbers, algal dry weight, and chlorophyll α were also assessed.

2.13.3 Fall experiment (October 31- November 13)

Four different initial OD_{680} values (0.2388, 0.5839, 1.0152, and 2.0693) were selected, and chlorophyll fluorescence efficiency measurements were carried out daily at 9 am, 2 pm, and 7 pm. OD_{680}, cell numbers, algal dry weight, and chlorophyll α were also evaluated.

2.13.4 Winter experiment (December 27-January 3)

Four different initial OD_{680} values (0.2096, 0.498, 1.1755, and 1.8976) were selected, and chlorophyll fluorescence efficiency measurements were conducted daily at 9 am, 2 pm, and 7 pm. OD_{680}, cell numbers, algal dry weight, and chlorophyll α were assessed.

2.14 Statistical Analysis

All experiments were conducted in triplicate, including sampling from three individual points and measurement was conducted in triplicate. The results obtained from these triplicates underwent statistical analysis using the one-way ANOVA function in IBM SPSS® software. For comparison, we employed Duncan' s multiple-range test, with a significance level (P-value) set at 0.05. The statistical significance results for $CaCO_3$, lipid, and chlorophyll α contents are individually reported in the supplementary information file.

3 Results and Discussion

3.1 Indoor experiment

3.1.1 Effect of urea on *N. oceanica* growth

After conducting a 10-day test on nitrogen sources, as depicted in Figures 1-a and 1-c, it became clear that the optimal nitrogen source shifts over time. From day 1 to day 4 (Figure 1-b), $NaNO_3$ at 1.0 g/L (Control) emerged as the most effective for *N. oceanica*. After day 4,

the 0.5 g/L urea as nitrogen source also conducive to *N. oceanica* growth same as control group (1.0 g/L $NaNO_3$), while 1 g/L $NaNO_3$ continued to sustain robust algal growth. However, an excess of urea (2 g/L) exhibited an inhibitory effect on *N. oceanica*.

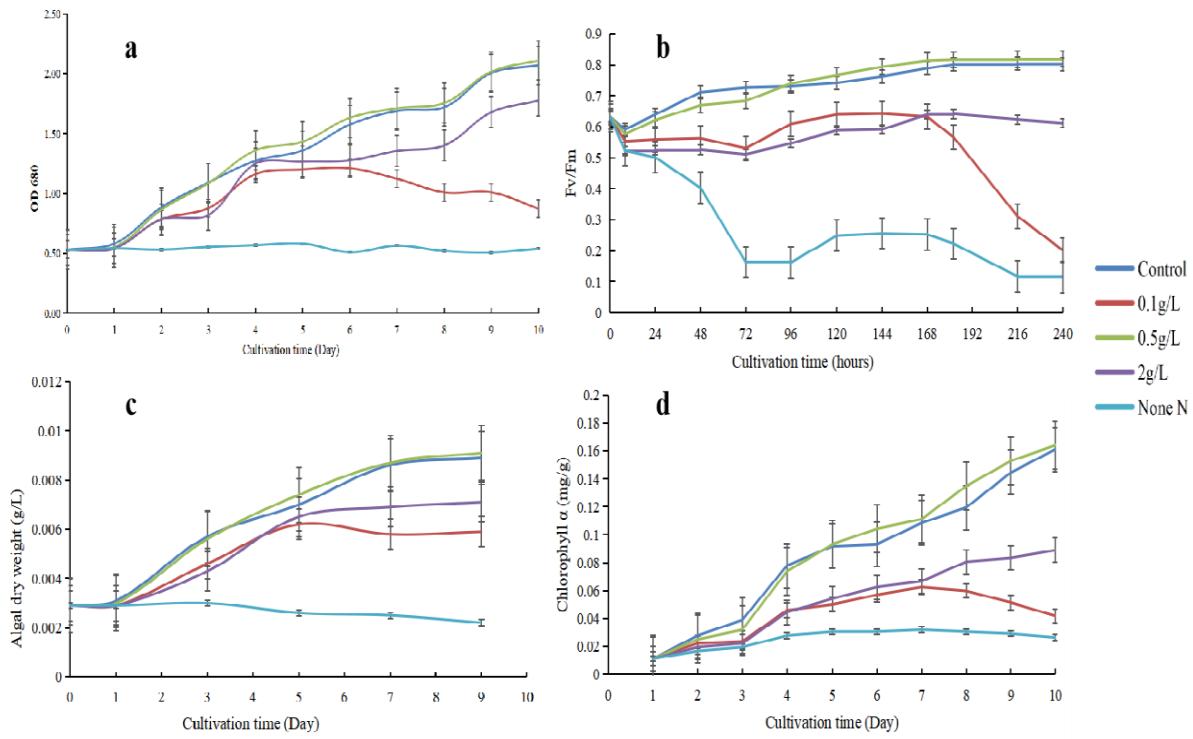


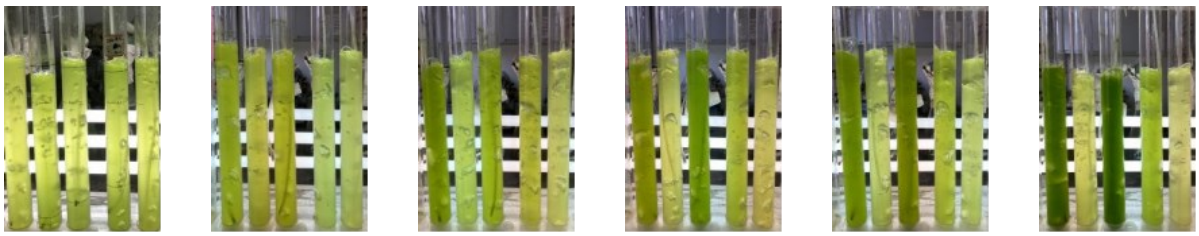
Figure 1 Bioproducts and chlorophyll fluorescence efficiency of *N. oceanica* under different urea concentrations. **a:** Optical density OD_{680}. **b:** Chlorophyll fluorescence efficiency Fv/Fm. **c:** Algal dry weight. **d:** Chlorophyll α. Control was 1.0 g/L $NaNO_3$.

However, the low urea concentration (0.1 g/L) exhibited growth-promoting effect in the first four days. Still, a decline started on day 6, reaching a low level by day 10. The cell growth with 2 g/L urea in the initial four days resembled that of the low-concentration urea culture, indicating an inhibitory effect on algae growth at 2 g/L. On the other hand, using 0.5 g/L urea, compared to sodium nitrate with the same molar mass of nitrogen, demonstrated a similar cell growth and did not exhibit inhibitory effects on algae. From a cost perspective,

0.5 g/L urea seemed more suitable for the cell growth of *N. oceanica* (Figure 1-a).

Figure 1-c shows dry algal weight increased effectively during the first five days at 0.1 g/L urea. However, after five days, the dry weight ceased to increase, indicating that the nitrogen source was essentially depleted, and the algae ceased to grow. This result suggests a cost-saving approach—implementing continuous nitrogen supplementation. Nitrogen can be replenished in the culture medium on the day 5 or earlier, around the 4th day, to achieve sustained growth. From the perspective of dry weight, 0.5 g/L urea still demonstrated favorable results. Figure 1-d reveals that 0.5 g/L urea promoted a higher chlorophyll accumulation than the control group with sodium nitrate. Moreover, urea's cost-effectiveness was higher than sodium nitrate in this context. On the other hand, 2.0 g/L urea inhibited the accumulation of chlorophyll α in *N. oceanica*. The efficiency of chlorophyll photosynthesis is presented in Figure 1-b. In the initial 8 h, the photosynthetic efficiency of algae was inhibited due to the recent inoculation into the new culture medium. The control group, using sodium nitrate as the nitrogen source, showed the quickest recovery of photosynthetic efficiency, followed by 0.5 g/L urea and then 0.1 g/L urea. However, 2.0 g/L urea continuously inhibited the photosynthetic efficiency of algae for the first 72 h, with Fv/FM slowly increasing only after 72 h, and the increase remained marginal until 240 h. At 0.1 g/L urea, the cultured algae's photosynthetic efficiency started declining after 144 h. Combining the results of chlorophyll α content (Figure 1-d), cell numbers (Figure 1-b), and daily photos (Figure 2), it is evident that nitrogen deficiency led to cell death and yellowing, which was the reason for Fv/FM value decreases.

Normal algae growth increased chlorophyll fluorescence efficiency to a good level (Fv/Fm>0.6) within 24 h after incubation under sufficient nutrient conditions. However, the chlorophyll fluorescence efficiency of algae was inhibited within 72 h safter inoculation under nutrient excess or deficiency (Figure 1-b). This provides valuable guidance for future monitoring of algae in commercial cultivation. Through chlorophyll fluorescence analysis, potential issues can be identified within the initial cultivation period (within 24 h), allowing for a prompt response and determining whether re-inoculation is necessary.



Day 0	Day 2	Day 4	Day 6	Day 8	Day 10

Figure 2 Color changes of *N. oceanica* under various nitrogen sources. In each picture and from left to right: NaNO3 (1 g/L), urea (0.1 g/L), urea (0.5 g/L), urea (2 g/L), and no nitrogen source.

Changes in algae during the cultivation process were presented in Figure 2. By day 10, only the control group and culture with 0.5 g/L urea showed deep green color, while the others exhibited light green or yellow color. This result has a similar trend with chlorophyll α. Combined with the results of chlorophyll fluorescence efficiency (Figure 1-b), 0.5 g/L urea and 1.0 g/L sodium nitrate will not inhibit *N. oceanica*, while low and high concentrations of urea showed inhibition on *N. oceanica* growth.

Urea is an inexpensive nitrogen-containing fertilizer that is water-soluble. However,

excessive use of urea can lead to environmental pollution, soil alkalization, and eutrophication of water bodies. Our results indicated that an appropriate urea concentration (0.5 g/L) can effectively cultivate *N. oceanica*. This contributes to cost savings in future industrial production and demonstrates that 0.5 g/L urea does not significantly impact the chlorophyll photosynthetic efficiency during the cultivation of *N. oceanica*. Hence, it can be used with confidence.

3.1.2 Effect of $NaNO_3$ and NH_4Cl on chlorophyll fluorescence efficiency and growth of *N. oceanica*

By combining the data from Figures 3-a, when the sodium nitrate concentration was 1 g/L, *N. oceanica* exhibited optimal cell growth, with an OD_{680} reaching 3.16 on day 7. However, 2 g/L sodium nitrate culture slightly inhibited *N. oceanica* cell growth. Due to an insufficient nitrogen source, the culture with 0.1 g/L sodium nitrate had an OD_{680} value of 2.77 on day 7.

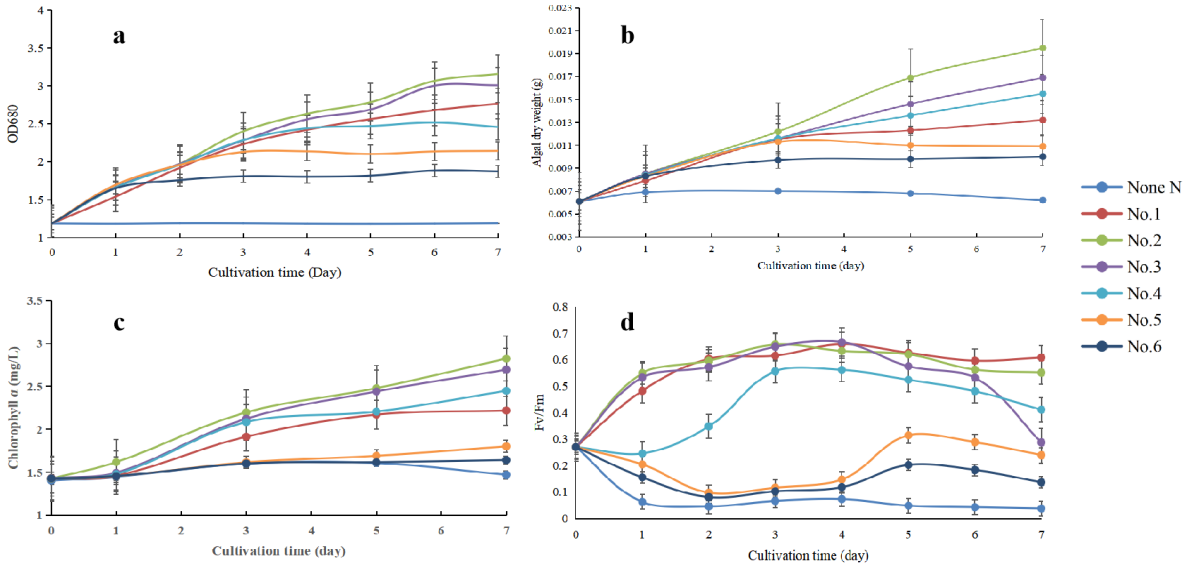


Figure 3 Bioproducts and chlorophyll fluorescence efficiency of *N. oceanica* under different

nitrogen sources. $NaNO_3$ 0.1 g/L (**No. 1**), $NaNO_3$ 1 g/L (**No. 2**), $NaNO_3$ 2 g/L (**No. 3**), NH_4Cl 0.16 g/L (**No. 4**), NH_4Cl 1.56 g/L (**No. 5**), NH_4Cl 3.18 g/L (**No. 6**). **a:** Optical density OD $_{680}$. **b:** Chlorophyll fluorescence efficiency Fv/Fm. **c:** Algal dry weight. **d:** Chlorophyll α.

A more potent growth inhibitory effect occurred at 3.18 g/L ammonium chloride (OD $_{680}$: 1.87) on day 7. *N. oceanica* reached the highest OD $_{680}$ value on day 6 at 0.16 g/L NH_4Cl (2.52) and then 3.18 g/L NH_4Cl (2.14) (Figure 3-a). However, a high ammonium chloride concentration (3.18 g/L) exhibited a pronounced inhibitory effect on algal proliferation. The growth of *N. oceanica* was also hindered at 1.56 g/L NH_4Cl. Cell dry weight results of *N. oceanica* are presented in Figure 3-b. Sodium nitrate (1 g/L) enhanced the yield of *N. oceanica*, reaching 0.0195 g on day 7. In contrast, the cultivation of *N. oceanica* at 0.16 g/L NH_4Cl only resulted in a dry weight of 0.0155 g. Figure 3-c revealed that the accumulation of chlorophyll α was inhibited when NH_4Cl were 1.56 and 3.18 g/L. This further impacted the growth and development of algae, as confirmed by the results from Figure 3-a and 3-b, the inhibitory effect could be associated with the nutrient excess caused by the high ammonium chloride concentration, affecting physiological algae status.

Sodium nitrate (1 g/L) rapidly restored chlorophyll fluorescence efficiency within the first day of cultivation, reaching a maximum Fv/Fm value of 0.62 on day 4 (Figure 3-d). Similarly, 0.1 g/L sodium nitrate did not exhibit inhibitory effects on chlorophyll fluorescence efficiency. However, 2 g/L sodium nitrate inhibited chlorophyll fluorescence efficiency after the fourth day, decreasing continuously from 0.666 to 0.287 from days 4 to 7 (Figure 3-d). These results suggest that the high concentration of algae obstructed chlorophyll

photosynthetic efficiency, leading to the observed decline (Figures 3-c and 3-d). Ammonium chloride (0.16 g/L) reduced algae photosynthetic efficiency in the initial three days, recovering to a relatively normal range by day 3. Higher NH_4Cl concentrations (1.56 and 3.18 g/L) consistently reduced *N. oceanica* photosynthetic efficiency. A brief increase was observed on days 5 and 6, followed by a subsequent decline. However, all three ammonium chloride concentrations had inhibitory effects on the growth and photosynthetic efficiency of algae. This indicates that ammonium chloride is not the most suitable nitrogen source for *N. oceanica* cultivating.

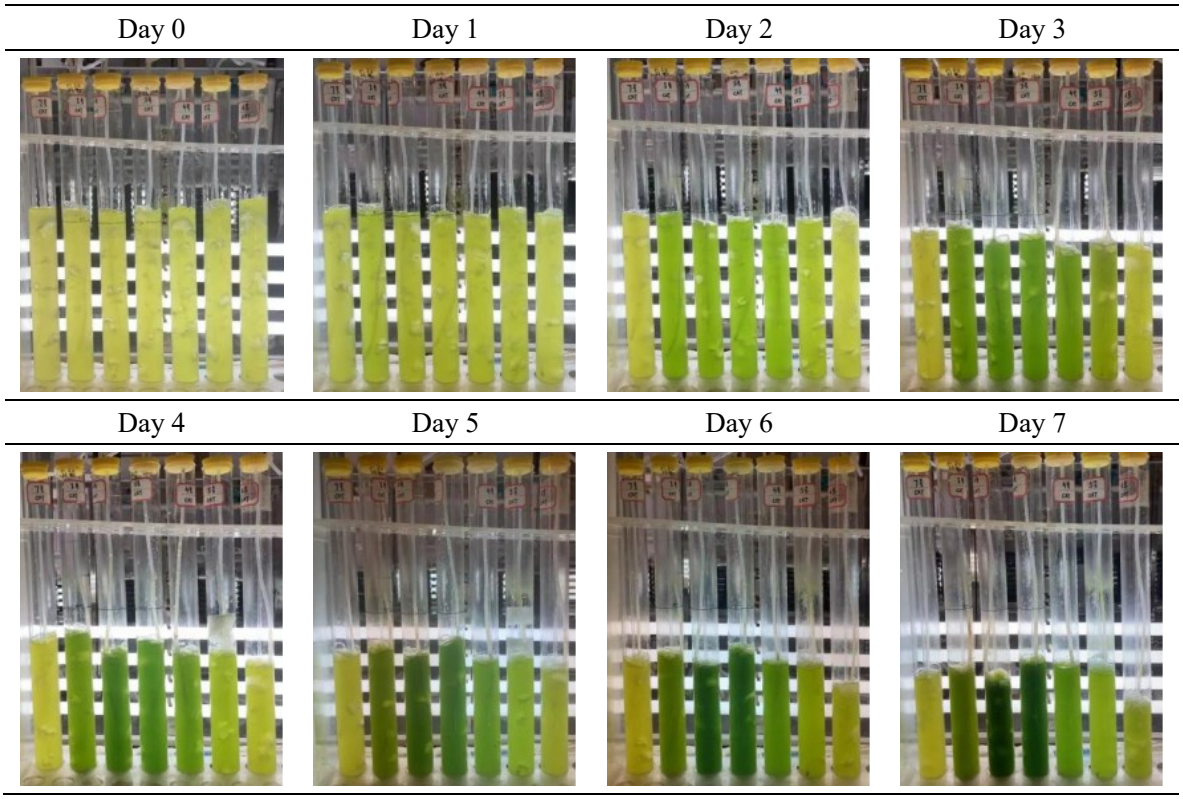


Figure 4 Color changes of *N. oceanica* under various nitrogen sources from days 1 to 7. In each picture, from left to right: no nitrogen source, 0.1 g/L of $NaNO_3$, 1.0 g/L of $NaNO_3$, 2.0 g/L of $NaNO_3$, 0.16 g/L of NH_4Cl, 1.56 g/L of NH_4Cl, 3.18 g/L of NH_4Cl.

As can be seen from Figure 4, when sodium nitrate is used as the nitrogen source, the

algae appear dark green on the day 10. When NH_4Cl is used as the nitrogen source, even low concentrations of NH_4Cl will cause the algae to appear dark green. Light green, combined with the chlorophyll fluorescence efficiency results and chlorophyll results, we can find that ammonium chloride as a nitrogen source has an inhibitory effect on the photosynthesis system of *N. oceanica*.

In future industrial-scale production of *N. oceanica*, careful consideration should be given to choosing nitrogen sources to reduce or avoid using ammonium chloride to ensure that the algae maintain a healthy growth status and optimal photosynthetic efficiency. This discovery holds essential guidance and insights for the large-scale cultivation and widespread application of *N. oceanica*. As researchers and industrial practitioners strive for sustainable development and efficient production, selecting nitrogen sources should be a focal point to advance algal biotechnology' s feasibility and successful application.

3.1.3 Comparative analysis of three optimal nitrogen concentrations for sodium nitrate, Urea, and chloride ammonium

Urea and sodium nitrate exhibited excellent growth-promoting effects, while the growth rate of *N. oceanica* was relatively slower when ammonium chloride was used as a nitrogen source (Figure 5-a). Cultivating with 0.5 g/L urea yielded a maximum OD_{680} value of 3.1555 on day 7. The OD_{680} value was 3.0882 when 1 g/L sodium nitrate was used. In contrast, OD_{680} was 2.8184 at 0.16 g/L ammonium chloride. During the same cultivation period, urea promoted *N. oceanica* growth pronouncedly. Sodium nitrate exhibited similar results, while ammonium chloride inhibited the growth of *N. oceanica* (Figure 5-a). These findings have practical

significance for optimizing cultivation conditions and enhancing biomass production.

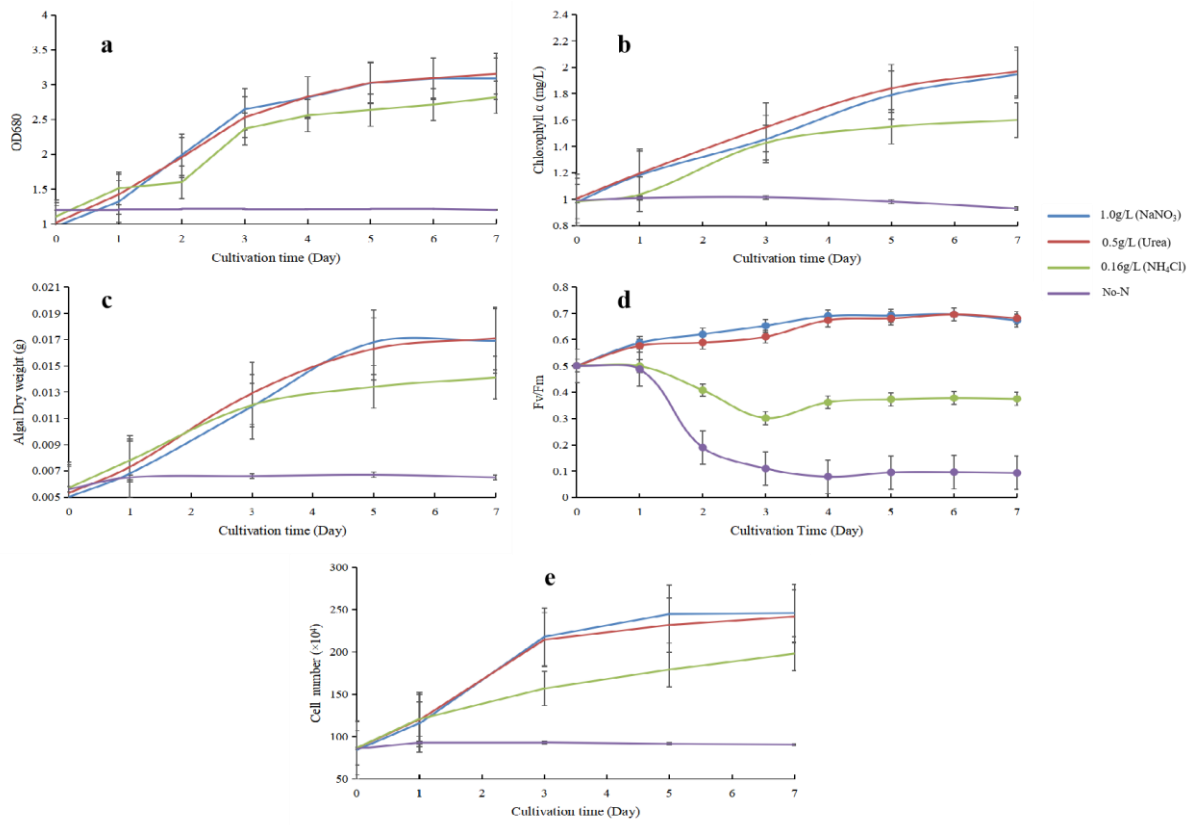


Figure 5 Bioproducts and chlorophyll fluorescence efficiency of *N. oceanica* under different nitrogen sources. **a:** Optical density OD $_{680}$. **b:** Chlorophyll α. **c:** Algal dry weight. **d:** Chlorophyll fluorescence efficiency Fv/Fm. **e:** Cell numbers.

Sodium nitrate enhanced the cell growth of *N. oceanica* at 0.5 and 1 g/L (Figure 5-e). The maximum cell growth of *N. oceanica* (2.46×10^6) was reached on the 7th day at 1 g/L sodium nitrate, while the maximum cell growth was 2.42×10^6 when urea was used at 0.5 g/L. However, 0.16 g/L ammonium chloride inhibited cell growth from the first day of cultivation. *N. oceanica* reached maximum dry weight of 0.0171 and 0.0169 g on day 7, respectively, when 0.5 g/L urea and 1 g/L sodium nitrate were used. However, the dry weight was 0.0141 on day 7 at 0.16 g/L ammonium chloride (Figure 5-b). Ammonium chloride inhibited

chlorophyll α accumulation in *N. oceanica*, even at a concentration as low as 0.16 g/L (Figure 5-c). However, 0.5 g/L urea and 1.0 g/L sodium nitrate did not inhibit chlorophyll accumulation. We observed the highest chlorophyll α accumulation of 1.9696 and 1.9477 mg/L on the 7^{th} day, respectively, when 0.5 g/L urea and 1.0 g/L sodium nitrate were used. The chlorophyll α accumulation at 0.16 g/L ammonium chloride on day 7 was 1.6014 mg/L. Urea (0.5 g/L) and sodium nitrate (1 g/L) did not inhibit photosynthetic efficiency in *N. oceanica*, which was gradually stabilized after day 3. However, 0.16 g/L ammonium chloride inhibited the photosynthetic efficiency with a declining trend in Fv/Fm from days 0 to 3 (Figure 5-d), suggesting that ammonium chloride could cause permanent damage to the photosynthetic system of *N. oceanica*. Figure 6 revealed that the green intensity of *N. oceanica* cultivated with ammonium chloride was not as pronounced as when *N. oceanica* was cultivated with sodium nitrate and urea. We observed a yellowish *N. oceanica* in the absence of nitrogen. This highlights the crucial role of nitrogen as an indispensable nutrient in algae cultivation and health. Moreover, chlorophyll fluorescence detection is a simple, convenient, and efficient method for monitoring the status of algal growth during cultivation.

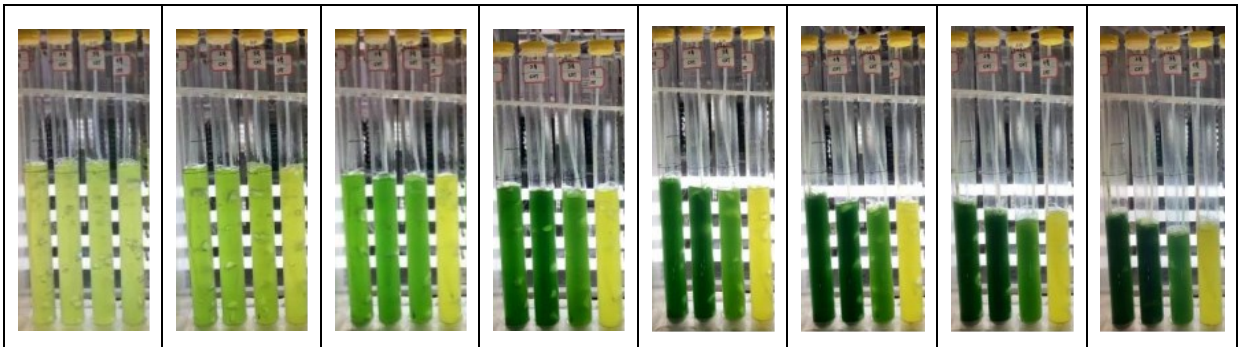


Figure 6 Color changes of days 1 to 7. In every picture, from left to right: 1 g/L $NaNO_3$, 0.5 g/L urea, 0.16 g/L NH_4Cl and no nitrogen source.

3.1.4 Effect of high ammonium chloride concentrations on photosynthetic efficiency

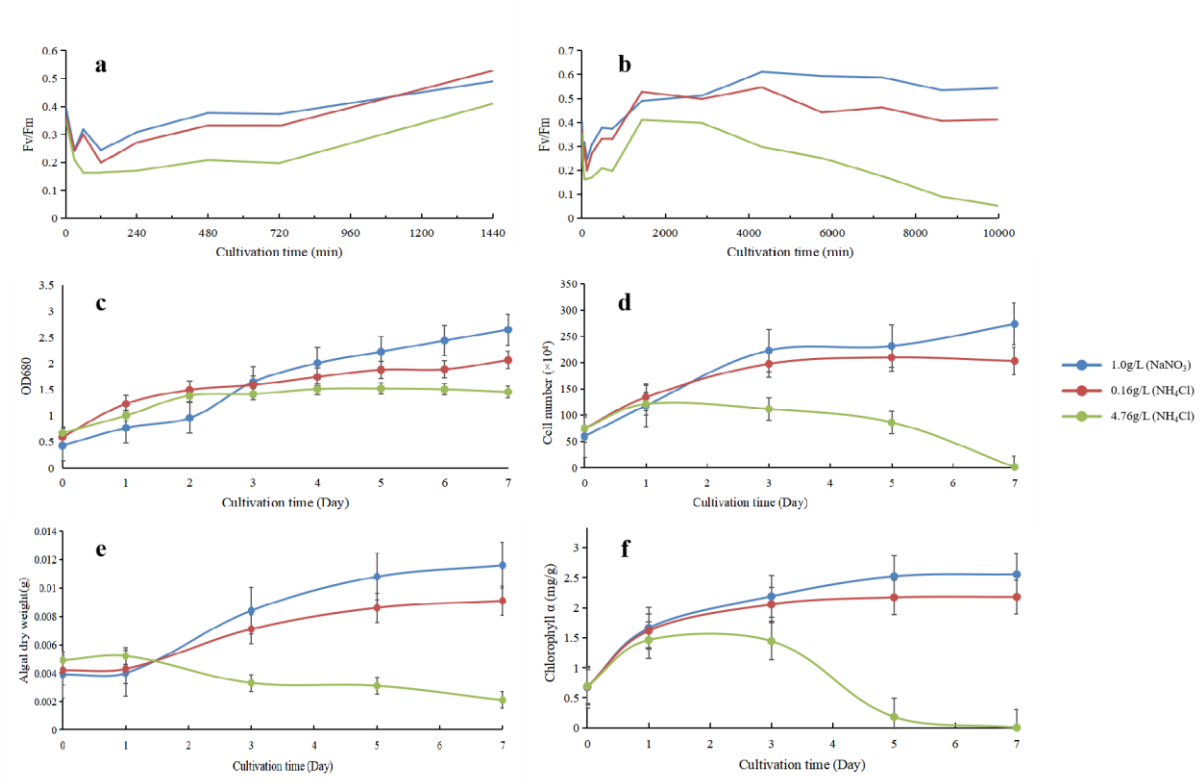


Figure 7 Bioproducts and chlorophyll fluorescence efficiency of *N. oceanica* under different ammonium chloride (0.16 g/L and 4.76 g/L). **a:** Chlorophyll fluorescence efficiency Fv/Fm of 24 h. **b:** Chlorophyll fluorescence efficiency Fv/Fm from day 0 to 7. **c:** Optical density OD_{680}. **d:** Cell numbers **e:** Algal dry weight. **f:** Chlorophyll α.

Ammonium chloride previously inhibited the photosynthetic efficiency of *N. oceanica*. Therefore, a higher ammonium chloride concentration was used, and the photosynthetic algae efficiency was intensively monitored during the initial inoculation phase. In Figure 7-b, 4.76 g/L ammonium chloride led to a gradual decrease in the photosynthetic efficiency of *N. oceanica* from 1440 min. The photosynthetic efficiency was also inhibited at 0.16 g/L ammonium chloride at 2880 min. For more detailed understanding of what transpired within the initial 24 h of inoculation, Figure 7-a displays changes in Fv/Fm over 1440 min. At the

60-minute, *N. oceanica* cultivated under high ammonium chloride concentrations did not exhibit a peak (i.e., photosystem II repair peak).

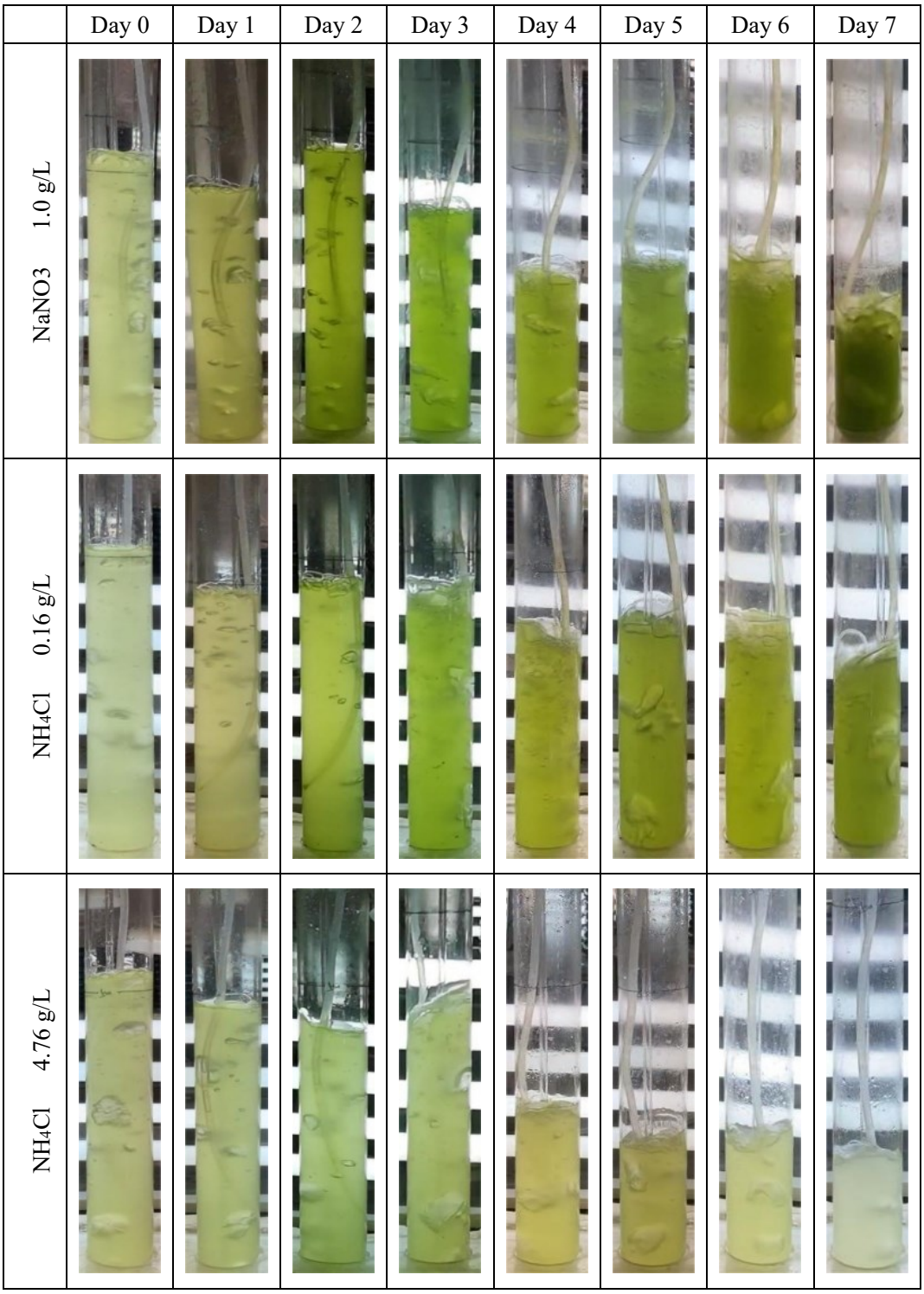


Figure 8 Color changes of *N. oceanica* under ammonium chloride as a nitrogen source.

In other words, the high concentration of ammonium chloride exerted a pronounced

inhibitory effect on the photosynthetic efficiency of *N. oceanica* from the initial stages, with the absence of the first peak (photosystem II repair peak). In Figure 7-c, ammonium chloride promoted the *N. oceanica* growth in the initial two days. However, 4.76 g/L ammonium chloride inhibited the *N. oceanica* growth from days 2 to 7. Ammonium chloride also inhibited the cell growth at 0.16 g/L. In Figure 7-d, 4.16 g/L ammonium chloride pronouncedly inhibited cell accumulation from the second day, with total inhibition on day 7. Furthermore, 0.16 g/L ammonium chloride inhibited the later stages of cell accumulation, with maximum cell count occurring on the sixth day at 2.10×10^6. The cell counts with 1 g/L sodium nitrate reached 2.73×10^6 on day 7.

In Figure 7-e, the dry weight on 7^{th} day was 0.0116 g when 1 g/L sodium nitrate was used. With a nitrogen source of 0.16 g/L ammonium chloride, the dry weight on the 7^{th} day was 0.0091 g, while under cultivation with a high concentration of ammonium chloride (4.76 g/L), the dry weight of *N. oceanica* decreased to 0.0021 g on day 7.

By combining Figures 7-f and 8, the 4.76 g/L ammonium chloride inhibitory effect on *N. oceanica* became more apparent. Initially, ammonium chloride enhanced the growth of *N. oceanica* in the early stages. However, this effect diminished over time. From the results of chlorophyll fluorescence in Figure 7-b, we can infer that using ammonium chloride as a nitrogen source could be detrimental to *N. oceanica*. Even though ammonium chloride can rapidly supply nitrogen initially, continuous cultivation of *N. oceanica* with ammonium chloride as the nitrogen source could lead to inhibitory effects.

3.1.5 Effect of salinity on *N. oceanica* photosynthetic efficiency

In the artificial cultivation process of *N. oceanica*, changes in salt concentration in the culture medium may occur due to evaporation, rainfall, and other factors. Analyzing and monitoring *N. oceanica* cultivated under various salt concentrations through chlorophyll fluorescence techniques is vital in practical production.

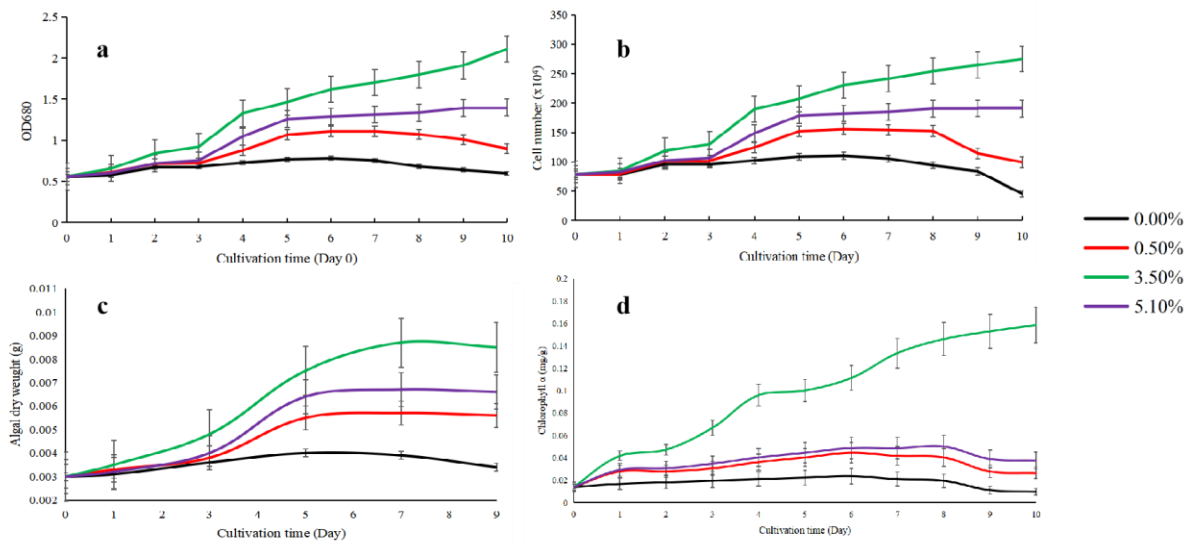


Figure 9 Bioproducts and chlorophyll fluorescence efficiency of *N. oceanica* under different salinity. **a:** Optical density OD $_{680}$. **b:** Cell numbers. **c:** Algal dry weight. **d:** Chlorophyll α.

Figures 9-a and 9-b display OD $_{680}$ and cell count numbers of *N. oceanica* under various salinity conditions. Salinity 3.5 % was more conducive to the growth of *N. oceanica*. *N. oceanica* exhibited greater tolerance under the higher salt concentration of 5.1 % compared to the low salt concentration of 0.5 %. When the salt concentration was 3.5 %, the cell count of *N. oceanica* on the 7th day was 2.75×10^{6}, whereas, at the high salinity (5.1 %), the cell count of *N. oceanica* was 1.91×10^{6}. The cell counts numbers followed the order from high to low: 3.5 % > 5.1 % > 0.5 % > 0 %. In Figure 9-c, after the 5th day, a cessation of increased dry weight was observed when *N. oceanica* was cultivated under 0.5 % and 5.1 % salt

concentrations, showing an inhibitory effect.

From Figure 9-d, when the salinity was 3.5 %, *N. oceanica* accumulated 0.1585 mg/L of chlorophyll α on the 10th day. In contrast, *N. oceanica* cultivated under 5.1 % salt concentration reached a maximum chlorophyll α content on the 8th day at 0.05 mg/L. Under 0.5 % salt concentration, the maximum chlorophyll α content was observed on the 6th day at 0.0445 mg/L. The synthesis of chlorophyll α was inhibited without salt in the culture medium.

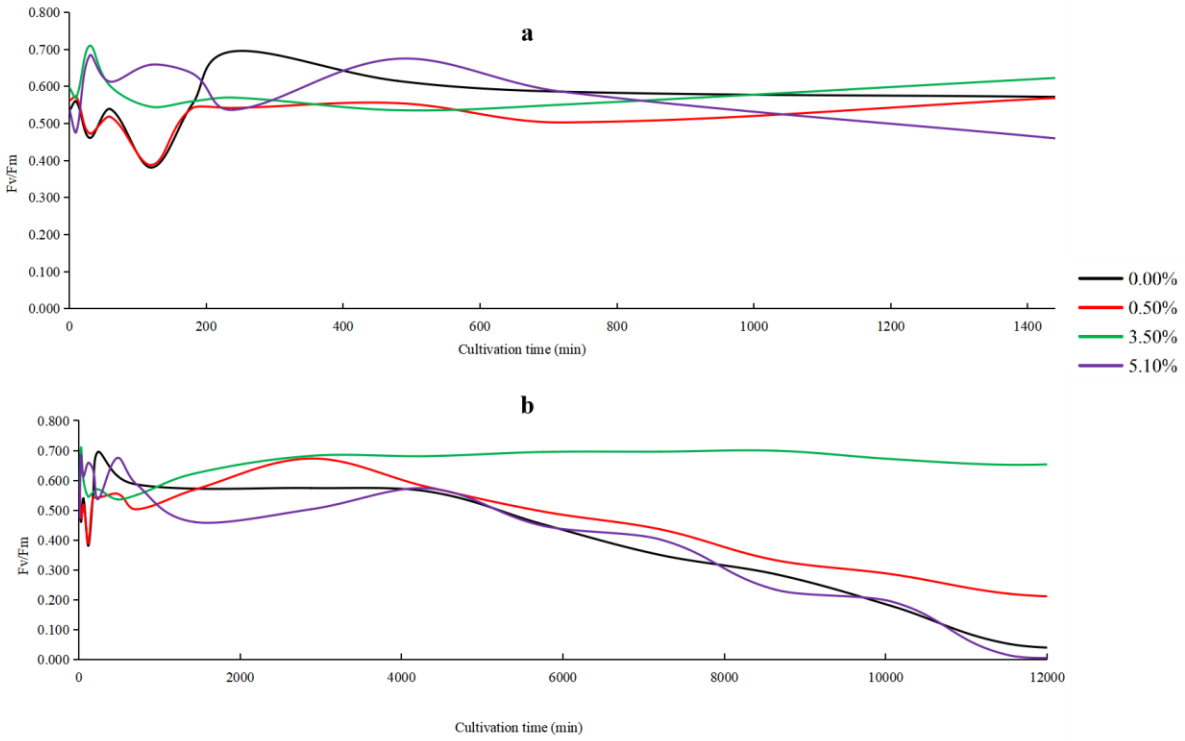
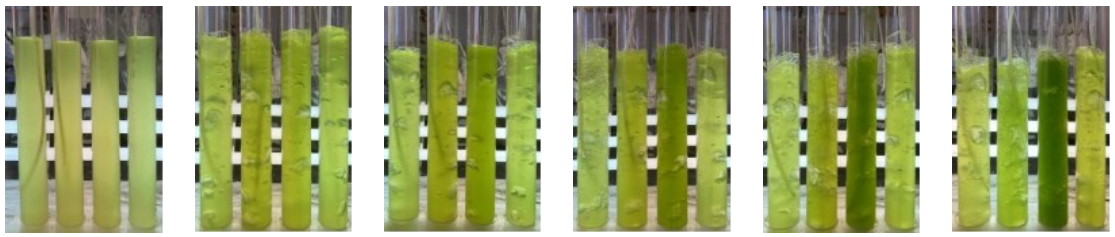


Figure 10 Fv/Fm of *N. oceanica* under salinity. **a.** The results of Fv/Fm in the initial 24 hours after inoculation. **b.** The 11 days results of Fv/Fm.

Figure 10 (a and b) presents the effect of salinity on *N. oceanica* chlorophyll fluorescence. The stress effect at a low salt concentration (0.5 %) was similar to that of salt-free algae culture within the initial 120 min. The effect of 3.5 % and 5.1 % salt

concentrations on the algae showed similarities in the first 60 min (Figure 10-a). Looking at the overall trend over 10 days, except for the 3.5 % salt concentration group, algae in the other groups were all affected and stressed. The photosynthetic efficiency was inhibited after 4320 min (day 3, Figure 10-b). After 10 days of cultivation, salinity at 0, 0.5 and 5.1% inhibited the maximum photosystem II photochemical efficiency.

In particular, a salinity of 5.1 % inhibited Fv/Fm results in 48 h (Figure 10-b). However, when algae were transferred to a new medium, whether the medium was suitable or not, it inhibited the maximum photosystem II photochemical efficiency.



A B C D E F

Figure 11 Color changes of *N. oceanica* under salinity. Day 0 (A), Day 2 (B), Day 4 (C), Day 6 (D), Day 8 (E), Day 10 (F). Each picture is 0 %, 0.5 %, 3.5 %, and 5.1 % from left to right.

Algae cultured with 3.5 % salinity appears greener than the other three cultures after day 2. This suggests that the optimal salinity concentration for the growth of *N. oceanica* is 3.5% (Figure 11). From Figure 11, where the algae still maintain a green appearance even in culture media with high salt concentration (5.1 %) or low salt concentration (0.5 %). This phenomenon could be misleading, and an accurate assessment of algae growth status becomes challenging without integrating this information with other data and chlorophyll fluorescence results. Hence, it is crucial to continually monitor changes in salt concentration in the culture medium during the practical large-scale cultivation of marine microalgae *N.*

oceanica. This monitoring should be complemented with chlorophyll fluorescence efficiency values (Fv/Fm) to evaluate their growth conditions comprehensively.

3.2 Outdoor experiment

The challenge of outdoor algae cultivation lies in the uncontrollable duration of sunlight, temperature fluctuations, and the impact of climate changes on the algae growth process. This challenge is particularly pronounced in small-scale cultivation. However, the advantages of small-scale outdoor cultivation are also significant. Firstly, it is characterized by meager costs. Secondly, the algae have adapted to the changing external environment, greatly enhancing their tolerance to environmental fluctuations. This adaptation reduces the time and expense of acclimating the algae to the environment when transitioning from initial inoculation to large-scale cultivation.

3.2.1 Investigating the impact of various initial OD_{680} levels on *N. oceanica* growth under outdoor spring conditions.

The climate type at the experimental site is characterized as subtropical monsoon climate. In spring, despite the gradual warming of the weather, there is a significant temperature difference between day and night, and the weather is unstable. Rainy and overcast conditions can lead to drastic temperature fluctuations, affecting algae growth.

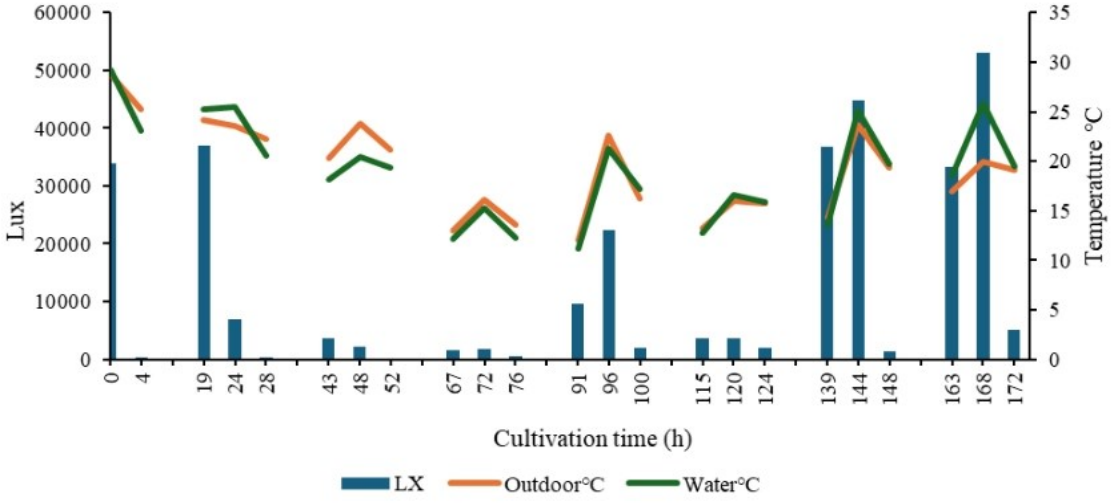


Figure 12 Outdoor weather changes of *N. oceanica* growth under spring outdoor conditions.

From Figure 12, on the seeding day (day 0), the air temperature was 28.7 ℃, and the water temperature was as high as 29.2 ℃. However, subsequent rainy weather caused a sharp drop in temperature on the third day, with the lowest air temperature reaching 13.6 ℃ at 18:00 on day 3 and the water temperature plummeting to 12.3 ℃. The temperature difference between air and water over these three days reached 16.9 ℃. The temperature gradually recovered over the next four days to an air temperature of 20 ℃ and a water temperature of 25.9 ℃. The climate variations in this experiment exhibit typical spring characteristics, making it representative of the season.

The impact of the subsequent climate changes on the photosynthetic efficiency of *N. oceanica* can be observed in figure 13-a. After the low temperatures on the 3rd day, the Fv/Fm values on the 4th day were affected.

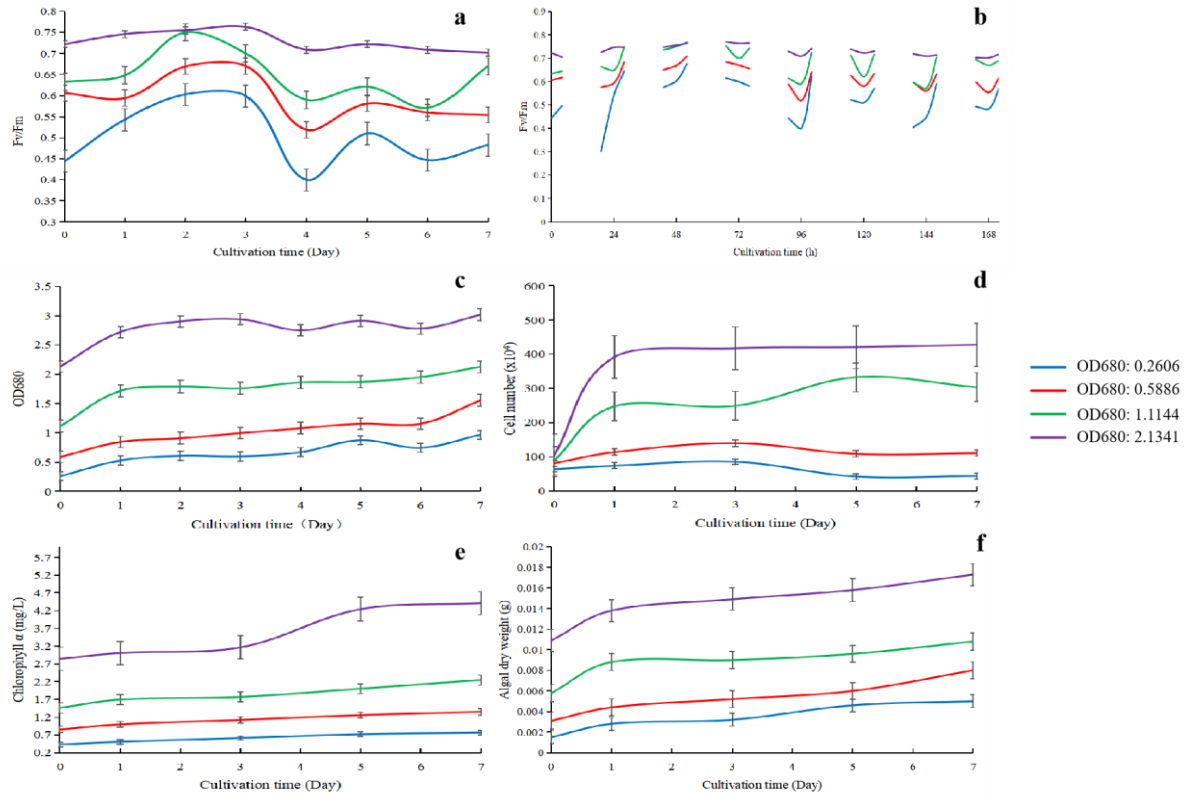


Figure 13 Bioproducts and chlorophyll fluorescence efficiency results of various initial OD $_{680}$ on *N. oceanica* growth under spring outdoor conditions. **a:** Chlorophyll fluorescence efficiency Fv/Fm. **b:** Chlorophyll fluorescence efficiency Fv/Fm of daily change. **c:** Optical density OD $_{680}$. **d:** Cell numbers **e:** Chlorophyll α. **f:** Algal dry weight.

All four groups of algae at different initial concentrations were affected, but the high concentration (OD $_{680}$: 2) showed less impact than the other three groups. The photosynthetic efficiency was influenced by climate changes in the following order, from high to low: 2 > 1 > 0.5 > 0.2 (Figure 13-a). From Figure 13-b, when the initial inoculation concentration was high (OD $_{680}$: 2), the chlorophyll fluorescence efficiency was less affected by external factors. However, when the initial inoculation concentration was OD $_{680}$: 0.2, the impact of external factors was stronger, especially during the morning and evening of the 2nd day, when the

photosynthetic efficiency underwent the most significant changes. Subsequently, it gradually returned to normal on the 2nd and 3rd days, but due to rainy weather and a decrease in temperature on these two days, on the 4th day, the chlorophyll fluorescence efficiency of the low-concentration group (0.2) is again stressed. On day 6, with the temperature rise, the chlorophyll fluorescence efficiency of the low-concentration group was again disturbed. Through this process, it can be inferred that the chlorophyll fluorescence efficiency of *N. oceanica* is disturbed when the environment undergoes drastic changes, and this impact becomes more pronounced when the initial inoculation concentration is low.

From Figure 13-c, the high-concentration inoculation (OD_{680}: 2) had a decrease in OD_{680} values on the 3rd day after the low temperature, and fluctuations were observed in the following days.

The cell numbers showed the most significant increase on the 1st day (Figure 13-d). Following rainy weather from days 2 to 3, the cell growth in the high-concentration group (OD_{680}: 2) stagnated and then gradually stabilized. This stagnation can be attributed to the high density of algae populations, leading to maximum saturation of resources. Similarly, the OD_{680}: 1 group experienced minimal growth during the rainy and cooler weather on days 2 and 3 but resumed growth after the 3rd day (Figure 12). In contrast, both low-concentration groups (0.5 and 0.2) exhibited decreased cell numbers in the following four days. These observations suggest that the density of inoculation played important role in determining the growth response to environmental changes.

The high-concentration group (OD_{680}: 2) rapidly accumulated chlorophyll α on day 3

(Figure 13-e). The rainy and cooler weather on days 2-3 inhibited the accumulation of chlorophyll α in *N. oceanica*. The remaining three initial inoculation concentration groups showed less noticeable accumulation of chlorophyll α. All four groups showed varying degrees of growth in dry weight accumulation but slowed growth (Figure 13-f). The growth rate was higher only on the 1st day, and the recovery was slower due to the rainy and cooler weather.

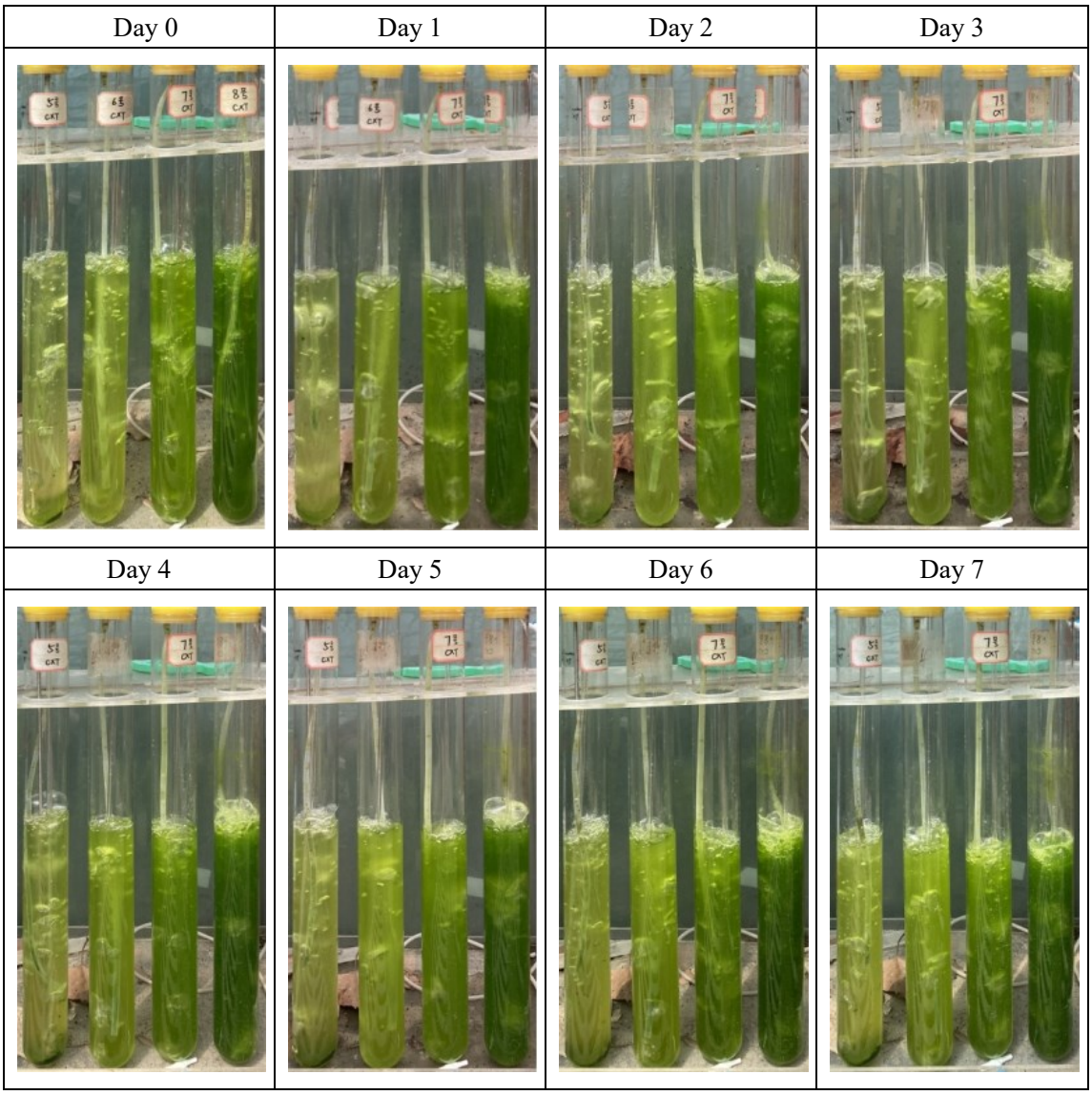


Figure 14 Color changes of *N. oceanica* under various initial optical densities in outdoor spring conditions. From left to right, the OD_{680} values are 0.2, 0.5, 1.0, 2.0.

From the daily photo records (Figure 14), comparing the photos taken on day 7 to those on day 1, there is a slight deepening of color in each concentration group. This indicates that the abrupt temperature change in spring could slow the growth and development of *N. oceanica* but does not entirely hinder its growth. The spring weather is characterized by frequent changes, increased rainfall, and larger temperature variations between day and night. In this experiment, there was more rain and more considerable temperature fluctuations. Our results indicate that during periods of fluctuating weather, algae was inhibited and, in some cases, even stagnated.

However, with the rise in temperature, *N. oceanica* gradually recovered and resumed growth. Moreover, results suggest that during spring cultivation, it is advisable to increase the initial inoculation concentration (at least OD_{680} >1.0) to prevent algae from being adversely affected by changes in climate, ensuring their normal growth. This experiment also indicates that *N. oceanica* has good resistance to low temperatures. Future targeted improvements could potentially expand its cultivable range of latitude. For example, expand to 38 ° or even 43 ° north latitude.

3.2.2 Investigating the impact of various initial OD_{680} levels on *N. oceanica* growth under outdoor summer conditions.

The experimental site was deep inland but featured numerous rivers and lakes. Frequent rain, sultry and humid conditions, and little temperature difference between day and night characterized the summer in this region. For the summer experiment (Figure 15). The weather was consistently overcast, with continuous rainfall in the first five days of inoculation. From

days 1 to 4, the overall temperature ranged between 28 and 24 °C.

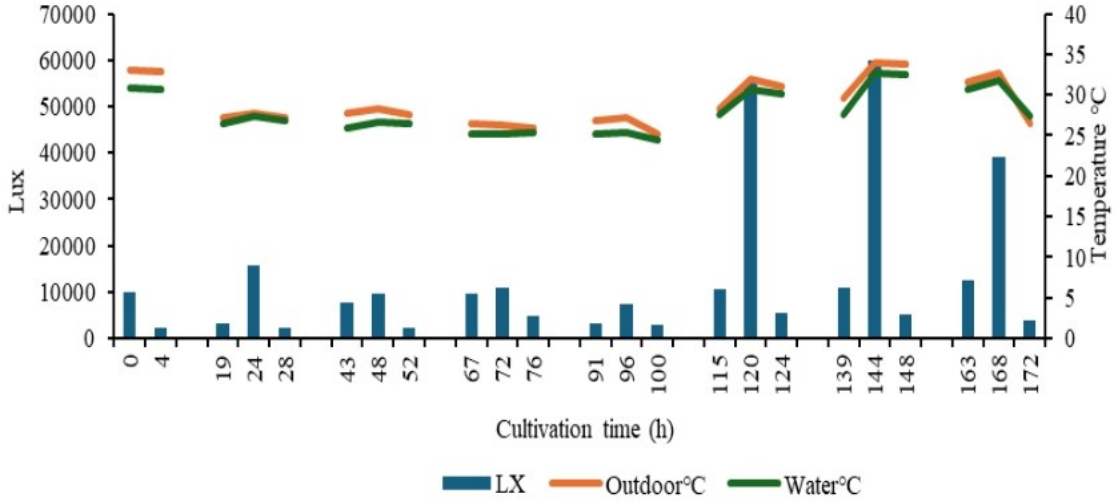


Figure 15 Outdoor light and temperature changes of *N. oceanica* growth under summer outdoor conditions.

However, the light intensity was low due to the persistent rain. On day 1, during the rainy weather, the maximum light intensity was 15,710 lux. From the fifth day, the weather cleared up, and on the sixth day, the temperature reached 33.9 ℃, the water temperature rose to 32.7 ℃, and the light intensity soared to 59800 lux.

We observed that the relatively warm and overcast weather at the initial inoculation stage decreased the photosynthetic efficiency of *N. oceanica* (Figure 16-a). However, as *N. oceanica* gradually adapted to the environment, its chlorophyll fluorescence efficiency gradually recovered to higher levels by day 4. Conversely, the sunny weather starting from day 5 with a sharp increase in light intensity inhibited the chlorophyll fluorescence efficiency of *N. oceanica*. Nevertheless, on day 6, despite the continued sunny conditions, the upward trend in the curve showed that *N. oceanica* is gradually adapting to the intensified light conditions.

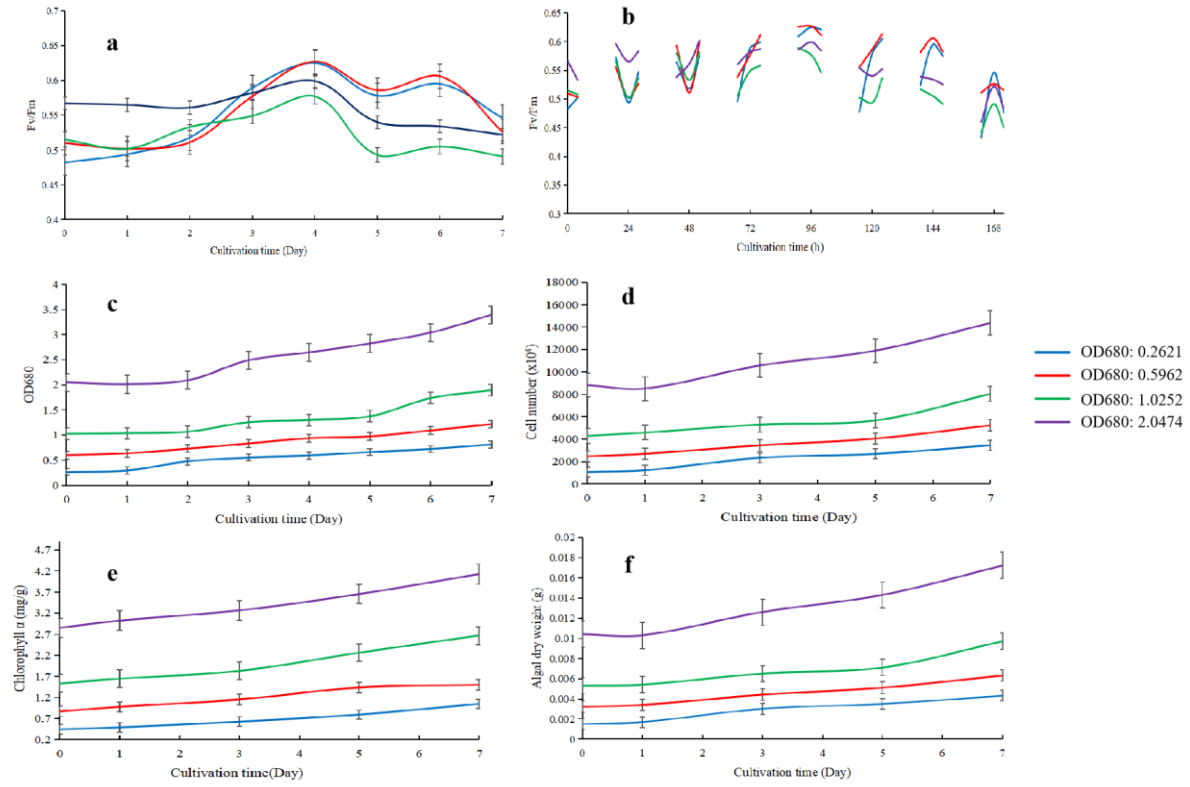


Figure 16 Bioproducts and chlorophyll fluorescence efficiency results of various initial OD $_{680}$ on *N. oceanica* growth under summer outdoor conditions. **a:** Chlorophyll fluorescence efficiency Fv/Fm. **b:** Chlorophyll fluorescence efficiency Fv/Fm of daily change. **c:** Optical density OD $_{680}$. **d:** Cell numbers **e:** Chlorophyll α. **f:** Algal dry weight.

From days 4 to 6 (Figures 16-a and 16-b), when the algae entered the high light exposure phase, the groups with low initial inoculation concentrations (OD $_{680}$: 0.2 and 0.5) exhibited higher chlorophyll fluorescence values. This contrasts with the two high-concentration groups (OD $_{680}$: 1 and 2), indicating that high-density algal cultures shade each other, leading to photosynthetic inefficiency. However, it is noteworthy that the higher concentration of algal cultures demonstrates greater environmental resilience despite the observed decrease in photosynthetic efficiency.

Figure 16-c showed the continuous overcast and rainy weather during summer. Even in

the case of high initial inoculation concentration with an OD_{680} of 2.0, where mutual shading among algae was more pronounced, there was still increased from OD_{680} 2.0474 to 3.394 on days 0 to 7. However, OD_{680} increased from 2.1341 to 3.0155 during spring on days 0 to 7 (Figure 13-c). The impact of overcast and rainy weather was more evident in algae with lower initial inoculation concentrations.

The cell number results showed that the high inoculation concentration (OD_{680}: 2.0) had a decrease in cell numbers on the first day of inoculation (Figure 16-d). However, cell numbers resumed continuous growth, increasing by day 7. But the growth of OD_{680}: 1.0 group was affected by overcast and rainy weather from days 0 to 5, showing a slow increase in cell numbers.

The overall growth trend of chlorophyll α was slow (Figure 16-e), whether in the high or low inoculation concentration groups. Because the growth inhibition caused by overcast and rainy weather. In the OD_{680}: 2.0 group showed high algal dry weight accumulation (Figure 16-f). The high-concentration group was less affected by overcast and rainy weather in the first three days and started growing from day 1. However, the dry weight accumulation was slow in the low-concentration group.

Day 0	Day 1	Day 2	Day 3
			
Day 4	Day 5	Day 6	Day 7
			

Figure 17 Daily pictures of different initial OD_{680} of *N. oceanica* growth under summer outdoor conditions.

From Figure 17, the overall color was of day 7 was darker than days 0. From this, the clear weather conditions are preferable during the initial stages of algae cultivation (days 1-3). This facilitates favorable growth conditions for the algae right from the beginning. Conversely, in the initial growth stages, continuous overcast and rainy weather could inhibit algae growth, delaying the period of rapid growth and impacting the overall production efficiency.

3.2.3 Investigating the impact of various initial OD $_{680}$ levels on *N. oceanica* growth under outdoor Fall conditions

The autumn climate characteristics in the experimental region exhibit significant temperature fluctuations, resembling a direct transition from summer to winter. Additionally, intermittent rainfall occurs, and the temperature difference between day and night increases, with a reduction in sunlight duration and intensity. The experimental duration was extended to 13 days to capture representative periods for this study.

The weather conditions (Figure 18) reflected typical climate features of the local area. Clear skies and comfortable temperatures characterized days 0-5 (around 25 ℃), while days 6-8 had a rapid temperature drop accompanied by overcast conditions from 22 to 6 ℃, and days 9-13 exhibited alternating patterns of rain and sunshine, reflecting fluctuating weather. From the initial inoculation on days 0 to 5 (120 h), the temperature decreased from 28℃ to 18℃.

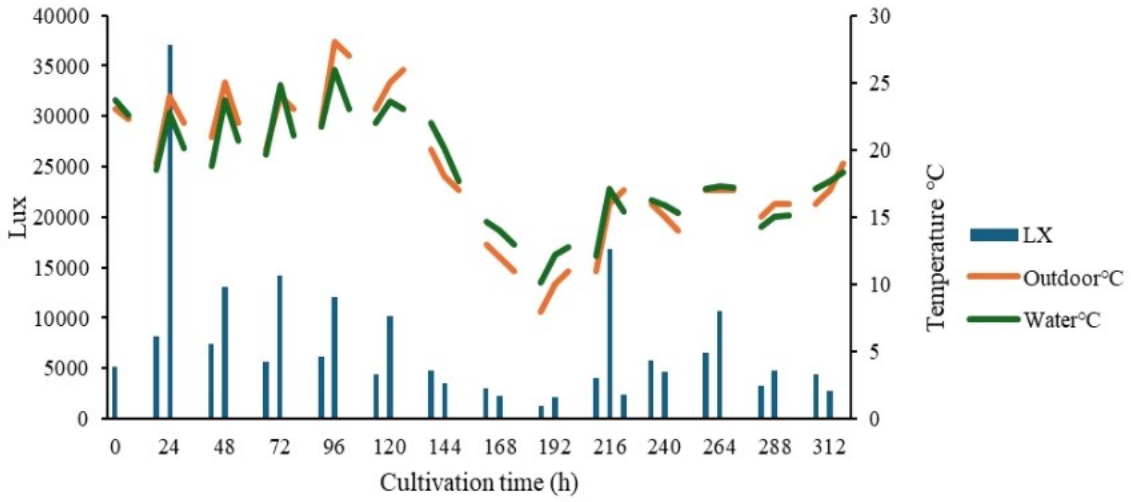


Figure 18 Daily weather fluctuations of *N. oceanica* growth under fall outdoor conditions. *N. oceanica* was tested at 9 am, 2 pm, and 6 pm daily. 2 pm on day 0 was set as 0 h.

Overcast conditions with strong winds brought about a rapid temperature decrease from day 6 (144 h). Rain began on day 7 (168 h), leading to a drastic temperature drop. By 9 am on day 8 (192 h), the temperature was only 8 ℃, and the water temperature was 10.1 ℃. The highest temperature on day 4 (96 h) reached 28 ℃, resulting in a temperature fluctuation 20 ℃ within four days. Days 9 and 11 (216-264 h) were clear, while days 10 and 12 (240-288 h) were rainy, and day 13 (312 h) was overcast. We can say that from days 9 to 13, a typical autumn fluctuating climate was represented.

Newly inoculated *N. oceanica* took two days to restore its photosynthetic system efficiency to relatively normal values from days 0 to 2 (Figure 19-a). However, the overall chlorophyll fluorescence efficiency in the high-concentration group remained lower. This can be due to the dense algal cells shading each other's light, coupled with the fact that the autumn sunlight intensity is not as strong as at different times, affecting the chlorophyll photosynthetic efficiency of the algae. Interestingly, the rapid temperature drops from days 6 to 8 seems to have little impact on chlorophyll fluorescence data, but a considerable decline occurred on sunny day 9. Similar trends were observed in spring experiments, indicating that damage does not immediately manifest but becomes apparent on sunny days following overcast and rainy weather. The chlorophyll fluorescence efficiency remained consistently low during the repeated overcast and rainy weather from day 9 to 11, only recovering on day 12.

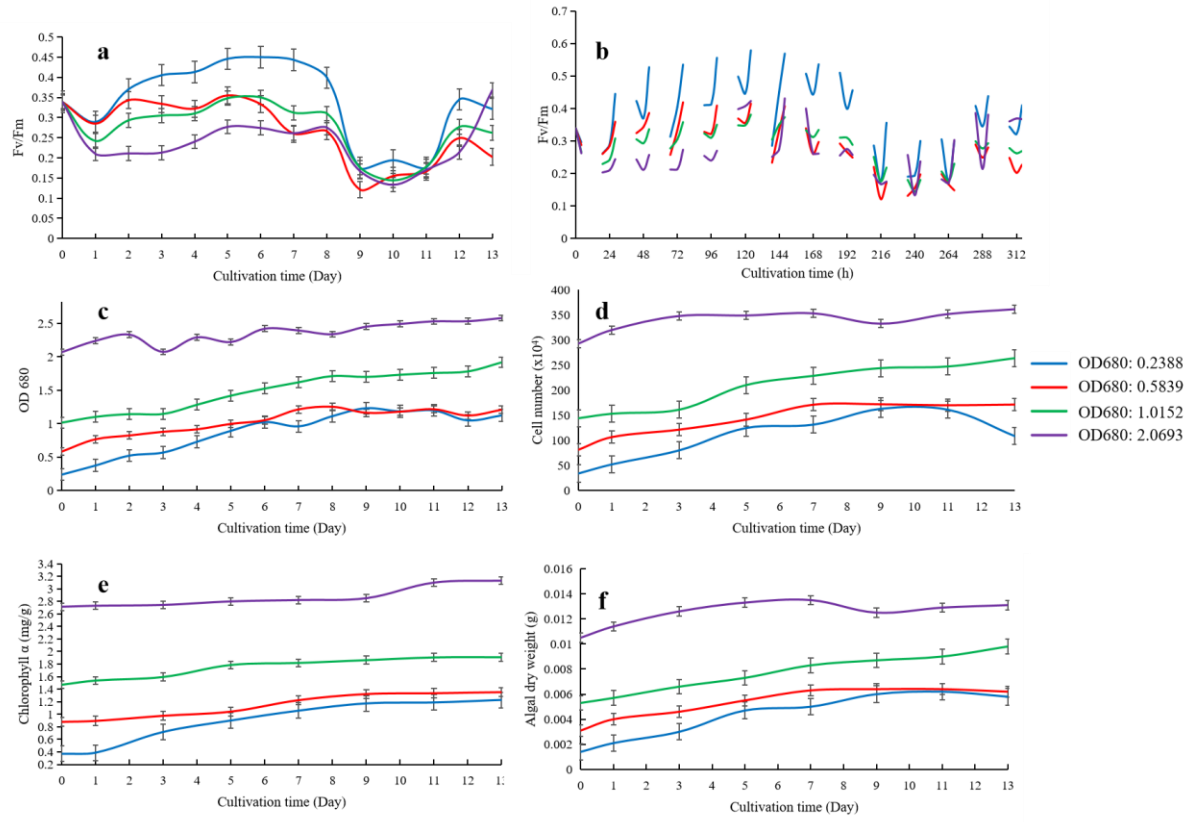


Figure 19 Bioproducts and chlorophyll fluorescence efficiency results of various initial OD $_{680}$ on *N. oceanica* growth under fall outdoor conditions. **a:** Chlorophyll fluorescence efficiency Fv/Fm. **b:** Chlorophyll fluorescence efficiency Fv/Fm of daily change. **c:** Optical density OD $_{680}$. **d:** Cell numbers **e:** Chlorophyll α. **f:** Algal dry weight.

Daily Fv/Fm measurements showed a decrease in photosynthetic efficiency when light intensity was high (14:00). This could be likely attributed to the algae activating self-protection mechanisms in response to excessive light energy and potential oxidative damage under intense light (Wang et al., 2021).

In Figure 19-c, we observed that the high-concentration group exhibited relatively slow growth while the low-concentration group showed rapid growth. But, after rapid temperature drop on day 8, the growth rate from days 9 to 13 considerably slowed, even showing a

declining trend. This indicates that the rapid temperature drop severely impacts the growth status of the low-concentration group.

The cell number results revealed that the high-concentration cultivation group (OD_{680}: 2) displayed a limited increase in cell growth. This is attributed to cell density approaching saturation, leading to growth inhibition in algae. On day 9, a decline was observed in the OD_{680}: 2 group, suggesting that rapid cooling could kill some cells.

However, cell numbers recovered in the subsequent days due to the high algal concentration. In contrast, the low-concentration group (OD_{680}: 0.2) had a decrease in cell count following the temperature drop on day 9. This shows that the ability to handle big temperature changes was weak when inoculation concentration was 0.2. The concentration of 0.5 also exhibited extremely slow or even stagnant cell growth after day 9.

The low-concentration inoculation group (OD_{680}: 0.2) had a stagnation in chlorophyll accumulation after the rapid temperature drop on the 9th day (Figure 19-d). Subsequently, the alternating sunny and rainy weather further inhibited chlorophyll accumulation in the low-concentration group.

There was no considerable increase in chlorophyll accumulation in the other inoculation concentrations in the first 7 days. This is attributed to the fact that, although sunny from days 0 to 7, the light intensity was not very high, leading to a lack of considerable growth.

The growth rate of the low-concentration inoculation group (OD_{680}: 0.2) was quite good in the first 9 days, but fluctuations were observed, indicating that lower initial inoculation concentrations are more sensitive to environmental changes (Figure 19-f). Even slight

variations could impact the dry weight accumulation of *N. oceanica*. After experiencing low temperatures on the ninth day, the dry weight accumulation showed stagnation and even a decline.

The experimental group with an initial inoculation concentration of 0.5 trended similarly to the 0.2 concentration group. In the high concentration group (2), there was a decline in dry weight on day 9, while the 1 concentration group did not show a decline but rather a slowdown in growth. We speculate that the decrease in the dry weight of the 2-concentration group is due to the inability of older *N. oceanica* cells in the high-concentration group to withstand rapid temperature drops. Moreover, the previous higher concentration hindered effective photosynthesis and nutrient accumulation. The rapid temperature drop led to cell death and a decline in dry weight. In contrast, the 1.0 concentration group, with an appropriate cell density, could undergo sufficient photosynthesis. The higher number of new cells and the accumulation of nutrients in each cell, even with a rapid temperature drop, only slowed their growth and development without causing death. Therefore, the dry weight results only show a slowdown in growth in the 1.0 concentration group.

Daily photo records showed a gradual increase in the cell density of *N. oceanica* from days 0 to 9 (Figure 20). However, after day 9, the cells in the culture started to turn yellow, indicating that the weather changes influenced their cell status. Thus, when cultivating algae strains in the fall, it is advisable to avoid low inoculation concentrations.

The inoculation concentration was $OD_{680} > 1.0$ can avoid the growth of *N. oceanica* from being inhibited due to the changes in fall weather, thereby avoiding losses.

Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
						
Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13
						

Figure 20 Color changes of different initial OD $_{680}$ of *N. oceanica* growth under fall outdoor conditions.

3.2.4 Investigating the impact of various initial OD $_{680}$ levels on *N. oceanica* growth under outdoor winter conditions.

The winter temperatures in the experimental area exhibited high humidity (Figure 21), low temperatures, and continuous overcast conditions, with more cloudy days than sunny ones. Days 2, 6, and 7 were sunny; during other periods, it was overcast. The highest water temperature recorded in this experiment was 13.2 ℃ on day 7, while the lowest water temperature occurred on day 2, reaching 3.3 ℃. The strongest illumination occurred on the 2nd day, measuring 22000 Lux.

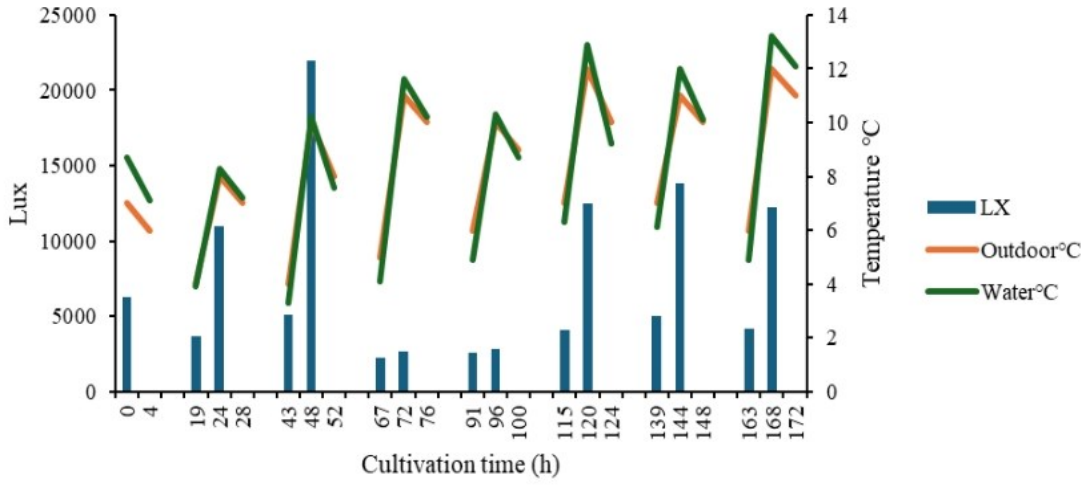


Figure 21 Daily weather changes of *N. oceanica*. The winter weather conditions were tested at 9 am, 2 pm, and 6 pm daily, and 2 pm on day 0 was set as 0 h.

Over the seven days, the chlorophyll fluorescence trend (Figure 22-a) showed that winter inoculation, characterized by low temperatures and low light intensity, inhibited the photosynthetic efficiency from days 0 to 1. Following a sunny day on day 2, the chlorophyll fluorescence efficiency in all concentration groups increased until day 5. This indicates that low temperatures and low light did not kill *N. oceanica*; instead, it seems to have induced a state of dormancy in the cells. With the appropriate enhancement of light and temperature, *N. oceanica* resumed its activity.

Compared to other seasons, most groups' Fv/Fm decline in wintertime (Figure 22-b). This is analogous to algae experiencing a daily transition from low to high temperatures during the winter, similar to the decrease in Fv/Fm values observed when temperatures rise again after a cooling period in autumn (Figure 19-a).

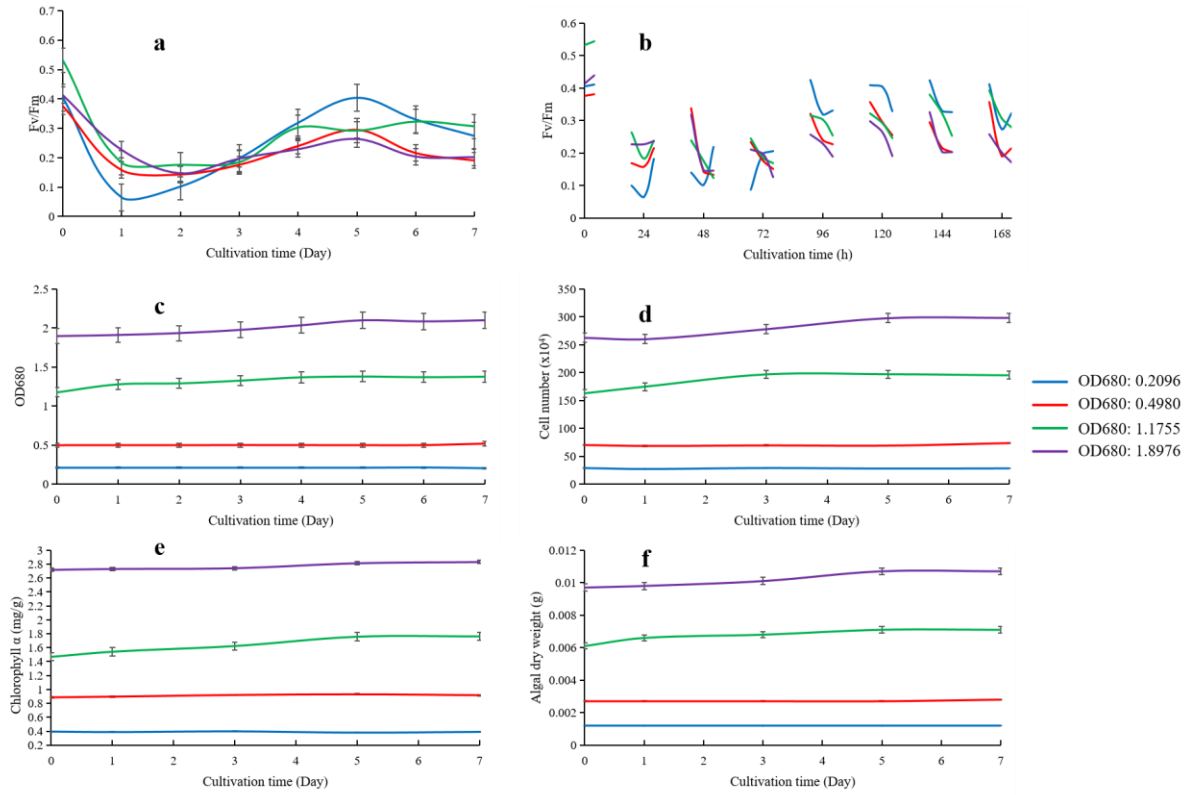


Figure 22 Bioproducts and chlorophyll fluorescence efficiency results of various initial OD $_{680}$ on *N. oceanica* growth under winter outdoor conditions. **a:** Chlorophyll fluorescence efficiency Fv/Fm. **b:** Chlorophyll fluorescence efficiency Fv/Fm of daily change. **c:** Optical density OD $_{680}$. **d:** Cell numbers **e:** Chlorophyll α. **f:** Algal dry weight.

We observed that the growth of *N. oceanica* was considerably inhibited, regardless of whether the initial inoculation concentration was high or low (Figure 22-c). The 1.0 and 2.0 groups showed slight growth, while the 0.2 and 0.5 concentration groups have almost stagnated.

Regarding cell growth (Figure 22-d), the high-concentration group still had increased cell numbers. The primary growth occurred after the sunny day on the 2nd day, indicating that *N. oceanica* possesses a level of cold adaptation. As long as a certain level of illumination is

maintained, it can continue to grow even under lower temperatures. However, the algal cell concentration needed to be sufficient. Even with a particular illumination level, normal growth could not be sustained if the cell concentration was not high enough.

The accumulation of chlorophyll α in the group with an OD_{680} concentration of 1 showed the best chlorophyll α growth (Figure 22-e). In the high-concentration group, the growth inhibition of chlorophyll α was attributed to the combination of low temperatures and the shading effect caused by the high cell concentration, hindering optimal light exposure. The low-concentration group was influenced by cold temperatures and low light intensity, resulting in limited accumulation of chlorophyll α. The trend in dry weight was similar to that of cell count (Figures 22-c and 22-f), indicating the suppression of low inoculation concentrations under winter climate conditions. On the other hand, high inoculation concentrations still exhibited a slow growth trend.

From Figure 23, the two low-concentration groups (0.2 and 0.5) showed no big changes. In contrast, the most significant changes were observed in the group with the initial inoculation concentration of OD_{680}: 1. For 7 days, it gradually turned green, aligning with the results obtained from the measurement of chlorophyll α accumulation (Figure 22-e).

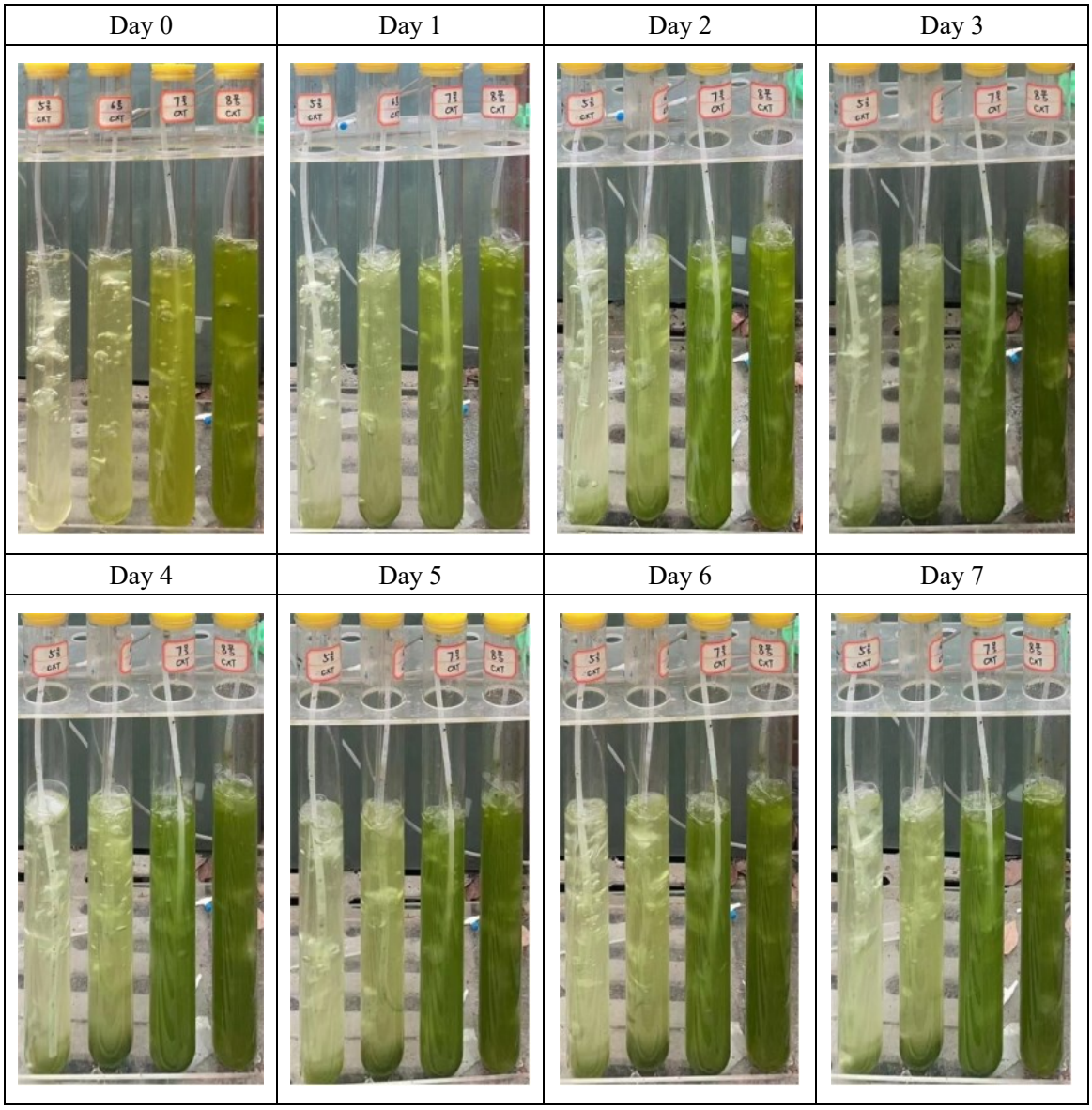


Figure 23 Color changes of different initial OD $_{680}$ of *N. oceanica* growth under winter outdoor conditions.

Conclusion

The investigation into the indoor and outdoor cultivation of marine microalgae *N. oceanica* indicated that using 0.5 g/L urea as the nitrogen source for *N. oceanica* is highly suitable. However, avoiding using ammonium chloride as a nitrogen source for *N. oceanica* is important, as it strongly inhibits its growth and development.

When cultivating *N. oceanica* in inland areas, the optimal salt concentration for growth

should be maintained below 3.5 %. To keep salinity within the 2.5 % to 3.5 % range to variations in salt concentration caused by evaporation and rainfall.

Increasing initial inoculation mitigated cloudy weather effects. Summer shading enhanced growth efficiency. Winter's low temperatures and light slowed growth, but *N. oceanica* thrived with OD_{680} exceeding 1.0. It's adaptable for year-round cultivation at 30 °N in China, saving costs. Chlorophyll fluorescence proved effective for real-time monitoring of growth inhibitors like salinity and temperature.

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Chapter 4. Growth and Chlorophyll Fluorescence Analysis of Algae *Nannochloropsis oceanica* in Outdoor 700 L and 150 L Water Tanks from Summer to Winter

Abstract

Cultivating marine microalgae inland can reduce influence from marine microorganisms. However, most marine microalgae are cultivated in coastal areas. In this experiment, two cultivation photobioreactors (PBRs) were compared for the semi-continuous cultivation of *Nannochloropsis oceanica* within 185 days. The new photobioreactor designed and tested showed good cultivation in partially shaded areas. Based on the innovative in-situ oxygen release rate (ORR) measurement method results, ORR was influenced by light intensity and temperature. The optimal temperature range for *N. oceanica* growth was 14-25 °C, showcasing cold tolerance and lipid accumulation at low temperatures. The maximum lipid content in 700-liter and 150-liter PBRs was 29 % and 28 %, respectively. The 150-liter PBRs (US $ 11.89 per kilogram) was more susceptible to environmental influences. Moreover, should avoid high temperatures and cold overcast days in initial inoculation.

Key words: *Nannochloropsis oceanica,* inland, outdoor, chlorophyll fluorescence, oxygen release rate

1 Introduction

At present, the cultivation of marine microalgae is mostly conducted near coastal areas (Lu et al., 2021). However, cultivating marine microalgae near the coast is susceptible to affect from other marine algae and interference from various factors such as bacteria in the sea (You et al., 2021), which can adversely affect both yield and quality. Inland regions have a more favorable environment for the cultivation of marine microalgae (Barclay et al., 1987). Even in open-air cultivation, the risk of influence from other algal species is reduced. Additionally, some inland areas may have large expanses of saline-alkali land that is unsuitable for traditional crop cultivation. Cultivating marine microalgae in these areas can enhance land use efficiency, to make barren land productive, for example, by using algae to produce oil (Liu et al., 2019).

Most outdoor algae cultivation PBRs are typically established on vast land areas (Yen et al., 2019; Gupta et al., 2015). However, this often results in the significant allocation of land exclusively for algae cultivation, while the same land, if repurposed for agriculture, forestry, industry, or residential use, frequently generates higher economic value. Furthermore, as research on algae advances, algae exhibit enormous potential in areas such as wastewater purification and the absorption and fixation of greenhouse gas carbon dioxide (Zhang et al., 2021; Shen; 2014; Kong et al., 2021; Molazadeh et al., 2019). In certain specific conditions, there is a need for compact, efficient, and easily transportable cultivation devices. This allows for microalgae cultivation in specific locations, such as rooftops (Kumar et al., 2023), spaces between buildings and windows or (Kim, 2022; Negev et al., 2019), in a more daring assumption, potential future lunar bases, space stations,

and similar environments (Detrell et al., 2020; Detrell et al., 2021; Detrell et al., 2022). Therefore, efficiently cultivating algae in limited spaces, even in small or unused lands, to create more economic value, is a crucial focus of current research.

The energy and business cycles exhibit a systematic relationship, and currently, new energy sources have become the primary driving force (Skare et al., 2021). Currently, 84% to 93% of funding in directions such as algae biodiesel production is allocated to basic costs, mainly stemming from energy consumption during cultivation, including electricity, water, and lighting (Maroušek & Gavurová et al. 2023). Translating algae technology into commercial dimensions poses a significant obstacle to the commercialization of microalgae, necessitating interdisciplinary research and discussion. Moreover, research on microalgae oil production is currently confined to laboratory scale, highlighting the importance of addressing the bottleneck of improving outdoor cultivation efficiency (Maroušek & Marouškova et al., 2023). Besides, currently common methods for outdoor cultivation of algae include the use of open pond reactors or raceway pond reactors (Borowitzka et al., 2012; Chisti, 2013). Alternatively, users may design their own cultivation tanks according to their needs. Once these cultivation tanks are constructed, they are difficult to move and involve high costs, and overlooked the cost of electricity usage, etc. In addressing this issue, a key challenge is to design compact and efficient cultivation systems that maximize the utilization of limited spatial resources. Such technological innovations not only contribute to the sustainability of algae cultivation but also offer more flexible for algae production in sectors like agriculture and aquatic feed. In the future, through these innovative cultivation systems will efficiently utilize limited land resources, promoting the sustainable

development of the algae industry.

During photosynthesis, chlorophyll molecules in the chloroplasts of plant cells absorb light energy. A crucial component of this process is Photosystem II (PSII), where light energy undergoes conversion into chemical energy. FV/FM is a specific measure that assesses the maximum efficiency of PSII in transforming light energy into chemical energy. (Krause et al., 1991; Krause et al., 1984).

Fv (variable fluorescence): This indicates the variance between the maximum fluorescence level in the dark-adapted state and the fluorescence level in the light-adapted state. Fm (maximum fluorescence): This represents the highest measurable fluorescence level when all PSII reaction centers are closed, typically in the dark-adapted state. The FV/FM ratio offers insights into the efficiency of PSII in capturing and converting light into chemical energy during photosynthesis. A higher FV/FM ratio suggests efficient PSII operation, while a lower ratio may indicate stress or damage to the photosynthetic apparatus. Researchers commonly employ FV/FM measurements to evaluate the health and performance of plants, particularly under varying environmental conditions or stress factors.

In this experiment, a new designed 700-liter (L) tank was compared with 150 L polyethylene (PE) plastic bucket obtained from the market for the outdoor cultivation of *N. oceanica*. Additionally, the chlorophyll fluorescence efficiency and growth status of *N. oceanica* over a six-month period in these two different cultivation PBRs were assessed. The aim was to identify a more cost-effective cultivation method and equipment for *N. oceanica*.

2 Methods and materials

2.1 Algal cultivation

To use f/2 medium to create artificial seawater for algae cultivation (Guillard, 1975), with 2.5% salinity, using 0.5 g/L urea as the nitrogen source.

2.2 New designed photobioreactor (PBR) for algal cultivation

In this experiment, an innovative toughened glass tank for outdoor cultivation was built. The tank was 5 meters long, 0.3 meters wide, and 0.66 meters high, featuring a sturdy 9 mm glass thickness. The outer periphery of the overall structure was reinforced with a steel frame structure, positioned 30 cm above the ground. Drainage outlets were installed at both ends of the bottom. It offered transparency on its front, back, and top sides. The tank had the capacity to hold up to 1000 L of algal solution, Air was provided by a WL-550 high-pressure scroll air pump (US $69.3, 550 watts: W, 220 Volt: V, Dongguan WeiLe Mechanical and Electrical Equipment Co., Ltd.) through 12 oxygen pump air stones (4 L/min/each). No additional CO_2 was supplied. The PBR individually occupies 1.5 square meters (m^2). Each PBR$_S$ was spaced 1 meter apart (i.e., aisle), so a single PBR occupies 6.5 m^2. This approach holds the promise of achieving efficient algae cultivation in confined spaces, thereby enhancing the economic viability of algae production without wasting land resources.

2.3 Semi-continuous cultivation (SCC)

For SCC, it was carried out every 15 days, collecting 100 L algal liquid from 150L and 700 L PBRs each time and adding new culture medium. In the event of special weather, the SCC time can be appropriately extended or shortened to observe the impact of inland

climate change on the outdoor culture of marine microalgae.

2.4 Algal cell optical density measurement

The optical density of the algae liquid was measured every day, and 5-milliliter (mL) of algae liquid was taken for each measurement, and the OD_{680} value was measured with UV spectrophotometer.

2.5 Algal cell count

Same as Chapter 3.

Or the algal solution was diluted to approximately 100 cells in each of the 25 central grids, and counts were performed for all central 25 grids：

$$\text{Cell/mL} = \text{Cell number} \times 10000 \times \text{Dilution factor}$$

2.6 Chlorophyll α measurement

Same as Chapter 3.

2.7 Algal dry weight measurement

Algal weight was measured on Day 0, 1, 2, and 3. First, the drying process was initiated by placing the sample in an oven for 24 hours. Cooled to room temperature in a drying dish equipped with a microporous filter membrane filter paper (0.45 μm pore size; diameter 50 mm, Jin Teng brand). The dish was pre-weighed (m1). Pumped 5 mL of the algal solution onto the filter paper. Subsequently, the sample was washed with a 0.65 M NH_4HCO_3 solution to eliminate salt residues. The sample was then placed into the oven set at 80 degrees Celsius (℃) for an overnight drying period. After that cooled to room temperature, and a precise weighing was conducted to determine the constant weight (m2). To calculate the final dry weight (DCW, in g/mL) of the algae, three measurements were

performed each time, and the results were averaged for accuracy. This comprehensive procedure ensured the accurate determination of the algal dry weight, providing reliable and reproducible results.

$$\text{Cell Weight } = \frac{m2 - m1}{5} \times 1000$$

2.8 The chlorophyll fluorescence assay

Same as Chapter 3.

2.9 Algal growth rate (GR) and Biomass productivity determination (BP)

The growth rate (μ_{max}) was taken as the exponential growth stage and calculated using the following formula:

$$\mu_{max} = \frac{\ln x_2 - \ln x_1}{t_1 - t_2}$$

The x_2 and x_1 represent the dry weight at time points t_2 and t_1.

Biomass productivity (milligram/liter/day: mg/L/day) was calculated using the following formula:

$$P = \frac{DW_X - DW_1}{T_X - T_1}$$

DW_X and DW_1 represent the dry weight (g/L) at time points T_X and T_1 respectively.

2.10 Lipid measurement

Lipid measurement was conducted using the chloroform-methanol extraction method (Chen et al., 2019). Weight W1 (10 mg) of fully ground dry algae powder and W2 of a pre-dried 1.5 mL centrifuge tube were prepared. Then, chloroform (385 μL), methanol (790 μL), and water (316 μL) were added to the mixture to achieve a final volume ratio of 1:2:0.8. The extraction solution was shaken and centrifuged to collect it in a 15 mL tube. The above

steps were repeated for the lower sediment three times, reducing the number of repetitions as necessary.

All the extraction solutions were combined, and chloroform and water were added to achieve a ratio of (1:1:0.9) (1185 μL of water, 1185 μL of chloroform). The mixture was shaken and mixed well then left to stand for 1-2 hours until clear layers appeared. The upper layer was the aqueous phase, containing salts and water-soluble substances, and the lower layer was the chloroform layer. The chloroform layer was collected into a centrifuge tube W2 that had been dried and weighed. It was evaporated at 105 °C to a constant weight and weighed again to obtain W3.

The oil content of microalgae was calculated as following formula:

$$X\% = \frac{W3 - W2}{W1} \times 100\%$$

X% was the percentage of lipids in the dry weight of microalgae, where W1 represented the dry weight of algal powder, W2 denoted the pre-dried and weighed centrifuge tube weight, and W3 was the weight of the centrifuge tube containing lipids.

2.11 Algal outdoor in-situ oxygen release rate (ORR) determination

To facilitate in-situ monitoring of algae photosynthetic oxygen evolution rates, a novel in-situ ORR detection method was devised. Sodium sulfite was employed to absorb oxygen from the water, and by measuring the oxygen evolution of algae over a specific time period, the algae' s oxygen evolution rate was measured. This method allowed for the in-situ assessment of the photosynthetic activity of algae. The specific steps were as follows: algal solution (100 mL) was taken into a 250 mL flask, and the dissolved oxygen meter (JPBJ-609L, INESA instrument, Shanghai, China) was placed. Then, 30 μL of saturated

sodium sulfite solution was added to ensure that the dissolved oxygen level in the sample decreased to around 1 mg/L, but not to 0 mg/L, to avoid excessive sodium sulfite affecting later measurement of algal oxygen release. The system was covered to block light and wait until the dissolved oxygen content gradually stabilized. Once stabilized, the light cover was removed, and the increase in dissolved oxygen content was recorded for 15 seconds to obtain the in-situ ORR (mg/L/s).

2.12 Statistical analysis

All experiments were conducted in triplicates. Statistical analysis was performed on the results obtained from all triplicates using the one-way ANOVA function of the IBM SPSS® software. The comparison method selected was the "Waller-Duncan" with the level of significance (P-value) set at 0.05.

3 Results and discussions

3.1 700 L tank data

Analyzing Figures 1-A and 1-B reveals that in the initial 50 days, temperatures were high with intermittent rains, leading to lower chlorophyll fluorescence levels. Additionally, Figure 2-B indicates a gradual rise in algae cell growth during this period, suggesting inhibition of *N. oceanica* growth by high temperatures, possibly due to adverse environmental conditions caused by high temperatures and rainfall.

After 55 days, both PBRs exhibited rapid and stable increases in chlorophyll fluorescence, corresponding to the changing weather conditions depicted in Figure 1-A. By day 60, Fv/Fm values generally rose and stabilized, reflecting the cooler and more favorable autumn weather conditions. These conditions were conducive to improved photosynthetic

efficiency in algae cells. Conversely, high temperatures and rainfall adversely affected the growth of *N. oceanica*, leading to a decrease in chlorophyll fluorescence. As temperatures decreased in autumn, algae experienced better growth conditions, resulting in increased chlorophyll fluorescence values.

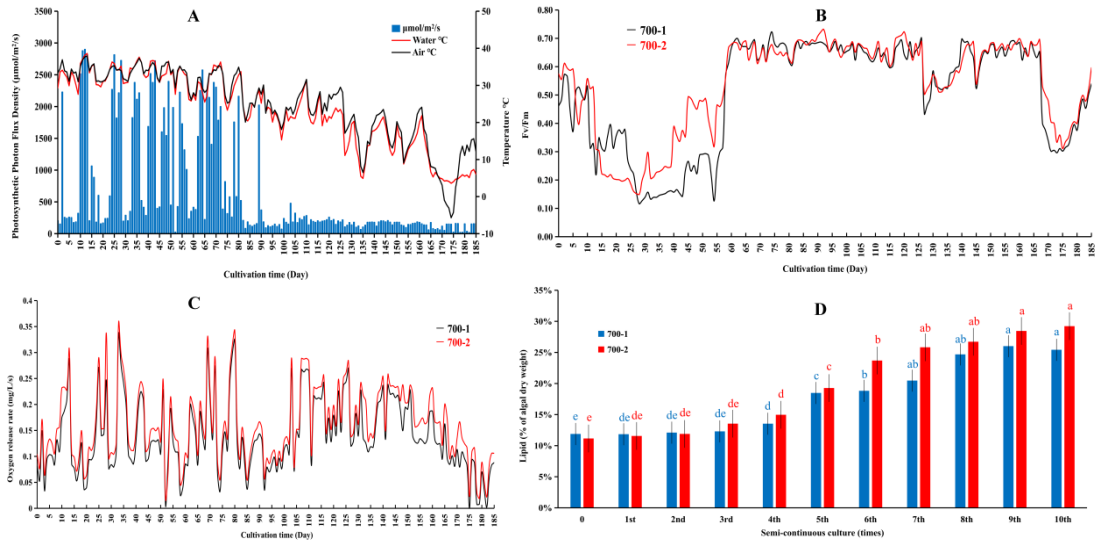

Figure 1 *Nannochloropsis oceanica* growth in 700 L tank under outdoor conditions. **A:** weather conditions. **B:** Chlorophyll fluorescence efficiency (Fv/Fm: Fv is variable fluorescence, Fm is maximum fluorescence). **C:** Oxygen release rate (mg/L/s). **D:** Lipid content (% of algal dry weight). **a, b, c, d, e** are marked as significant differences in lipid content at each SCC run. The same letters indicate no significant differences, and different letters indicate significant differences. Data are reported as mean ± standard deviation of triplicates.

Analyzing daily weather conditions, including water temperature in the culture tank and real-time air temperature (Figure 1-A), aided in determining optimal outdoor culture conditions. The 1st significant cell concentration (SCC) occurred on day 40 (Figure 2-A). Tank 700-1 removed 100 L of culture medium, then adjusted the volume to 700 L by adding

water and nutrients, while tank 700-2 only added nutrients. The 2nd SCC, conducted on the 55th day, showed a decline in OD $_{680}$ value on the 62nd day, indicating rapid growth but insufficient nutrition. Consequently, the 3rd SCC was advanced to Day 65. Nutrients were replenished on day 75 after insights from the 2nd SCC. OD $_{680}$ peaked on day 81 (1.2968 in tank 700-1 and 1.0844 in tank 700-2) before rapidly declining due to a sudden temperature drop during rainy days. The water temperature dropped from 33.7 ℃ on day 81 to 24.2 ℃ on day 82, facilitated by the semi-enclosed system allowing rainwater to dilute the algae solution concentration.

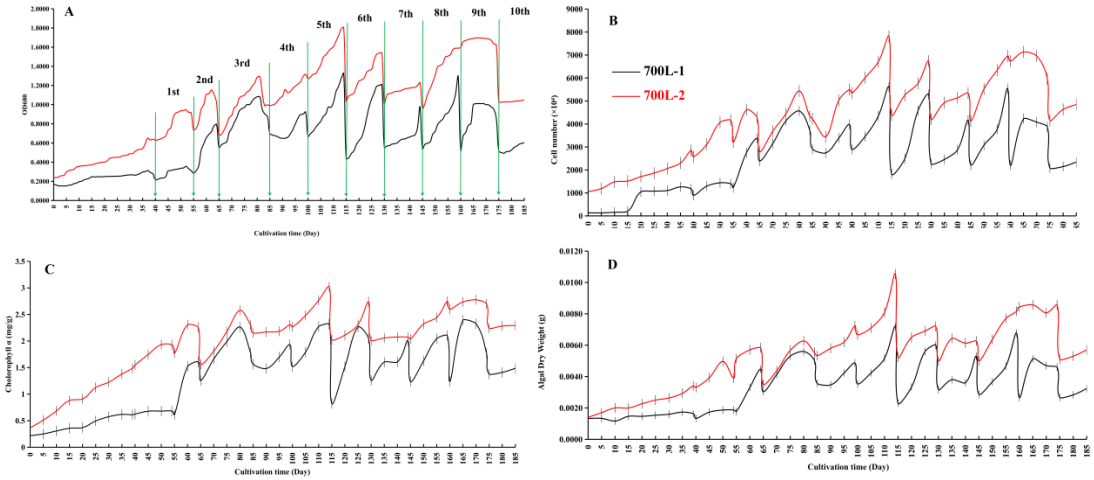

Figure 2 Bioproducts of *N. oceanica* growth under outdoor conditions in 700 L photobioreactor. **A:** Optical density OD $_{680}$. **B:** Cell numbers. **C:** Chlorophyll α. **D:** Algal dry weights. Data are reported as mean ± standard deviation of triplicates.

On the 85th day, the 4th SCC was conducted. However, after day 85, the temperature dropped due to continuous rain. This weather affected low-concentration algae noticeably, causing a decline, while high-concentration algae were less affected and continued to thrive. After October 1st (Day 93), persistent rain led to a temperature drop. On the 99th day, the maximum water temperature was only 15.3 ℃. From algal OD $_{680}$ results, temperature

fluctuations had impact in the early growth stages. After algae adapted the growth conditions, they could still grow even with decreasing temperatures. On day 100, in the 5th SCC allowed to observe the GR of different concentrations of algae at low temperatures and determine whether high-concentration algae solutions could achieve higher cell concentrations at low temperatures.

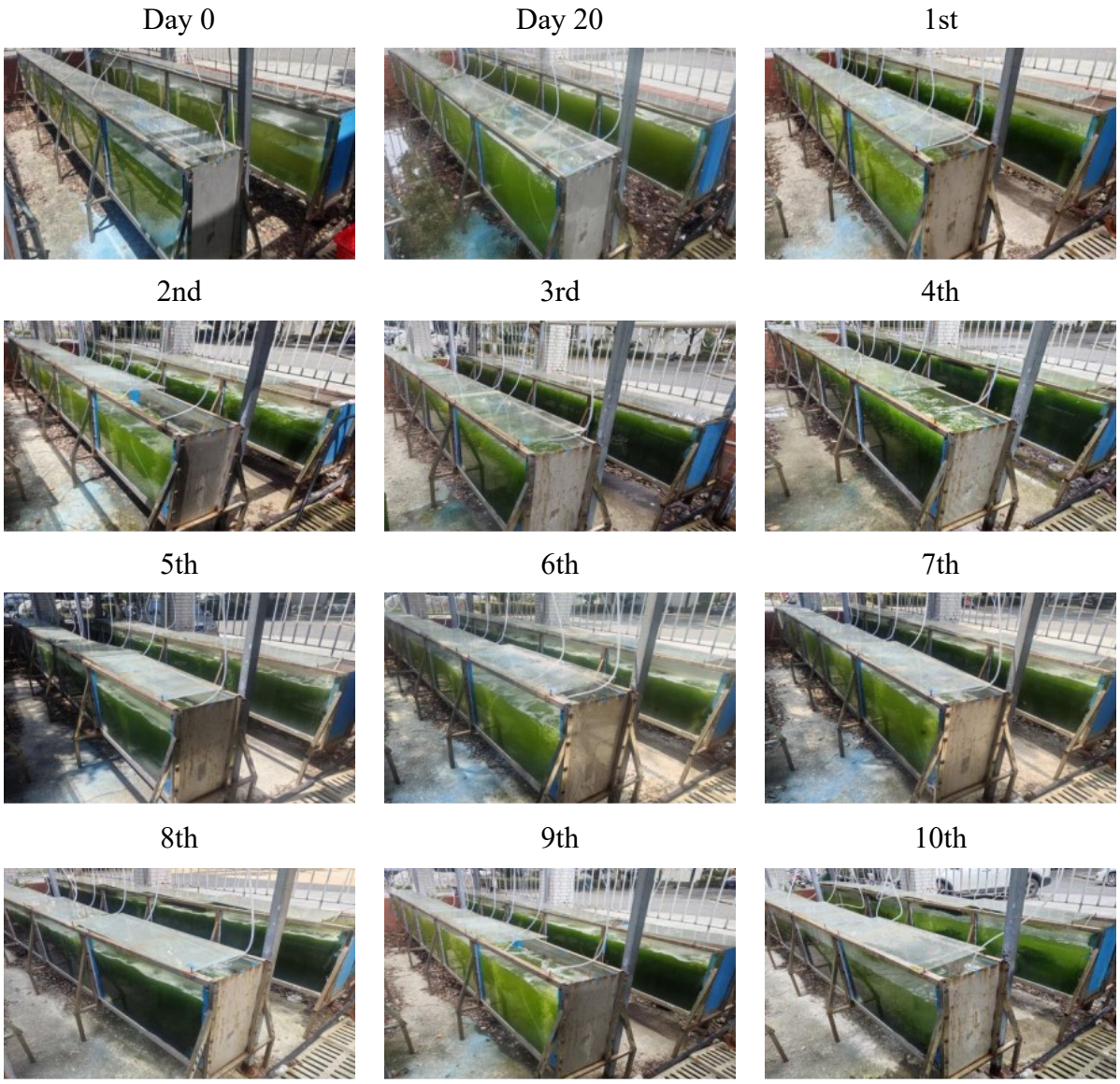

Figure 3 Pictures of the growth of *N. oceanica* with different initial OD_{680} levels in a 700 L tank under outdoor conditions, the one in the front of the picture is 700-1, and the back is 700-2 front tank is 700-1 . Except for day 0 and day 20, the rest of the pictures were taken

on the last day of each semi-continuous cultivation.

On day 115, the 6th SCC began with temperatures averaging around 27 °C, accompanied by mostly sunny conditions, leading to increased algae growth. However, rain started on Day 127, causing a 10 °C temperature decrease and slowing down algae growth. By Day 130, the 7th SCC had commenced. The rainfall from Day 131 to 133, resulting in a decrease in air temperature from 22.2 to 6.5 °C, and water temperature dropped from 21.1 to 4.9 °C. On the 145th day, the 8th SCC began with an average temperature of 16.12 ± 3.65 °C and an average water temperature of 14.31 ± 3.04 °C during the subsequent 15 days of cultivation. Despite initial temperature fluctuations, the OD_{680} values demonstrated fast growth from Day 145 to Day 159, as depicted in Figure 2-A.

On Day 160, the 9th SCC commenced, coinciding with a temperature drop from 24.1 °C to -5.7 °C by Day 174. Initially, there was growth in OD_{680} values, but as temperatures decreased, growth plateaued. Below 6 °C, algal OD_{680} values declined. On the 175th day, the 10th SCC began with an initial temperature of -4.3 °C during inoculation and a water temperature of 4 °C. Subsequently, temperatures rose to 15.6 °C by Day 184, but OD_{680} results showed a very slow increase in growth during this period.

The highest OD_{680} value occurred on the Day 114 which was the 5th SCC at 700-2, reaching 1.8076. According to Figure 2-A, the GR of the initially cultured algae was slow from day 0 to 40, attributed to factors such as rain, reduced sunshine, and higher temperatures (e.g., reaching 38.6 °C on day 13). The unstable temperature fluctuated violently, inhibiting algal growth. After 40 days, the SCC began, and the high-concentration algae began to grow rapidly. Between days 40 and 51, the weather was mostly cloudy with

occasional sunny, characterized by relatively stable temperatures and the absence of violent fluctuations.

The highest cell number occurred on the 114th day in the 700-2 group, at 78.08 $\times 10^6$. The weather conditions were clear, with water temperature of 21.2 ℃ and an air temperature of 23.1 ± 3.66 ℃. Through the results of Figures 2-B and 2-C, a similar overall trend throughout the 185 days of cultivation was observed. Starting from day 40, the average water temperature and air temperature during the 1st SCC (days 40-54) were 33.53 ± 2.50 and 34.18 ± 2.03 ℃, respectively (Table 1). From Figures 2-B, 2-C, and 2-D regarding cell numbers, chlorophyll α content, and dry weight. The growth of *N. oceanica* was continuously inhibited by high temperatures. This indicated that *N. oceanica* growth was suppressed when the temperature exceeded 33 ℃.

Table 1 The average water temperature and air temperature at each semi-continuous culture stage. *

Semi-continuous	0	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
Water ℃	33.41± 2.71	33.53± 2.50	29.48± 3.16	29.41± 5.08	23.42± 3.63	23.1±3.66	20.71± 4.56	15.01± 5.50	14.31± 3.04	8.83± 6.18	5.85± 2.41
Air ℃	33.95±2.02	34.18± 2.03	30.45± 3.06	30.15± 4.65	24.58± 3.28	24.85± 3.24	24.87± 3.98	17.31± 5.64	16.12± 3.65	7.9±9.36	6.48± 6.16

*Data are represented as mean ± standard deviation of each semi-continuous cultivation.

During the 2nd SCC (Days 55-64), the average temperature decreased to 30.45 ± 3.06 ℃. Cell numbers, chlorophyll α content, and dry weight showed rapid growth, indicating around 30 ℃ was optimal for *N. oceanica* growth, regardless of initial

inoculation concentrations. From the 2nd to the 10th SCC, average air temperature gradually decreased. *N. oceanica* tolerated low temperatures, able to grow above 5 ℃ (Figures 1-A, 2-A, 2-B, 2-C, and 6). The most suitable water temperature was around 25 ℃. On the 114th day, the highest chlorophyll α content (3.02 mg/L) was observed in the 700-2 culture.

Table 2 Semi-continuous cultivation of *N. oceanica*: highest growth rate and biomass productivity under 700 L outdoor cultivation. *

Semi continuous	Growth Rate (μ_{max}: d^{-1})		Biomass productivity (mg/L/day)	
	700-1	700-2	700-1	700-2
0	1.86 ± 0.61 ^{e}	2.17 ± 1.12 ^{a}	2.65 ± 0.84 ^{e}	5.71 ± 1.72 ^{a}
1st	5.20 ± 5.2 ^{cde}	4.09 ± 0.21 ^{a}	8.69 ± 4.12 ^{de}	16.67 ± 0.52 ^{a}
2nd	10.35 ± 0.4 ^{ab}	3.41 ± 0.6 ^{a}	30.00 ± 2.63 ^{bc}	18.83 ± 2.98 ^{a}
3rd	6.62 ± 2.21 ^{bcd}	5.00 ± 3.16 ^{a}	27.11 ± 9.28 ^{bc}	22.35 ± 11.3 ^{a}
4th	8.31 ± 3.68 ^{bc}	4.36 ± 3.16 ^{a}	33.19 ± 13.77 ^{bc}	27.62 ± 21.33 ^{a}
5th	5.06 ± 1.14 ^{cde}	4.22 ± 1.84 ^{a}	25.95 ± 6.04 ^{bc}	35.79 ±17.27 ^{a}
6th	9.93 ± 3.01 ^{ab}	4.54 ± 3.47 ^{a}	38.32 ± 8.4 ^{ab}	26.9 ± 18.03 ^{a}
7th	4.61 ± 0.99 ^{cde}	4.92 ± 3.93 ^{a}	18.99 ± 5.12 ^{cd}	27.29 ± 20.16 ^{a}
8th	6.14 ± 0.45 ^{bcde}	3.50 ± 0.44 ^{a}	27.86 ± 1.69 ^{bc}	22.62 ± 3.22 ^{a}
9th	13.52 ± 2.96 ^{a}	1.59 ± 1.11 ^{a}	49.33 ± 2.07 ^{a}	13.33 ± 9.31 ^{a}
10th	3.24 ± 0.74 ^{de}	1.17 ± 0.63 ^{a}	9.33 ± 2.07 ^{de}	6.33 ± 3.39 ^{a}

*Data are represented as mean ± standard deviation of triplicates.

The letters **a**, **b**, **c**, **d**, **e**, etc. following the data indicate significant differences. Data with the same letter indicate no significant difference, while data with different letters indicate significant differences.

Combining the results of dry weight from Figure 2-D, the calculations for the GR and BP of each SCC stage were calculated (Table 2). Under low initial inoculum concentration conditions, higher GR were observed. Correlating these observations with weather data, indicating that the temperature range of 5-30 ℃ was conducive to the cultivation of *N.*

oceanica. The maximum dry weight value occurred on the 114th day, specifically at the end of the fifth SCC, and its dry weight value, when converted, was 10.5 g/L.

From Table 2, it was observed that when the algae concentration in the culture medium was higher, there was not much significant difference in the GR and BP at various SCC stages. This indicated that higher algal concentration provided better resistance to environmental seasonal changes. Furthermore, the highest BP was observed in the 9th SCC stages at 49.33 ± 2.07 mg/L/day, with temperature ranges concentrated between 14 to 25 ℃ during these periods. Therefore, *N. oceanica* can get a higher BP within this temperature range. These research findings provide valuable guidance for optimizing *N. oceanica* cultivation conditions and biomass productivity. Lipid accumulation remained low during the summer period (0-3rd of SCC) due to the hot and intense sunlight (Figure 1-D). However, as temperatures decreased in the fall, lipid accumulation increased. From the 5th SCC onwards, lipid accumulation continued to rise, showing an inverse correlation with decreasing temperatures. This suggests that outdoor low-temperature cultivation promoted lipid accumulation. The maximum lipid content, reaching 29 % of algal dry weight, occurred in the 10th SCC, consistent with previous research (Yuan et al., 2019).

The in-situ ORR results reflected photosynthesis efficiency and plant or algae responses to environmental changes. ORR in high-concentration inoculation group 700-2 surpassed low-concentration group. Figure 1-B's chlorophyll fluorescence efficiency, along with weather conditions in Figure 1-A, illustrated illumination's impact on ORR. Overcast or rainy weather decreased ORR, while intense illumination tended to increase it.

From Figure 3，pictures were taken on the last day of each SCC, showing clear cell

density and health status through color variations. On the 10th SCC (Figure 3), poor overall growth conditions were roughly observed, with instances of cell adhesion, particularly after low temperatures. The 700 L tank, positioned between two buildings, allowed effective cultivation even in areas with weaker sunlight. Transparency on three sides facilitated this. Additionally, the SCC method enabled continuous *N. oceanica* production.

3.2 150 L tank data

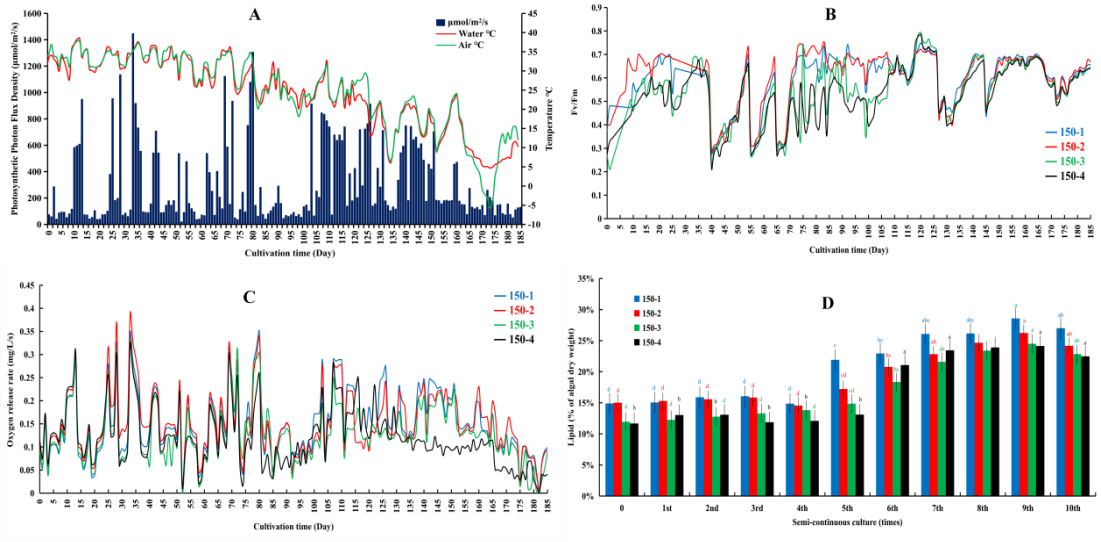

Figure 4 *N. oceanica* growth in 150 L tank under outdoor conditions. **A:** weather conditions. **B:** chlorophyll fluorescence efficiency (Fv/Fm: Fv is variable fluorescence, Fm is maximum fluorescence). **C:** Oxygen release rate (mg/L/s). **D:** Lipid content (% of algal dry weight). **a, b, c, d** are marked as significant differences in lipid content at each SCC run. The same letters indicate no significant differences, and different letters indicate significant differences. Data are reported as mean ± standard deviation of triplicates. Data are reported as mean ± standard deviation of triplicates.

From the weather results of the 700 L tank in Figure 1-A, it can be observed that there were differences compared to the weather results of the 150 L tank (Figure 4-A). The

distance between the positions of the 150 L tank and the 700 L tank was only 10 meters. However, the 700 L tank was situated in a location with more tree coverage and closer to a building, resulting in less sunlight exposure time compared to the 150 L tank. It can be seen that, before day 100, the light intensity measured at the location of the 700 L tank was higher than that at the location of the 150 L tank. This was because during the summer cultivation period, a sunshade canopy was installed above the 150 L tank to reduce excessive light and high temperatures.

The light intensity of 150 L PBRs higher than 700 L PBRs after 100 days, due to the sun's angle changing during fall and winter, providing longer sunlight exposure with less obstruction. As fall progressed and trees shed leaves, obstruction further decreased. Comparing weather conditions, Fv/Fm, and oxygen evolution rate results (Figures 4-A, 4-B, 4-C), it's evident that under similar conditions, the low inoculum concentration group exhibited lower oxygen evolution rates compared to the high inoculum concentration group (Day 100-185). Additionally, the 150 L PBRs with a sunshade canopy showed higher ORR during summer sunlight (Day 34), suggesting that strong light inhibits algal cells and shading helps decrease the damage. Fv/Fm results (Figure 4-B) indicate that chlorophyll fluorescence efficiency in the small 150 L tank was inhibited after each nutrient renewal. However, this impact was less pronounced in 700 L PBRs (Figure 1-B). Despite an additional cycle on Day 25 due to its smaller volume, SCC in the 150 L tank with a sunshade net facilitated good growth even under strong light exposure (Figure 5-A).

Furthermore, when temperatures dropped, the rain shelter provided insulation, allowing *N. oceanica* to continue growing well even in cooler conditions. The highest OD

$_{680}$ value, 2.1662, was recorded on the 19th day in the 150-2 group. Cell count results (Figure 5-B) showed that during summer, the shading canopy promoted rapid cell growth. However, as fall brought decreased sunlight intensity, the rain shelter began to inhibit cell growth. Interestingly, as leaves fell, improving light conditions, cell growth increased. Although the rain shelter provided insulation, the quick temperature decreased significantly impacted cell numbers. The maximum cell numbers, 6.03×10^8 cells, was recorded on Day 144 in the 150-1 group. Chlorophyll α accumulation remained stable in the first 100 days due to shading canopies and rain shelters. However, chlorophyll α accumulation increased after temperature decreased (Day 130-150). Low temperatures during initial inoculation could inhibit chlorophyll α accumulation (Day 160 to 185). The highest chlorophyll α content, 4.00 mg/L, was recorded on Day 159 in the 150-1 group.

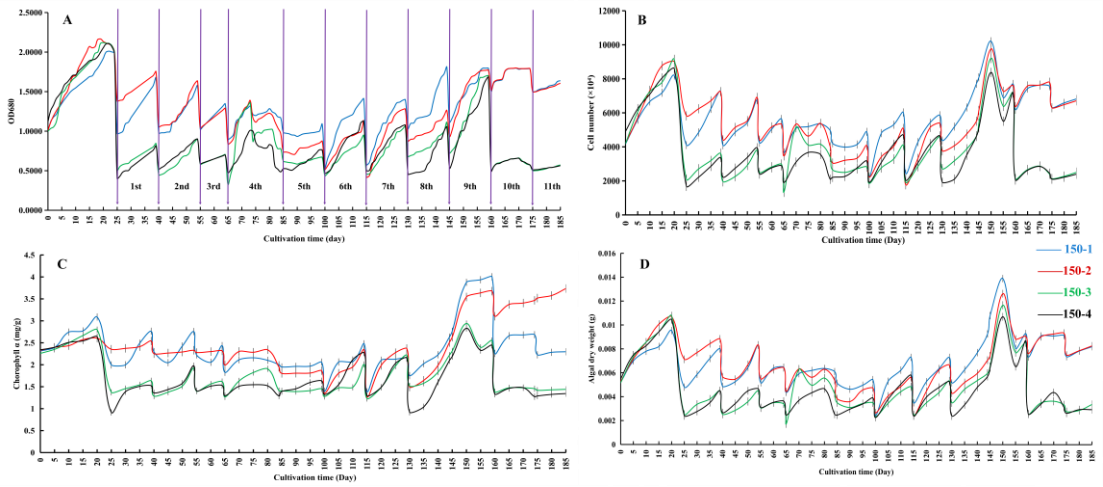

Figure 5 Bioproducts of *N. oceanica* growth under outdoor conditions in 150 L photobioreactor. **A:** Optical density OD $_{680}$. **B:** Cell numbers. **C:** Chlorophyll α. **D:** Algal dry weights. Data are reported as mean ± standard deviation of triplicates.

The highest GR (Table 3) was observed on the 4th SCC at 21.85 ± 2.61 d^{-1}. In 150 L PBRs, it was showed that when the algae concentration was high, the difference of GR in

each period was not significant, indicating better environmental tolerance of high-concentration algae. Similar trends were observed in the BP results (Table 4), in the 150 L PBRs, the highest BP (104.00 ± 24.59 mg/L/day) was recorded on the 9th SCC.

Table 3 Semi-continuous cultivation of *N. oceanica*: highest growth rate under 150 L outdoor cultivation.*

Semi continuous	Growth Rate (d^{-1})			
	150-1	150-2	150-3	150-4
0	3.02 ± 0.7 ^{a}	4.05 ± 1.19 ^{bcd}	3.70 ± 0.89 ^{cd}	3.61 ± 0.77 ^{ab}
1st	7.01 ± 2.99 ^{a}	1.55 ± 0.36 ^{d}	4.92 ± 3.24 ^{cd}	6.41 ±3.01 ^{ab}
2nd	4.03 ± 2.68 ^{a}	4.42 ± 1.46 ^{bcd}	7.05 ± 3.67 ^{bcd}	7.19 ± 3.18 ^{a}
3rd	7.13 ± 4.13 ^{a}	2.33 ± 0.27 ^{bcd}	2.82 ± 1.00 ^{d}	3.01 ± 1.47 ^{ab}
4th	5.79 ± 2.75 ^{a}	7.28 ± 1.49 ^{ab}	21.85 ± 2.61 ^{a}	5.63 ± 2.74 ^{ab}
5th	7.02 ± 4.16 ^{a}	6.8 ± 5.39 ^{abcd}	2.15 ± 0.5 ^{d}	3.97 ± 1.39 ^{ab}
6th	8.85 ± 3.44 ^{a}	7.05 ± 4.05 ^{abc}	6.53 ± 1.75 ^{bcd}	7.36 ± 3.04 ^{a}
7th	5.43 ± 2.47 ^{a}	7.26 ± 0.68 ^{ab}	5.47 ± 0.41 ^{bcd}	6.37 ± 4.37 ^{ab}
8th	4.18 ± 0.31 ^{a}	3.55 ± 0.63 ^{bcd}	3.85 ± 1.08 ^{cd}	6.21 ± 0.57 ^{ab}
9th	4.97 ± 2.93 ^{a}	10.58 ± 2.22 ^{a}	11.59 ± 0.65 ^{b}	8.06 ± 3.91 ^{a}
10th	4.29 ± 4.65 ^{a}	4.73 ± 3.13 ^{bcd}	9.08 ± 5.31 ^{bc}	5.54 ± 2.63 ^{ab}
11th	3.3 ± 2.08 ^{a}	1.79 ± 0.91 ^{cd}	1.88 ± 4.93 ^{d}	1.32 ± 1.08 ^{b}

*Data are represented as mean ± standard deviation of triplicates.

The letters **a**, **b**, **c**, **d**, **e**, etc. following the data indicate significant differences. Data with the same letter indicate no significant difference, while data with different letters indicate significant differences.

Dry weight followed the same trend as cell count. In the 8th SCC, on day 150, *N. oceanica* reached its highest dry weight of 13.9 g/L in tank 150-1. The weather was clear, with water and air temperatures at 19.2 ℃ and 20.2 ℃, respectively. Comparatively, results from Table 2 indicate that even with shading canopy, the 150 L tank exhibited good growth rates during the hot summer months with intense illumination. In the 150 L-1 group, the maximum GR occurred during the 6th SCC and was 8.85 ± 3.44 per day (d^{-1}). In the 150-2

group, the maximum GR was 10.58 ± 2.22 d^{-1}, also appearing during the 9th SCC. The 150-3 group reached its maximum GR of 21.85 ± 2.61 d^{-1} during the 9th SCC. Similarly, in the 150-4, the maximum GR occurred during the 9th SCC and was 8.06 ± 3.91 d^{-1}.

The BP in the 150 L tank was higher than 700 L tank, especially during the summer when the shading canopy was used. This effective increase in BP extended the cultivable period for *N. oceanica*. However, in both the 700 L and 150 L tanks, SCC conducted at temperatures below 5 ℃ inhibited growth, resulting in stagnation or decline. The 9th SCC in the 150-1 group showed the highest BP at 62.00 ± 37.22 mg/L/day, while in 150-2, it was 104.00 ± 24.59 mg/L/day. Similarly, both 150-3 and 150-4 reached their peak BP values during the 9th SCC, recording 102.67 ± 9.18 mg/L/day and 68.1 ± 34.12 mg/L/day, respectively.

Table 4 Semi-continuous cultivation of *N. oceanica*: highest biomass productivity (BP) under 150 L outdoor cultivation.*

Semi continuous	Biomass productivity (mg/L/day)			
	150-1	150-2	150-3	150-4
0	21.67 ± 5.47 ^{a}	30.33 ± 6.09 ^{bc}	28.00 ± 5.81 ^{c}	27.72 ± 4.33 ^{b}
1st	41.39 ± 12.87 ^{a}	12.14 ± 2.21 ^{bc}	14.05 ± 4.71 ^{c}	21.85 ± 10.61 ^{b}
2nd	24.52 ± 14.57 ^{a}	24.13 ± 10.04 ^{bc}	22.12 ± 12.28 ^{c}	26.19 ± 10.57 ^{b}
3rd	41.93 ± 22.51 ^{a}	14.15 ± 3.14 ^{bc}	8.89 ± 1.72 ^{c}	10.44 ± 4.86 ^{b}
4th	32.22 ± 15.25 ^{a}	38.00 ± 3.58 ^{b}	76.33 ± 19.64 ^{b}	17.00 ± 3.9 ^{b}
5th	33.46 ± 19.1 ^{a}	27.74 ± 21.24 ^{bc}	7.11 ± 1.21 ^{c}	11.81 ± 2.56 ^{b}
6th	34.76 ± 4.62 ^{a}	23.9 ± 5.72 ^{bc}	20.95 ± 2.75 ^{c}	25.24 ± 3.74 ^{b}
7th	27.14 ± 11.3 ^{a}	30.24 ± 0.37 ^{bc}	20.24 ± 2.42 ^{c}	21.48 ± 12.41 ^{b}
8th	29.29 ± 1.92 ^{a}	19.76 ± 3.69 ^{bc}	17.62 ± 4.25 ^{c}	23.57 ± 1.11 ^{b}
9th	62.00 ± 37.22 ^{a}	104.00 ± 24.59 ^{a}	102.67 ± 9.18 ^{a}	68.1 ± 34.12 ^{a}
10th	34.59 ± 36.83 ^{a}	39.33 ± 23.28 ^{b}	29.67 ± 18.81 ^{c}	18.00 ± 8.2 ^{b}
11th	25.00 ± 14.94 ^{a}	7.67 ± 10.05 ^{c}	6.00 ± 15.28 ^{c}	3.00 ± 1.55 ^{b}

*Data are represented as mean ± standard deviation of triplicates.

The letters **a**, **b**, **c**, **d**, **e**, etc. following the data indicate significant differences. Data

with the same letter indicate no significant difference, while data with different letters indicate significant differences.

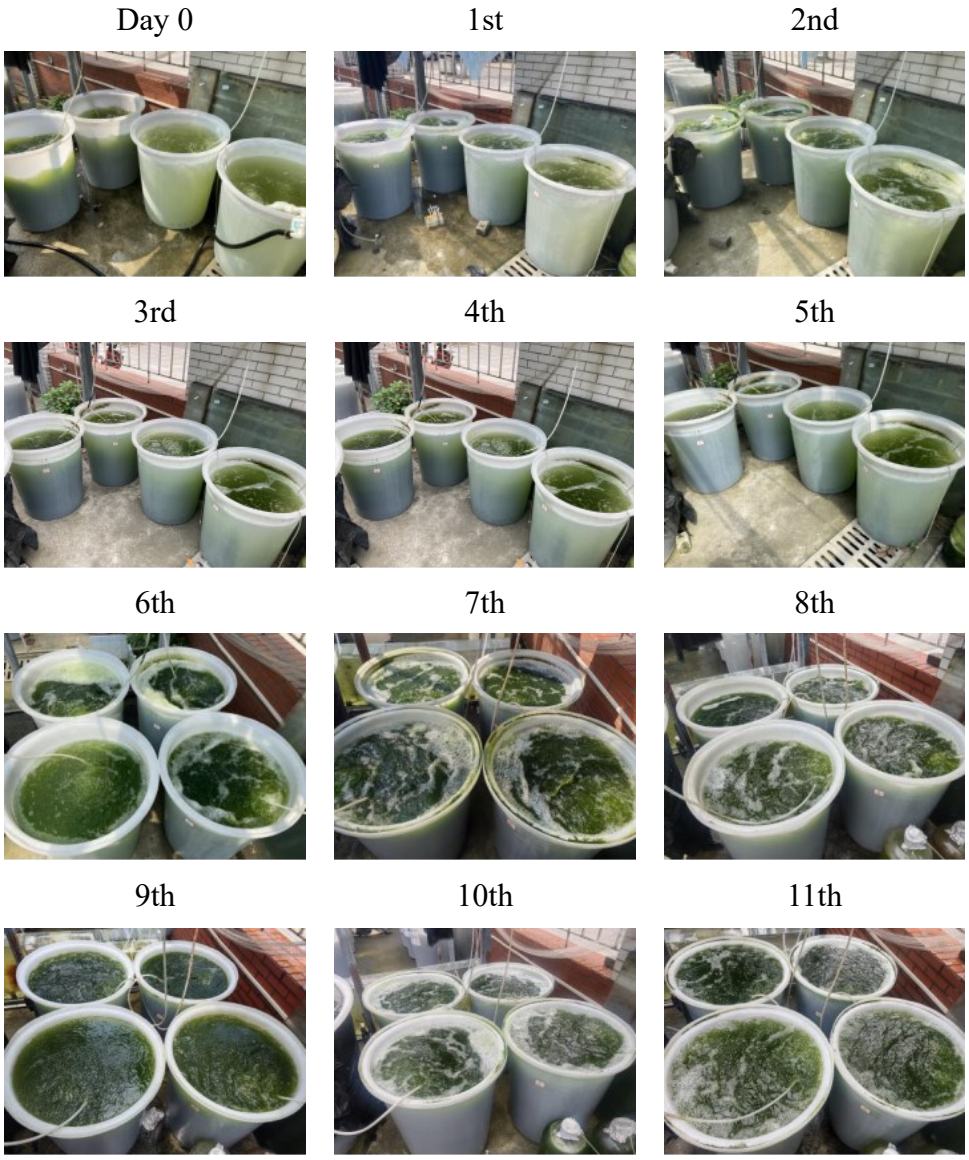

Figure 6 Picture of the growth of *Nannochloropsis oceanica* with different initial OD_{680} levels in a 150 L tank under outdoor conditions.

Lipid accumulation under 150 L PBRs was illustrated in Figure 4-D. The lipid accumulation was lower in the summer when temperatures were higher. However, as entered the autumn and winter seasons, with decreasing temperatures, the lipid accumulation gradually increased. This trend was similar to the results in the 700 L PBRs,

indicating that lower temperatures were more conducive to inducing lipid accumulation in *N. oceanica*. In the 150 L PBRs, the maximum accumulation of lipids occurred during the 10th SCC, accounting for 28 % of algal dry weight.

In Figure 6, the final states of each SCC on the last day were shown. The 150 L bucket, with only its opening exposed to sunlight, demonstrated minimal impact on growth as long as the stirring rate was maintained. However, the drawback of an open design was that rainwater was prone to entering the tank. To prevent rainwater ingress, a rain shelter could be added. Yet, small volume cultivation PBRs could not withstand prolonged exposure to rain, necessitating the addition of a rain shelter or transparent cover.

The hydro costs during cultivation can be calculated, with electricity costing $ 1.83/day in the experimental area, totaling US dollar ($) 338.55 over 185 days. A single 550-watt WL-550 high-pressure scroll air pump can provide air mixing for either 12 of 700 L PBRs or 56 of 150 L PBRs, with electricity costs of $ 28.21 and $ 6.05 for each, respectively. Water was priced at $ 0.54 per ton, with consumption at 425 L ($ 0.23) for each 150 L PBR and 1700 L ($ 0.92) for each 700 L PBR. According to Table 2 and 4, the average algal yield was 371.16 g for 1700 L (700 L PBRs) and 527.78 g for 425 L (150 L PBRs). The costs for cultivating *N. oceanica* in 700 L and 150 L PBRs were 12.74 g/$ and 84.04 g/$, respectively. Based on maximum BP, the cost of 700 L and 150 L PBRs was 20.27 g and 298.09 g/$, respectively. If calculated at average BP as the lowest cost, the price would be $ 11.89 per kilogram (kg); if calculated at maximum BP as the lowest cost, the price would be $ 3.35/kg. Comparing with the current price of fish feeds such as Black Soldier Fly Larvae at $5.43/kg (Maroušek & Strunecký et al., 2023), *N. oceanica* remains

competitively priced. However, careful cultivation timing is crucial. Currently, *N. oceanica* is being sold on the Alibaba website for $ 20.19 to 35.34/kg, whereas the basic price obtained in this experiment was $ 11.89/kg, indicating its high competitiveness.

From the results, it can be seen that both yield and electricity costs were the main factors influencing prices. Electricity was primarily used to power high-pressure scroll air pumps to provide agitation for the algae. Thus, two issues should be addressed in the future: 1. Pay attention to the climate during cultivation to increase yield. 2. Developing a method for agitation without electricity could significantly lower cultivation costs in the future.

Conclusion

The *N. oceanica* cultivable temperature range was 5-30 ℃, the optimal cultivation temperature range was 14-25 ℃. It showed cold tolerance and ability to accumulate more lipid at low temperatures. The novel in-situ ORR measurement method can further monitor real-time growth conditions. The 150 L tank demonstrated higher GR (11.60 ± 0.007 d^{-1}) and BP (104.00 ± 0.04 mg/L/day). But new designed 700 L tank was not susceptible to environmental influences. The algae price was US $ 11.89 /kg, which was cheaper than the current market price. The consumption of electricity increases the cost of cultivating microalgae. Using smaller PBRs at the same hydro price is more cost-effective.

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Chapter 5. Conclusion and future studies

1 Conclusion

This experiment focused on cultivating two marine microalgae, *Chrysotila (Pleurochrysis) dentata* and *Nannochloropsis oceanica*, to advance algae research in addressing environmental, climate, food, and energy challenges.

Chrysotila (Pleurochrysis) dentata, known for its ability to fix carbon dioxide and high lipid content of 33.61%, holds promise (Marsh, 1999; Marsh, 2003; Chen et al., 2019). Optimal indoor cultivation conditions include an 18L/6D photoperiod, pH 8.5, and 2.5% salt concentration. A positive correlation between coccolith thickness and light intensity was also discovered, suggesting a novel approach for carbon sequestration.

To address pollution concerns in algae cultivation, we developed an Algal-Bacterial Symbiotic System (ABSS) with *C. dentata* and *Nitratireductor aquibiodomus*. Results showed varying algae growth with different algal-bacterial ratios, informing open cultivation monitoring. An optimal ratio of 8:2 was found, and co-cultivation with Nannochloropsis oceanica benefited *C. dentata*' s growth.

Incorporating PET microplastics into the symbiotic system, the impact on microbial growth was investigated. Optimal microplastic levels (5-15 mg/L) facilitated algae growth, but higher concentrations hindered growth by blocking light. Notably, algae attached to microplastics entered the food chain when consumed by predators.

For *Nannochloropsis oceanica*, small-scale indoor and large-scale outdoor cultivation experiments were conducted at 30 °N latitude. The findings from chlorophyll fluorescence analysis suggest using 0.5 g/L urea as the nitrogen source in commercial cultivation,

avoiding ammonium chloride due to its adverse impact on photosynthesis. Maintaining salt concentration between 2.5 % and 3.5 % is crucial due to natural evaporation and rainfall.

The designed 700 L tank, featuring three transparent sides, facilitated favorable cultivation of *Nannochloropsis oceanica*, even in partially shaded areas. Outdoor experiments, conducted across summer, autumn, and winter, explored the cultivability of *N. oceanica* in temperatures ranging from 5-30 ℃, with the optimal range identified as 14-25 ℃. The study introduced real-time monitoring techniques, including chlorophyll fluorescence analysis and a novel in situ oxygen release rate measurement method. Results demonstrated that the new 700 L tank design enabled efficient algae production under various light conditions. Notably, *N. oceanica* showed cold tolerance, with maximum lipid contents of 29.22 % and 28.57 % observed in the 700 L and 150 L setups, respectively.

The 150 L LLDPE plastic bucket offered advantages of lower initial costs, higher growth rates, and biomass productivity. However, its susceptibility to environmental influences, particularly in smaller water volumes, led to significant fluctuations in chlorophyll fluorescence during semi-continuous cultivation. The larger 700 L tank showed greater resilience to climatic fluctuations but had drawbacks due to its larger size, making transportation inconvenient and maintenance costs relatively higher. In summary, shading measures during intense light and hot summer days, along with insulation below 5 ℃, are recommended to enhance year-round outdoor cultivability, especially for smaller setups.

This experiment, studying *Chrysotila (Pleurochrysis) dentata* and *Nannochloropsis oceanica*, offers insights into algae production and applications in inland and indoor settings. It introduces a novel microalgae cultivation system and a new real-time oxygen release rate

monitoring method, advancing algae cultivation and commercial production.

2 Future studies

Chrysotila (Pleurochrysis) dentata has significant potential for carbon sequestration due to its ability to synthesize calcium carbonate (Moheimani, 2005; Nimer & Merrett, 1993; Sikes et al., 1980). However, a fascinating research direction is how to stimulate continuous growth and shedding of *Chrysotila (Pleurochrysis) dentata*' s shell after it has fully formed, ensuring sustained carbon sequestration. This can be achieved through external interventions to induce the shedding of the calcium carbonate shell or through genetic engineering by modifying key synthetic codons in the V unit and R unit to alter its structure (Chen et al., 2019). From our current research, the addition of phenol to the culture medium can induce calcium carbonate precipitation in the culture medium of *Chrysotila (Pleurochrysis) dentata* (Figure 1), but the specific mechanism remains unclear and requires further investigation.

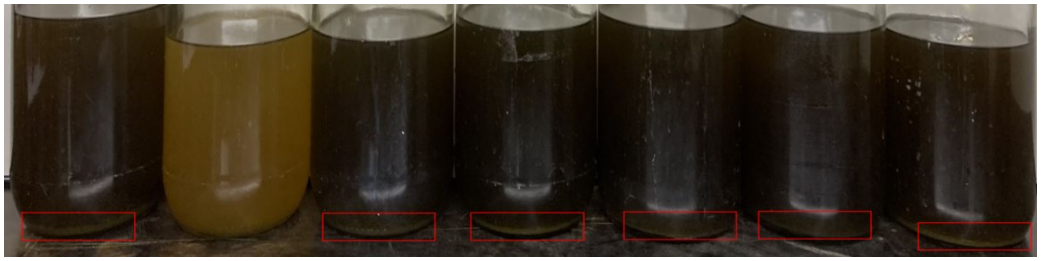

Figure 1 Addition of phenol to the culture medium of *Chrysotila (Pleurochrysis) dentata* results in the precipitation of calcium carbonate at the bottom (highlighted in the red box). Additionally, the overall color of the algae is darker compared to the culture without phenol.

But phenol is toxic. In the future, we hope to find an environmentally friendly reagent that can facilitate coccolith shedding without harming *C. dentata*, thereby enabling more carbon fixation.

At the current stage, outdoor cultivation of microalgae still faces numerous challenges (Xu et al., 2009; Yu et al., 2022; Brennan et al., 2010). The uncertainty of climate significantly affects algae yield, and many locations and regions are not suitable for traditional algae cultivation systems (Novoveská et al., 2023; Khan et al., 2018; Qari et al., 2017). The development of low-cost, light-permeable, and environmentally resilient cultivation devices holds practical significance (Kose et al., 2017). In the future, we aim to design a cultivation system that provides adequate shading and insulation. Through comparative studies on the tolerance of different volumes of cultivation containers to environmental changes during outdoor semi-continuous cultivation, we hope to identify the optimal cultivation volume—one that is less affected by climate changes and exhibits higher biomass production.

Since low temperatures can induce lipid accumulation in *Nannochloropsis oceanica*, but at the same time reduce the growth rate of algae, there is a need to find the optimal low temperature that induces lipid accumulation while maintaining a suitable growth rate. Then developing a new year-round algae production model, with spring and summer seasons dedicated to biomass production and autumn and winter seasons focused on lipid production.

Furthermore, during our experimental process, we observed the need for optimization of microalgae harvesting methods. Finding a rapid, efficient, and cost-effective way to harvest algae is a significant challenge that requires resolution.

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