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Engineering whiting events in culture: A microalgae-driven calcium carbonate and biomass production process at high pH and alkalinity with the marine microalga *Nannochloropsis oceanica* IMET1

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ABSTRACT

The impact of whiting events on global inorganic carbon cycle and climate change is debatable at the current ocean pH. This work engineered whiting events in a high pH, high alkalinity microalgal culture for carbon dioxide capture and storage. When growing the marine microalga *Nannochloropsis oceanica* IMET1 in photobioreactors, culture alkalinity more than doubled, increasing from 72.5 mg L^{-1} to a maximum level of 159.6 mg L^{-1}. At the same time, culture pH increased from 7.9 to 10.1 with concomitant calcium carbonate production. X-ray diffraction analysis revealed the precipitated calcium carbonate was primarily monohydrocalcite. The lab culture was scaled up to a 340-L bioreactor, in which *Nannochloropsis* ash-free dry weight productivities ranged from 25.1 to 51.4 g m^{-2} d^{-1} and a maximum monohydrocalcite productivity of 133.4 g m^2 d^{-1} was recorded. In this system, *Nannochloropsis* biomass contained about 23.9 % lipids and the eicosapentaenoic acid content was about 1.8 %. Together, these results suggest the microalgae-driven calcium carbonate and biomass production process efficiently captures and stores atmospheric carbon dioxide in the form of calcium carbonate while producing valuable bioproducts. Study of the bacterial communities associated with the *Nannochloropsis* culture identified four dominant species *Maricaulis maris*, *Marinisubtilis pacificus*, *Gracilimonas* sp., and an uncultured bacterium in the OD1 phylum with interesting features that warrant further investigation.

1. Introduction

Clouds of fine grained calcium carbonate ($CaCO_3$) precipitated in marine or freshwater environment during or after phytoplankton blooms have long been observed in nature and described as whiting events [1,2]. In some places, whiting events occur frequently at a large scale covering the great lakes, e.g., Lake Michigan (58,000 km^2) or smaller regions in oceans, e.g., the Gulf Coast of Florida and Bahama Banks [3–6]. Whiting events also can occur repeatedly over time: whiting events were visible on MODIS on NASA's Terra satellite images for 13.8 % of days near the Gulf Coast of Florida over a 13-year time span [3,4]. However, the impact of whiting events on global inorganic carbon cycle and climate change is still debatable, because whitings may be a source or a sink for inorganic carbon and organic carbon in oceans and lakes depending on how they are formed and deposited [7,8].

Globally, it is suggested that phytoplankton, including microalgae and cyanobacteria, can sequester 1100 Tmol carbon (C) per year, while calcification algae (i.e., $CaCO_3$ forming) sequester up to 47 Tmol C per year [9]. In the ocean, $CaCO_3$ precipitation is often initiated by calcifying organisms in a process known as the carbonate pump (also known as carbonate counter-pump) through Reaction (1): $Ca^{2+} + 2HCO_3^- \rightleftharpoons CO_2 + CaCO_3 + H_2O$ [10]. At the current ocean pH (~8.1) bicarbonate is the dominant carbonate species and calcification processes lead to formation of $CaCO_3$ from Reaction (1), during which about 0.6 CO_2 is released to the surrounding water per $CaCO_3$ produced [7,9]. As a result, $CaCO_3$ formation through this reaction in the ocean has been suggested to have no effect on capturing atmospheric CO_2 on short geological time scales (<100 years) [7,11].

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In contrast, emerging microalgal technologies that harness the power of photosynthesis have the potential to increase culture pH and alkalinity, thereby fostering carbon capture and utilization (CCU) through biological means [12]. *Nannochloropsis* spp. has attracted much industrial and academic interest because of their ability to grow rapidly, synthesize large amounts of lipids including high-value omega-3 fatty acids such as eicosapentaenoic acid (EPA), and tolerate changing environmental conditions [13,14]. Maintaining axenic culture of algae has been a major challenge for the algal biotechnology industry, particularly for high-value nutraceutical and pharmaceutical markets [15]. However, for algal carbon sequestration and biofuel production applications, it is not economically viable nor necessary to grow axenic algae cultures, because other microbes such as the bacterial community associated with microalgae may contribute to culture stability and algal growth stimulation [16,17]. Moreover, previous work on the microbially induced calcium carbonate precipitation (MICP) process has identified bacterial species that contribute to $CaCO_3$ precipitation [18,19]. Prior work focused on bacterial processes directed by urea hydrolysis, denitrification, or sulfate reduction [20,21], while the role of bacteria in microalgae-driven carbonate precipitation process has not yet been investigated in depth.

In this work, our engineering principle is inspired by whiting events. Through an ecosystem-inspired biotechnology approach [22], we aim to engineer a microalgae-driven calcium carbonate and biomass production (MadCAP) process for carbon capture, usage and storage. We chose to grow *N. oceanica* strain IMET1 in bioreactors nonaxenically, a well-characterized strain for which a genome sequence and genetic manipulation tools are available [23–26]. We developed the MadCAP process with *N. oceanica* in the lab scale (1 L) and pilot scale bioreactors (340 L). The MadCAP process resulted in a large increase in culture pH and alkalinity, leading to simultaneous production of $CaCO_3$ and algal biomass. Further analysis revealed precipitated $CaCO_3$ was in the form of monohydrocalcite. Substantial amounts of monohydrocalcite and omega-3 fatty acids-rich algal biomass could be produced if properly scaled up. The bacteria closely associated with *N. oceanica* IMET1 and the free-living bacterial communities were studied, with the novel OD1 strain identified with some interesting features that need further study.

2. Materials and methods

2.1. Algae culturing and measurements in 1-L cultures

For lab scale 1-L culture experiments, *N. oceanica* IMET1 was maintained and cultured in modified f/2 medium with a final Ca^{2+} level of 4.1×10^{-3} M and EDTA level of 1.17×10^{-5} M [27]. The saltwater medium was adjusted to 17 ppt and the final concentration of $NaNO_3$ and $NaH_2PO_4 \bullet H_2O$ was adjusted to 0.9 g L^{-1} and 40 mg L^{-1}, respectively. The seed culture was maintained at room temperature under continuous light illumination of 10 - 20 μE m^{-2} s^{-1} in Erlenmeyer flasks. Optical density, cell density, dry weight and pH were measured to assess algal growth. Optical density was measured by using a Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA) at 750 nm (OD_{750}). Cell density was measured using a hemocytometer (Hausser Scientific). For algal biomass dry weight measurements, algal biomass was collected on a GF/F glass fiber filter with 45 mm diameter (pore size 0.7 μm, Whatman) under vacuum, washed with distilled water, dried at 100 °C for overnight and weighed on an analytical balance.

For the first two laboratory-scale studies, a 1-liter closed recirculating algal culture system was carried out in a horizontal culture chamber under continuous light illumination of 50 μE m^{-2} s^{-1} at room temperature with aeration by air sparging through air stones (an aeration rate of 0.15 vvm). Algae were grown in the algae side of the horizontal culture chamber separated by a Whatman Nuclepore Track-Etch polycarbonate membrane with 1 μm pore size. Different lines were set up with appropriate ports for media, algae sampling, filtrate sampling, and air venting to prevent contaminations of the clear side by the algae side (Fig. S1). After Day 13, the system was maintained in semi-continuous culture by sampling about 60 ml culture and replenishing with equal amounts of fresh f/2 medium every two days to maintain culture pH at about 10. Algae culture samples were collected from the algae side, and algal fluid samples were collected from the fluid (clear) side of the chamber. Measurement of water chemistry indexes (such as pH, salinity, calcium and total alkalinity) of algal fluid were performed by ZooQuatic Laboratory, LLC. To collect precipitate, at the end of the experiment 280 ml algal fluid was passed through a 3 μm polycarbonate filter, then an 1 μm polycarbonate filter in order to comparatively analyze the quantity and quality of the precipitate on each filter.

In the third lab-scale study, the culture was maintained in a 1-liter Pyrex bottle under continuous light illumination of 50 μE m^{-2} s^{-1} at room temperature with aeration by air sparging through air stones (an aeration rate of 0.15 vvm). This study was conducted to determine whether addition of 1.17×10^{-5} M EDTA in the f/2 medium was essential for growth and for maintaining high pH in the *Nannochloropsis* cultures. Because EDTA in the culture medium is known to chelate with metal ions (such as Fe^{3+} and Ca^{2+}), its presence may affect calcium carbonate formation. The f/2 media was prepared with 1.17×10^{-5} M EDTA and with no EDTA (0 M).

In the fourth lab-scale study, sodium bicarbonate was added to the cultures to determine if it would enhance the MadCAP process. The cultures were maintained in 1-Liter Pyrex bottles under the same conditions as the third study. Various concentrations of sodium bicarbonate solution were added at the beginning of the experiment when the cultures were inoculated (0 M, 0.02, 0.05, and 0.1 M sodium bicarbonate).

2.2. Bacterial communities associated with N. oceanica strain IMET1

For characterization of associated bacteria, *N. oceanica* strain IMET1 was grown with ambient air in the laboratory with either no addition of $NaHCO_3$ or 0.02 M $NaHCO_3$. Microalgal cultures and associated bacteria (20 ml samples) were vacuum-filtered through a 0.45 μm cellulose acetate filter (Advantec) with a Nalgene Reusable Filter Unit (Thermo Scientific), and the filtrate was collected. DNA from both size fractions was extracted using the DNeasy Powerwater kit (Qiagen) and sent for sequencing. DNA was sequenced on an Illumina paired-end platform (MiSeq 2 ×300) at the BioAnalytical Services Laboratory (BASLab) at IMET. Bioinformatic analysis was performed with QIIME 2 2023.5 [28]. Raw forward and reverse sequence data were demultiplexed, quality filtered, and merged using the q2-demux plugin followed by denoising with DADA2 [29]. Taxonomy was assigned to amplicon sequence variants (ASVs) using the q2-feature-classifier [30] with the classify-sklearn naïve Bayes taxonomy classifier trained from the newest Silva database (version 132 – full length). Sequences were uploaded to the NCBI blastn program and identities were confirmed using a percent identity alignment of 99 % or higher.

2.3. Algae culturing and measurements in the 340-L bioreactor

For the two scaled-up studies, a custom-built 340 L-photobioreactor system (Fig. S2) was used for the experiment. LED lights housed in the center of the bioreactor provided continuous illumination for the culture. The light channel and intensity of the LED lights was controlled by a custom program that also was used to fill and empty the bioreactor. Aeration was provided with ambient air through an air pump, and the flow rate (an aeration rate of about 0.02 vvm) was controlled by air valves and monitored by an air flow meter. For this system, the f/2 media (with 34 ppt salinity and 400 mg L^{-1} Ca^{2+}) was made with artificial seawater supplied by Aquaculture Research Center at UMCES IMET. Cell density, optical density, pH and dry weight were measured to monitor the growth; aeration and culture level in the bioreactor were recorded to monitor the operation. The area productivity was calculated using an bioreactor area ratio of 1:1.2 to represent the bioreactor "facility" square footprint [31]. Because of the size and non-axenic nature

of the bioreactor experiments, there were no replicates for each experiment. Instead, these experiments were repeated independently in an effort to determine similar trends and observations were found.

2.4. Collection and analysis for calcium carbonate precipitates

Algal fluid was collected from the 1 L-growth chamber as described above. For cultures in the 340 L-bioreactor, all solid matter in a culture suspension was first collected by centrifugation at 2000x *g* for 10 min. The pellets were then resuspended in distilled water. To separate $CaCO_3$ precipitate crystallines from microalgal biomass in the suspension, low force centrifugation was employed owing to the weight density of the $CaCO_3$ particles being greater than *Nannochloropsis* cells. Controls are included as the medium with no algae. The separation was achieved by repeating low speed centrifugation (50×*g* - 230×*g*) and washing with distilled water until the supernatant became clear. Finally, the washed pellets were freeze-dried and weighted.

The precipitated carbonates were examined under dissection microscope. The composition of the crystalline precipitate was determined by X-ray diffraction (XRD) analysis. The proportion of carbonates in the precipitate was estimated using a custom-built 'Acid Test' chamber that measured the CO_2 (CO_2Meter K30 10,000 ppm CO_2 Sensor) evolved when 90 % phosphoric acid was mixed with a known mass of precipitate. The CO_2 evolved from the precipitate was compared to a standard curve developed for $CaCO_3$ ($CaCO_3$(mg) = 0.0114 *CO_2(ppm) + 0.1503, R^2 = 0.99) with proportional correction based on the molecular weight of $CaCO_3$ and the crystalline carbonate identified by XRD.

2.5. Lipid content measurement and fatty acid analysis

Total lipids were extracted from algal biomass from the 340-L bioreactor experiment using Biogogno's method [32]. In brief, 100 mg dry algal biomass was treated with 2 ml methanol/DMSO (9:1, v/v) for 1 h at 50 °C followed by another hour at 0 °C with stirring. The mixture was spun down, and the first batch of supernatant was collected. The

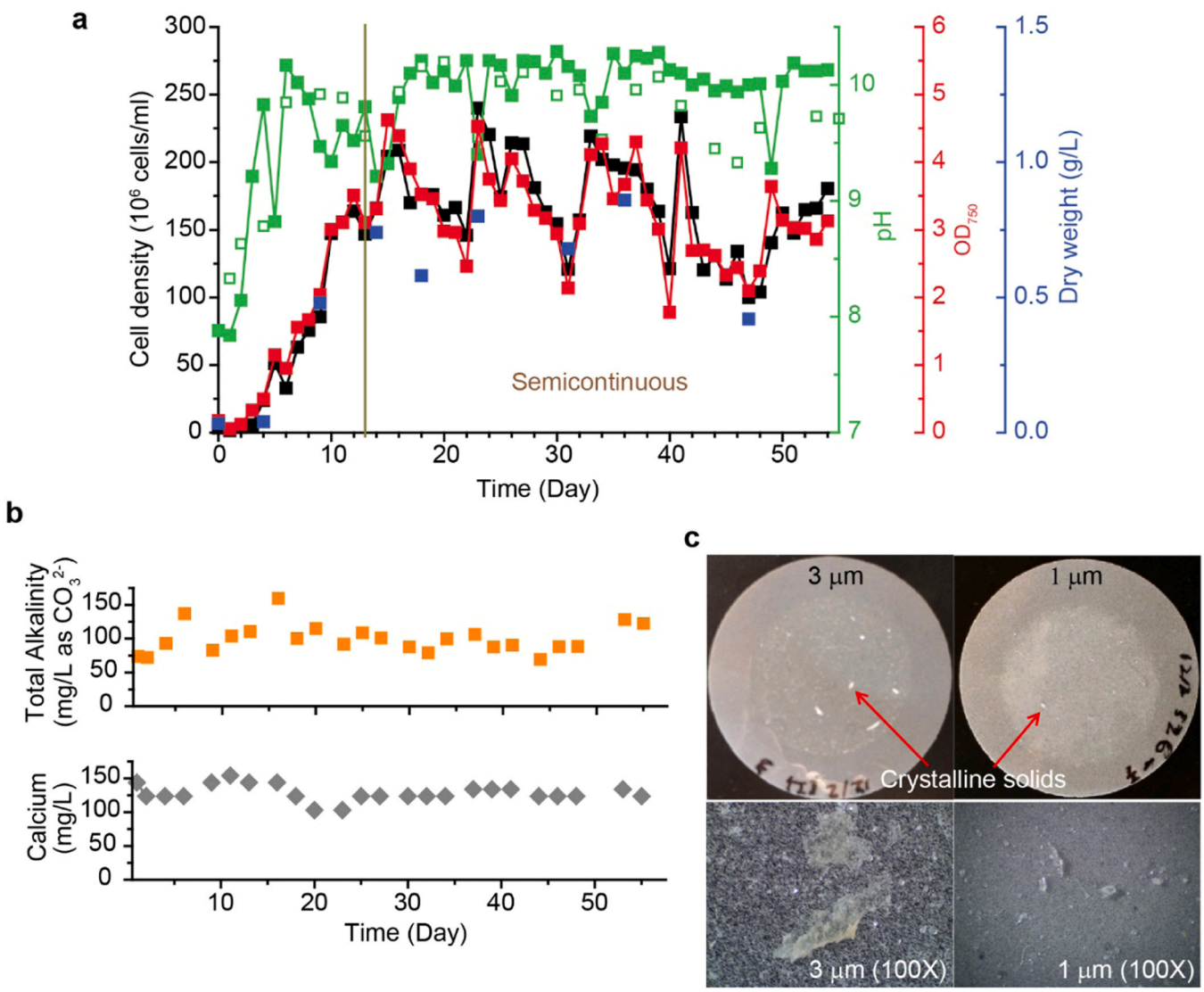

Fig. 1. Growth and culture medium chemistry of *Nannochloropsis oceanica* IMET1 grown in the first experiment in horizontal culture chambers (Fig. S1) with f/2 media and 17 ppt salinity. (a) Cell density (10^6 cells ml^{-2}, black), optical density (OD_{750}, red), biomass dry weight (g L^{-1}, blue), pH on the algae side (open green square) and pH on the clear side (solid green square) over time. The vertical bar at day 13 indicates the start of semicontinuous growth conditions. (b) Total alkalinity (mg L^{-1} as CO_3^{2-}), and calcium concentration (mg L^{-1}, gray) in the *Nannochloropsis oceanica* IMET1 culture fluid. Algae fluid samples were collected from the fluid side of the culture chamber for the measurements. (c) Precipitate collected on 3 µm and 1 µm polycarbonate filters. The lower panel shows the crystalline images on the 3 µm (left) and 1 µm (right) filters under a dissection microscope.

residual biomass was then treated with 4 ml hexane/diethyl ether (1:1, v/v) for 1 h at 0 °C with stirring, and the supernatant was collected and combined with the first batch of supernatant. The treatment was repeated until the residual biomass lost color. Water was added into the collected solvent mixture (the collected supernatant) to make a mixture of methanol/hexane/ether/water 1:1:1:1 for phase separation. The organic phase containing lipids was collected in a pre-weighed vial and evaporated under a gentle stream of N_2 gas. Total lipids content was calculated as the weight of the lipid extracts divided by the processed dry biomass. For fatty acid analysis, fatty acids in total lipids were converted to fatty acid methyl esters (FAMEs) with 1 % H_2SO_4 in methanol at 85 °C for 1.5 h, dissolved in hexane, and then profiled using TSQ Triple 8000 GC-MS System (Thermo Scientific). FAME standards (Sigma-Aldrich) and heptadecanoic acid (C17:0) (Sigma-Aldrich) were used as the external and internal standards, respectively.

3. Results

3.1. *Nannochloropsis* culture maintained high pH and transiently increased alkalinity

In a laboratory-scale microalgae driven calcium carbonate and biomass production (MadCAP) process, pH increased rapidly when algae grew to exponential stage in the first horizontal bioreactor experiment (Fig. S1). In a culture of *N. oceanica* IMET1 grown under 17 ppt salinity, pH increased from 7.9 to 10.2 while cell density increased 10-fold within 6 days after inoculation (Fig. 1a). After day 13, the culture was shifted to semicontinuous growth conditions. During this stage, the culture was able to maintain pH 10.0 ± 0.3 for 42 days with little fluctuation (Fig. 1a).

The salinity, total alkalinity and calcium concentration in the 1 L-*N. oceanica* IMET1 culture were monitored over time. The salinity increased slightly from 17.1 ppt to 18.9 ppt, while total alkalinity gradually increased by about 2.2-fold, i.e., from 72.5 to 159.6 mg L^{-1} on day 16, and then gradually decreased afterwards but kept in a range between 88 and 128 mg L^{-1} (Fig. 1b). Calcium concentration remained relatively stable during the first 16 days and maximum reduction of about 28.6 % was observed on day 20, indicating some calcium being either consumed by algae or precipitated.

The high pH algal fluid was tested for the presence of calcium carbonate precipitation. White and yellowish solids were visible on the 3 μm and 1 μm polycarbonate filters (Fig. 1c). Microscopic analysis revealed some crystalline solids on the filters (Fig. 1c, lower panel), indicating formation of carbonate precipitation. It was clear that the culture system needed to be scaled up to collect more precipitates for further analysis.

Similar results showed when *N. oceanica* IMET1 was grown under 34 ppt salinity in the second experiment in the horizontal culture chamber. In this culture, pH started at 7.8 and reached 10.2 within 4 days of batch culture. Afterwards *N. oceanica* IMET1 was cultured under semi-continuous conditions and the culture maintained at pH 10.1 ± 0.1 for 23 days (Fig. S3).

A third experiment was conducted in 1-L Pyrex bottles to determine how EDTA influenced pH and culture growth, and whether we can further increase culture pH by removing EDTA. Algae growth in the absence of EDTA was much slower (Fig. 2). The cell density and optical density of the IMET1 culture grown in the presence of EDTA were 4.4-fold and 5.7-fold higher, respectively, than that in the absence of EDTA (Fig. 2a). In this experiment, the culture grown in the presence of EDTA reach pH 10.0 within 3 days, and was able to maintain high pH (pH >10.5). When grown in the absence of EDTA, the culture pH increased slightly from 8.6 to 8.7 within 3 days and then remained stable, but was not able to increase beyond pH 8.8 throughout the entire growth course (Fig. 2b). These results suggest EDTA is necessary for maintaining *Nannochloropsis* growth and high culture pH, agreed with previous work that EDTA in algal culture medium maintains a constant

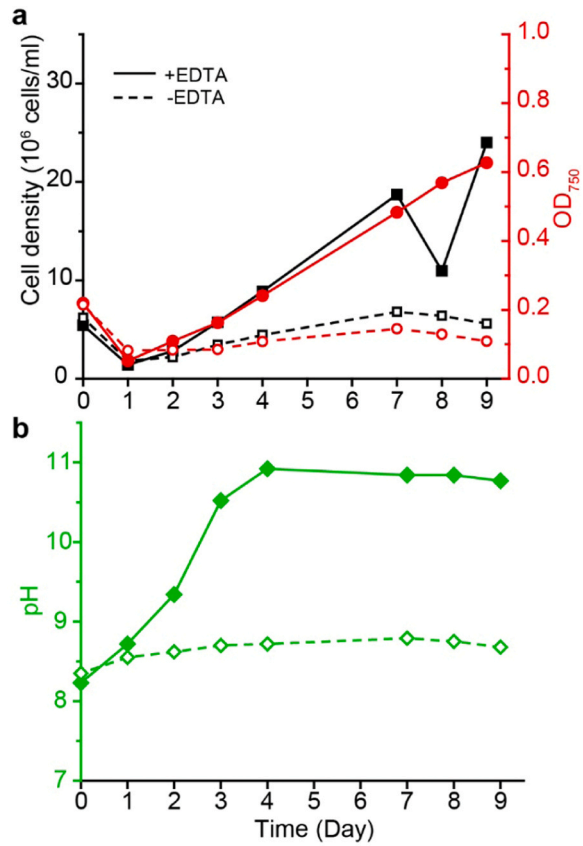

Fig. 2. The culture of *Nannochlorpsis oceanica* IMET1 grown in 1-L bottles with f/2 media in the presence of EDTA (solid line and solid symbols) or the absence of EDTA (dashed line and open symbols). Measurements of (a) cell density (black squares) and optical density (red circles) and (b) pH (green diamonds) were made over time.

and controlled supply of Fe [33], essential for sustained algal growth.

The fourth lab-bench experiment was designed to explore the effect exogenous bicarbonate on the MadCAP formation (Fig. 3). Compared with cultures supplemented with sodium bicarbonate, the culture without sodium bicarbonate (0 M) grew better. The cell density of the culture without sodium bicarbonate addition was over 2-fold higher than the cultures supplemented with 0.02 M and 0.1 M sodium bicarbonate after day 9 (Fig. 3b). Besides the culture density, the culture pH without sodium bicarbonate increased rapidly from 8.6 at day 2 to 9.9 at day 4 and remained stable at 10.6 at day 6 and beyond (Fig. 3c). However, in this experiment the calcium carbonate precipitate in the culture without sodium bicarbonate addition was too low to measure (Fig. 3e). The cultures supplemented with 0.02 M and 0.1 M sodium bicarbonate were able to reach and retain a culture pH above 10 after day 4, and the precipitate yield was elevated by the addition of sodium bicarbonate (Fig. 3e). XRD analysis showed that the precipitate crystalline was composed of calcium carbonate (100 % monohydrocalcite) from the culture supplemented with 0.02 M and 0.1 M sodium bicarbonate. Precipitates formed in the culture supplemented with 0.5 M sodium bicarbonate consist of largely magnesium carbonate (96.5 % nesquehonite) and small portion of calcium carbonate (3.5 % monohydrocalcite) (Fig. 4 and Fig. S4a, S4b and S4c). However, 0.5 M sodium bicarbonate supplementation completely inhibited algae growth while pH of the culture increased only slightly from 9.4 to 9.7 (Fig. 3a, b and d), suggesting this treatment imposed stress on *Nannochloropsis* culture.

3.2. Bacterial communities associated with cultures of *N. oceanica* IMET1

To analyze the bacterial communities of *N. oceanica*, two fractions of

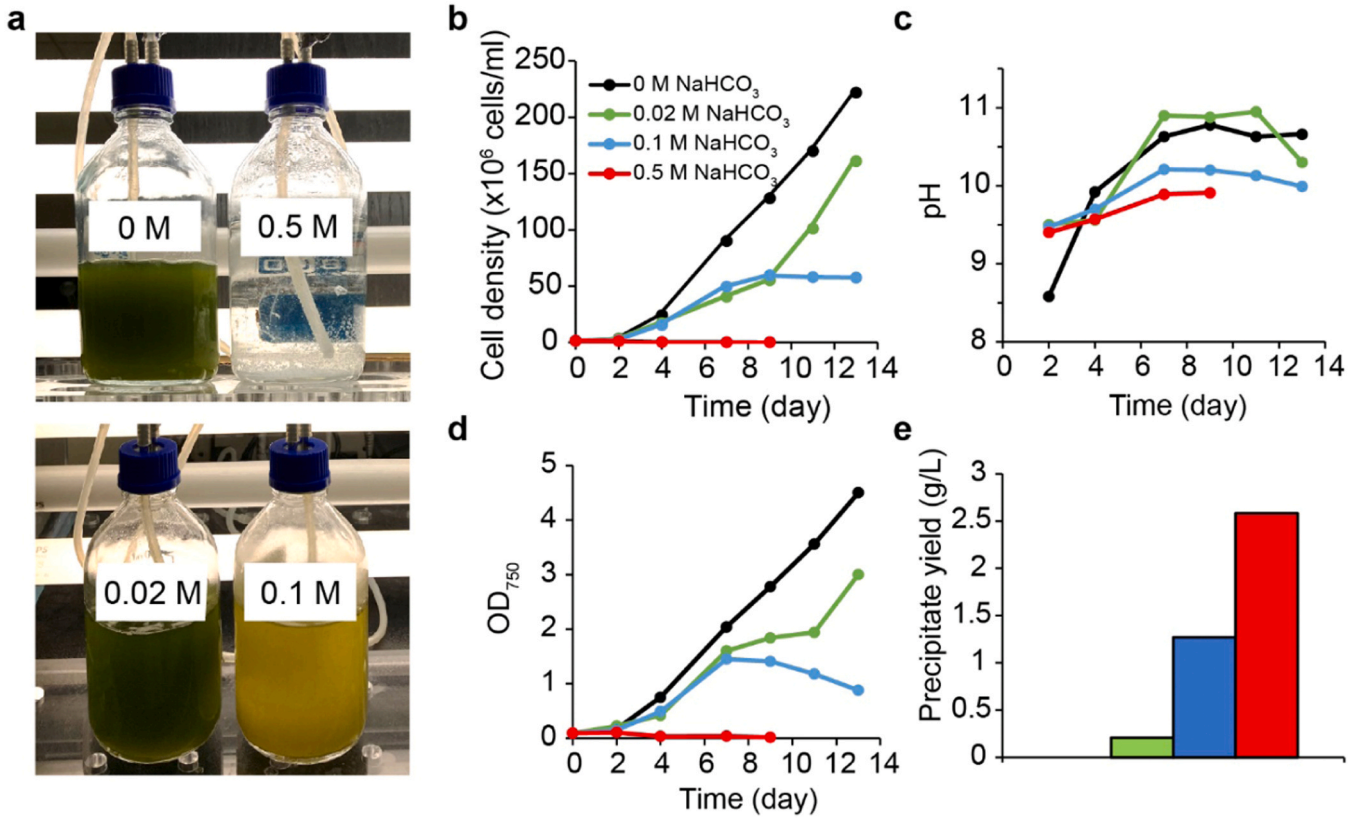

Fig. 3. *N. oceanica* IMET1 cultures under ambient air and supplemented with sodium bicarbonate. (a) *N. oceanica* IMET1 were cultured in f/2 (17 ppt salinity) supplemented with 0, 0.02, 0.1 or 0.5 M sodium bicarbonate. (b) Cell density, (c) pH and (d) optical density of the cultures were followed over time. (e) Precipitates were collected and the yield was measured at day 13. The yield of the precipitates from the culture without sodium bicarbonate addition (0 M) was not detectable.

samples from the fourth lab-bench experiment were collected and analyzed separately, including the bacteria closely associated with *N. oceanica* IMET1 (obtained on 0.45 μm-filter fractions together with the algae) as well as the free-living bacterial communities (0.45 μm-filtrates subsequently concentrated on 0.2 μm filters). As expected, the 0.45 μm fraction was dominated by chloroplast 16 S rRNA gene sequences from *N. oceanica*. To better reveal the 16 S rRNA gene sequences derived from bacteria, all chloroplast sequences were removed bioinformatically. The resultant bacterial communities are shown in Fig. 5.

The bacterial community of *N. oceanica* IMET1 was dominated by four species. In the bacterial community closely associated with *N. oceanica* strain IMET1, *Maricaulis maris* and *Gracilimonas* sp. were dominant on Day 0 and Day 4 regardless of whether microalgae were supplemented with $NaHCO_3$ or not. *Marinisubtilis pacificus*, a member of the Microbacteriaceae family, also was found at a lower relative abundance. Interestingly, after days 0 and 4, all samples were completely dominated by a novel uncultured bacterium clone. Preliminary phylogenetic analysis (Fig. S5) suggests that this novel bacterium may be a member of the uncultured OD1 phylum.

3.3. Scaling up the Nannochloropsis culture in a large-scale bioreactor

To scale-up, a batch culture of *N. oceanica* IMET1 was initiated in the 340 L-bioreactor and then the volume was increased stepwise. At the beginning, 4 L of the IMET1 seed culture was inoculated into 80 L of f/2 media. The initial culture was grown under gentle aeration (< 0.5 L/min) and low intensity of blue light (ca. 20 μmol m^{-2} s^{-1}) (Fig. S2c) to avoid cell death due to shear force and high-light stress. Cell density and OD_{750} increased from 2.55×10^6 cells/ml and 0.069 at day 0 to 14.2×10^6 cells/ml and 0.385, respectively, on day 10 (Fig. 6a and b). pH increased rapidly from pH 8.6 (at day 0) to pH 9.1 (at day 2) within the first 2 days and reached a peak of pH 9.3 at day 8 (Fig. 6c). At day 10, 100 L fresh media was added into the bioreactor to increase the culture volume to ca. 160 L (Fig. 6f) that diluted the culture to 5.84×10^6 cells/ml, with OD_{750} at 0.17 and pH at 7.5 (Fig. 6a–c). Aeration was increased gradually to promote algae growth. After 2 days of culture dilution, its pH increased to 9.0 (at day 12), and the cell density were also increased to the level prior to dilution (12.3×10^6 cells/ml). After day 12, the cell density and the optical density continued to increase, and pH remained between 8.9 – 9.1 (Fig. 6a–c). At day 17, when the cell density and optical density reached 16.8×10^6 cells/ml and 0.54, respectively, 80 L of the culture was removed from the bioreactor and 80 L of fresh media was refilled to maintain a semi-continuous culture and dilute the culture. At this time, the light source changed from blue light (20 μmol m^{-2} s^{-1}) (Fig. S2c) to white light (50 μmol m^{-2} s^{-1}) (Fig. S2d) to increase the light intensity. After day 17, cell density and optical density of the culture rapidly increased. In 3 days (at day 20), cell density and optical density bounced back to the levels as prior to the dilution at day 17 (Fig. 6a and b). Cell density continued to increase to reach the maximum of 29.1×10^6 cells/ml at day 23 (Fig. 6a), and then another 100 L of the culture was replaced with fresh media. The culture again responded rapidly after the refill of fresh media. At day 30, 150 L fresh media was filled in to increase the water level to full tank (ca. 80 in; 320 L) (Fig. 6f). The filling of fresh media resulted in a drop in cell density, optical density and pH, but the culture recovered in 3 days, as in the case of previous dilutions. Overall, *N. oceanica* IMET1 grew rapidly while increasing culture pH in the scaled-up bioreactor, and the semi-continuous culture status was maintained in the 340 L-bioreactor system.

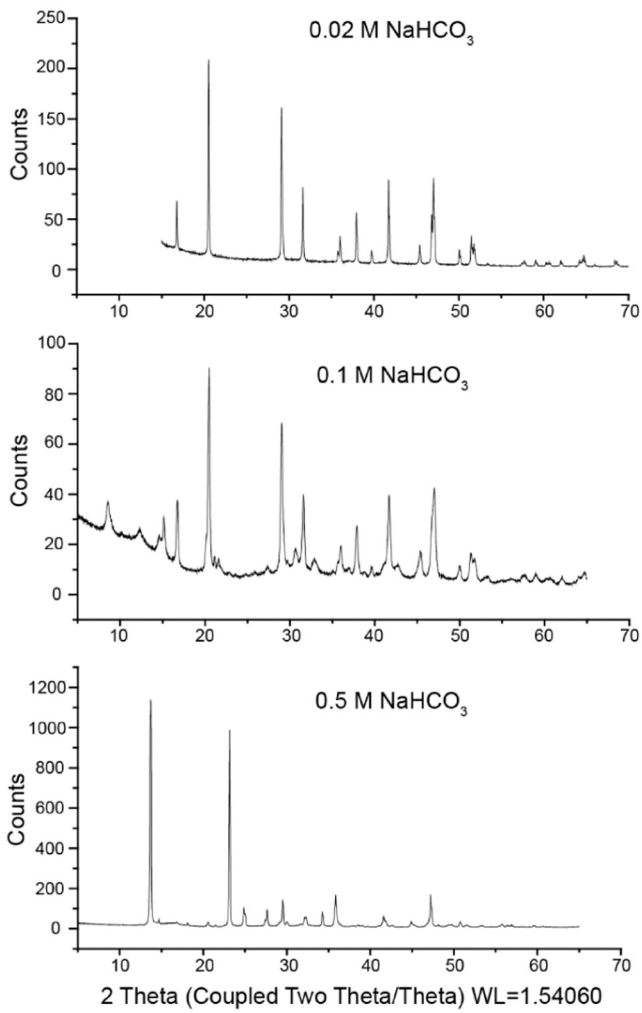

Fig. 4. Compositions of precipitate crystalline from the culture supplemented with sodium bicarbonate were determined by XRD analysis. The crystalline in the precipitates collected from the culture supplemented with 0.02 M and 0.1 M sodium bicarbonate were 100 % calcium carbonate (monohydrocalcite). The crystalline in the precipitates collected from the culture supplemented with 0.5 M sodium bicarbonate contained 96.5 % magnesium carbonate (Nesquehonite) and 3.5 % calcium carbonate (monohydrocalcite).

3.4. MadCAP in the scaled-up bioreactor

In the second bioreactor experiment, we attempted to grow IMET1 in the 340 L-bioreactor so that it would reach a high culture pH (pH ≥10) that would facilitate calcium carbonate formation. In the first test of the bioreactor, the culture pH was stable at ~ pH 9 (Fig. 6c). In this test, the light intensity next to the light compartment in the 340 L-bioreactor was 50 μE m^{-2} s^{-1}, and at the mid-point of the culture the light intensity was only 10–20 μE m^{-2} s^{-2} (Fig. 7a), hence the low culture pH level likely was due to light limitation. Possible limiting factors include low light transmission through the plastic material over the LED light strips that had become cloudy, low light input, long light path, etc.

To improve the illumination in the 340-L bioreactor, we replaced the LED light strips (Fig. 7b) with higher powered-LED lights (Fig. 7c). As a result, the light intensity in the headspace in the bioreactor chamber (i.e. above the culture level) was 680 μE m^{-2} s^{-1} at the point adjacent to the light compartment (the near point; Fig. 7a) and 450 μE m^{-2} s^{-1} at the mid-point (Fig. 7a) between the light compartment and the wall of the bioreactor (Table S1). Moreover, the light intensities in an algal culture with self-shading effect of cells in a diluted culture at day 0 were 320–350, 80–100 and 12–35 μE m^{-2} s^{-1} at the near, the middle and the far points (as indicated in Fig. 7a) from the light compartment, respectively (Table S1). Under the improved lighting conditions, pH of the culture in the bioreactor was able to increase from 9.0 to 10.0 within 5 days (Fig. 8; Table 1); cell density increased 1.8-fold from 26.6 × 10^6 to 47.2 × 10^6 cells ml^{-1}, and optical density (OD_{750}) increased 2.7-fold from 0.69 to 1.84 (Fig. 8; Table 1). The improved lighting conditions brought the maximal biomass dry weight to 0.5 g L^{-1} and the AFDW to 0.34 g L^{-1} on Day 19 (Fig. 8c). The area productivity of the algal dry weight and AFDW were 30.0 - 72.0 g m^{-2} d^{-1} and 25.1 – 51.4 g m^{-2} d^{-1}, respectively, during the 19-day growth course (Fig. 8e). On Day 19 at the end of the experiment, the area algal dry weight and AFDW productivity were 39.8 g m^{-2} d^{-1} and 25.2 g m^{-2} d^{-1}, respectively (Fig. 8e).

Precipitates were collected from the culture grown in the second 340-L bioreactor experiment. In this batch, the starting pH was 8.8, and the pH increased to 10.0 on day 2 and 10.6 on day 3 (Table 1). The yield of precipitates was 0.012, 0.20 and 0.03 g L^{-1} from the samples take on day 0, day 2 and day 3, respectively (Table 1). The precipitate was examined under dissection microscope (Fig. 9a), and the composition of the crystalline components was assessed by X-ray diffraction (XRD) analysis that indicated the crystalline components in precipitates were monohydrocalcite (Fig. 9c and Fig. S4d). When immersing the precipitate in 90 % phosphoric acid on our Acid Test chamber, the concentration of CO_2 evolved from precipitates (e.g., Fig. 9b) collected on day 0, 2, and 3 indicated that approximately 75 %, 69 %, and 61 %, respectively, of the precipitate mass was primarily monohydrocalcite, while it is worth noting these samples were not completely dried so there may be small amount of water left. Considering the bioreactor facility footprint of 0.156 m^2, system volume of 320 L, and precipitate content on Day 2, a maximum monohydrocalcite productivity of 133.4 g m^{-2} d^{-1} was achieved.

3.5. Lipid production in the scaled-up bioreactor

Nannochloropsis is known to produce high level of lipids and has abundant omega-3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA). We evaluated total lipid content and fatty acid profile in *Nannochloropsis* biomass grown in the second run of the 340 L-bioreactor. Under the growth condition in our system, *Nannochloropsis* contains 23.9 ± 0.9 % lipids of the dry biomass. The lipids consisted of eleven fatty acids that included seven different PUFAs (Fig. 10). The top three most abundant fatty acids were, palmitic acid (C16:0), palmitoleic acid (C16:1Δ9) and EPA (C20:5) (Fig. 10). The EPA content in our culture was 1.8 ± 0.04 % dry weight and 7.1 ± 0.5 % over the total lipids.

4. Discussion

Our MadCAP experiments were set up to mimic phytoplankton-induced whiting events in nature. Whiting events are often seen in freshwater lakes and certain water columns of oceans at a scale visible on satellites, ranging from about 0.1- 226 km^2 in the Bahama Banks to about 58,000 km^2 in Lake Michigan [5,6]. Here, we showed that our laboratory and pilot scale MadCAP processes precipitated $CaCO_3$ from *Nannochloropsis* culture in the form of monohydrocalcite (Fig. 9), which is readily transformable to calcite and aragonite that can be used as cementitious materials [34,35], while *Nannochloropsis* biomass can be used as feedstocks for bioproducts and biofuels [14]. We grew *N. oceanica* in bioreactors from 1 L to 340 L, demonstrating our system is highly scalable while the non-axenic *Nannochloropsis* culture maintained its productivity at high pH and alkalinity during the course of our experiments (Figs. 1 and 8).

Under the current ocean pH (8.1), $CaCO_3$ precipitation by calcifying organisms such as microalgae and macroalgae through Reaction (1) is known to release CO_2 to the surrounding water [7,9,10]. As a result, CO_2 removal and addition through photosynthetic organisms in the ocean is suggested to have no effect on total alkalinity [36]. On the other hand,

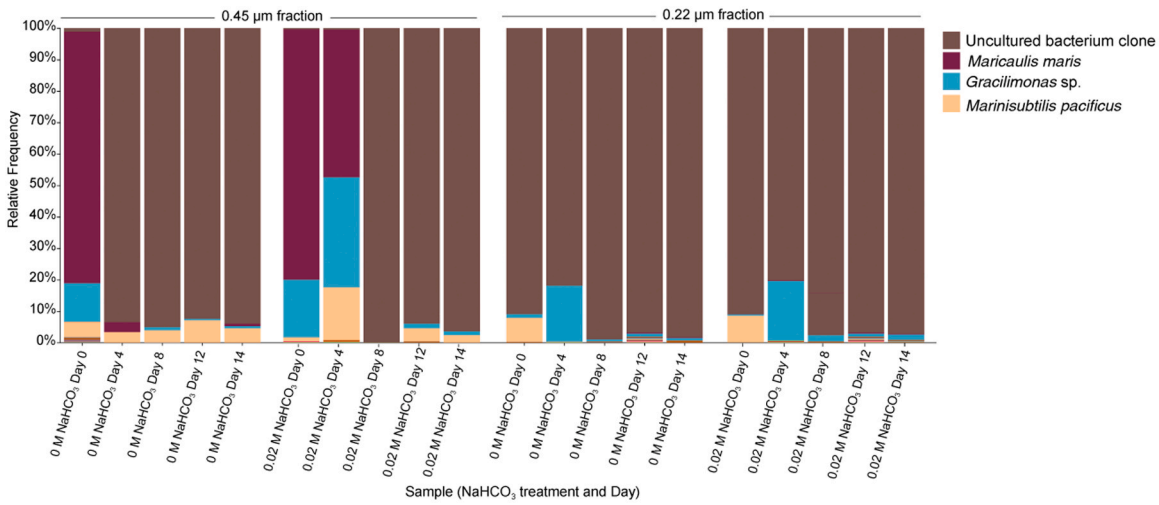

Fig. 5. Relative abundance of closely-associated prokaryotic community (0.45 μm fraction – first ten bars) and free-living prokaryotic community (0.22 μm fraction – remaining ten bars) of *N. oceanica* IMET1. Sequencing was targeted on the V3/V4 hypervariable region of the 16 S rRNA gene. The culture was grown over 14 days with ambient air in 1-L Pyrex bottles during the fourth experiment with sodium bicarbonate additions, with either no addition or 0.02 M addition of $NaHCO_3$. 16 S rRNA gene sequences derived from chloroplasts have been removed bioinformatically.

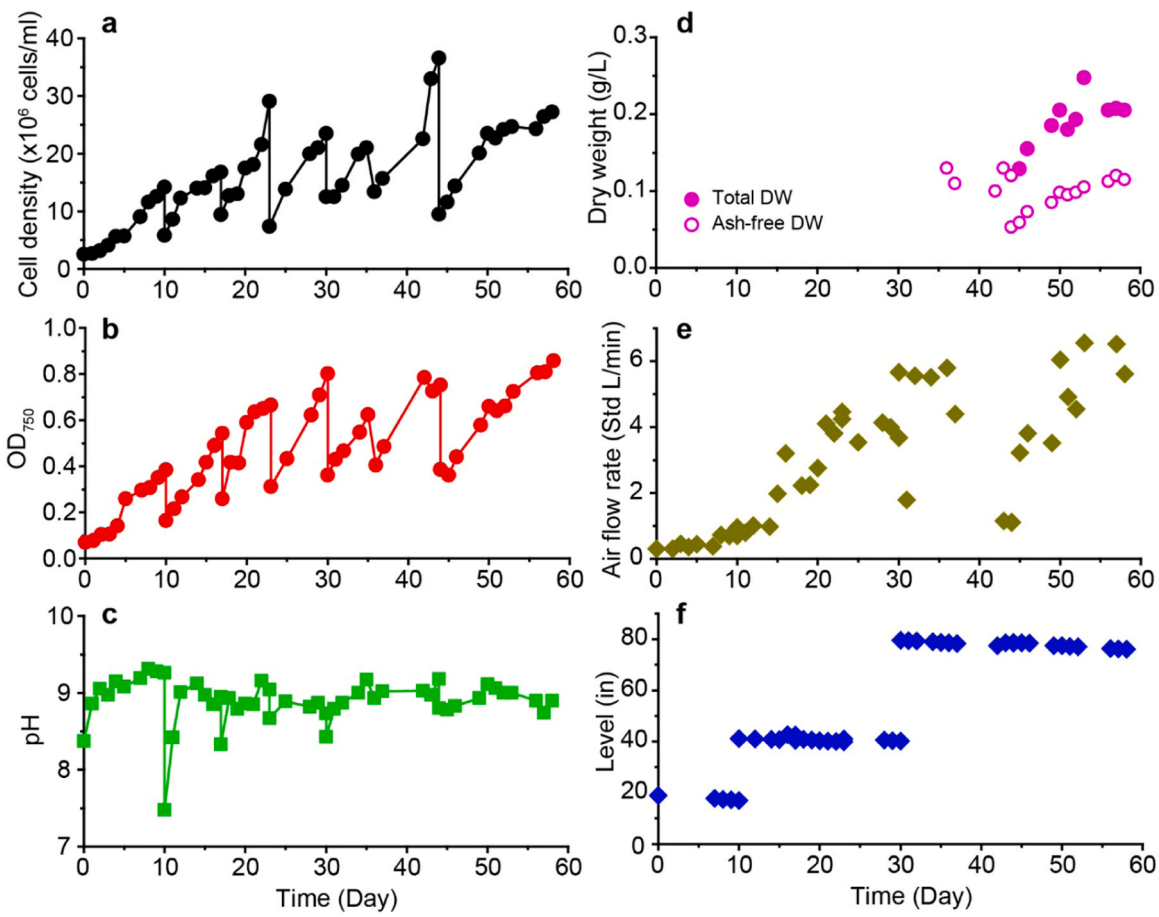

Fig. 6. Growth and bioreactor indexes for the *Nannochloropsis oceanica* IMET1 culture in the first 340-L bioreactor experiment. (a) Cell density, (b) optical density, (c) pH, and (d) dry weight changes were measured to indicate growth. (e) Aeration intensity and (f) water level were recorded to monitor the operation of the bioreactor.

algal growth is known to couple with assimilation of N and P, such as NO_3^- and HPO_4^{2-}, which can uptake H^+ or liberate OH^-, resulting in increase in culture pH and alkalinity [36]. We demonstrated that in our MadCAP process, *Nannochloropsis* cultures grown with NO_3^- and HPO_4^{2-} were able to increase culture alkalinity from 72.5 mg L^{-1} to a maximum level of 159.6 mg L^{-1} while maintaining culture pH at over 10 for 23 days (Figs. 1 and 2). At this pH, culture alkalinity is dominated by carbonate ions [37] and $CaCO_3$ is primarily formed through Reaction (2): $CO_3^{2-} + Ca^{2+} \rightleftharpoons CaCO_3$. This leads to net sequestration of CO_2 in the form of $CaCO_3$, in contrast to Reaction (1) that leads to CO_2 release. In line

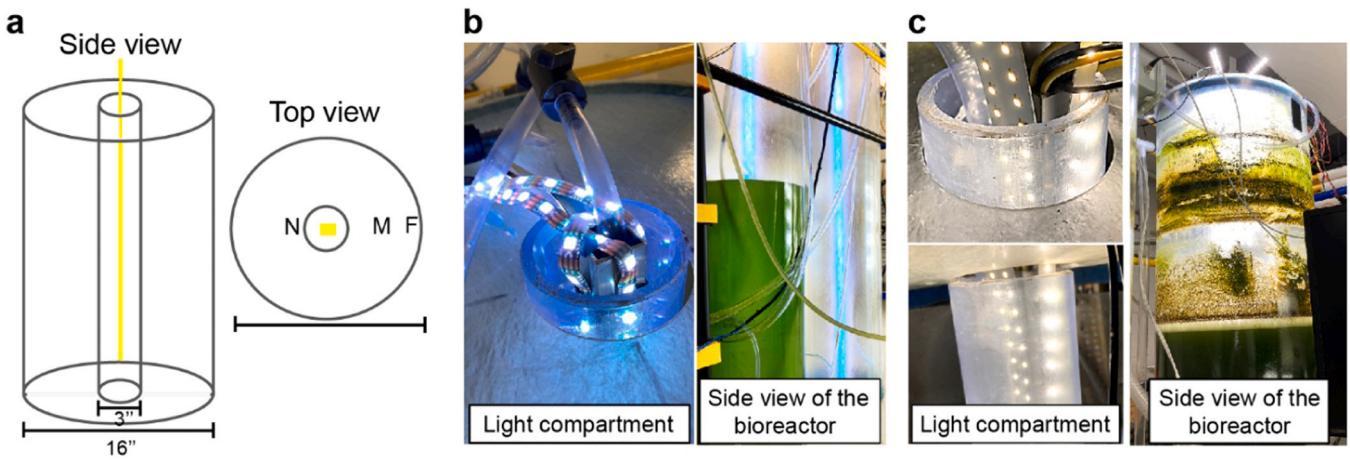

Fig. 7. Improvement in lighting of the 340 L-bioreactor. (a) The bioreactor was designed as a 16-inch diameter cylinder with a 3-inch diameter cylindrical lighting compartment in the center. Both bioreactor and the lighting compartment were made of acrylic plastic. The illumination intensity in the bioreactor was measured near the lights (N), at the mid-point between the lights and outside wall (M), and the farthest point from the light source (F). The bioreactor was illuminated by (b) strips of low power LEDs or high power-LEDs. (c) The LED light strips were tied to an air-cooled square aluminum core in the middle of the light compartment.

with this, recent work showed that increase of pH and total alkalinity was the key contributor of carbonate precipitates in the Bahamian carbonate mud, rather than phytoplankton-driven whitings at current ocean pH [8].

$CaCO_3$ precipitation in seas and lakes may be due to biotic factors such as phytoplankton bloom and viral lysis or abiotic such as sediment resuspension and abrasion of ooids [7,8]. As such, there are debates whether whitings are a source or a sink for carbon. Recently, an algal-bacterial co-culture was found to promote $CaCO_3$ formation in saline environment, with the halophilic microalga *Dunaliella salina* and its mutualistic halophilic bacteria *Nesterenkonia* sp. [38]. This work suggests whitings are likely a sink for carbon in such environment as the co-cultivation stimulated $CaCO_3$ precipitation by about 55 % and promoted the assimilation of CO_2 by 50 % [38]. It was estimated that with the productivity of their system, 2.3 Tg C yr^{-1} carbon sequestration is achievable in global saline lakes [38]. In this work, we also showed the MadCAP as a carbon sink to capture and store CO_2 in the form of monohydrocalcite, a hydrated amorphous calcium carbonate often found in recent sediments in saline lakes [35]. It is worth noting that pending further analysis with SEM, the crystallites of monohydrocalcite in this study seemed not similar as compared with those previously reported [39], possibility due to different precipitation methods. We achieved a peak monohydrocalcite productivity of 133.4 g m^{-2} d^{-1} growing under continuous light (Fig. 8). Assuming this productivity, we estimate a C capture rate of 13.6 g m^{-2} d^{-1} or 4950.8 tons km^{-2} yr^{-1}. To reach the same 2.3 Tg C yr^{-1} carbon sequestration with monohydrocalcite, we need only 464.6 km^2 land, just a fraction of the global saline lakes area (6×10^6 km^2). However, it must be noted that the scaling up process for algal bioreactors at such scale would be much more complex and costly, and no commercial facility has reached that level yet [40]. When scaling up we propose to start with small scale bioreactor/mesocosm experiments where local seawater is used to grow algae to produce high pH, high alkalinity water and algae. In addition to carbon capture and storage, monohydrocalcite has been found to effectively absorb environmental contaminants, such as phosphorus, arsenic, and lead [41,42], thus may be advantageous over traditional carbon sequestration methods by remediation of these toxic compounds.

Using highly alkaline conditions to grow alkaliphilic algal cultures has been proposed as a way to improve carbon utilization efficiency and cost-effectiveness of algal biotechnology [43–46]. Biocarbonate has been used in previous work to lower carbon supply cost, harvesting cost, as well as labor and energy costs [43–46]. In this study, while addition of bicarbonate promoted $CaCO_3$ formation, it didn't increase culture pH and compromised algal growth when grown with air (Fig. 3). *N. oceanica* IMET1's growth without bicarbonate supplement promotes an increase in the seawater culture medium pH and alkalinity, and thus facilitates sequestration of CO_2 in the form of $CaCO_3$ when aerated with air. Addition of $NaHCO_3$ can result in supersaturation of $CaCO_3$ crystal polymorphs and hydrates, and its buffering capability will help maintain pH in the algal culture. Our recent preliminary tests found when grown with high CO_2 flue gas (6–12 % CO_2), adding $NaHCO_3$ resulted in a higher growth rate in *N. oceanica* IMET1, suggesting use of this strain for direct carbon capture from power plant flue gases with high concentrations of CO_2 (6–12 %).

N. oceanica IMET1 is a model oil-producing microalga and known to produce the high value omega-3 fatty acid EPA in its lipidome [24,26, 47,48]. EPA-rich *Nannochloropsis* biomass has been proposed to be used in the aquaculture as well as nutraceutical industries, while the biorefinery idea of using *Nannochloropsis* spp. to produce EPA, other bioactive compounds, and biofuels has been proposed [13,14]. Our work showed that *N. oceanica* IMET1 is also a promising alga for permanent carbon sequestration in the form of monohydrocalcite ($CaCO_3$) while producing EPA. The EPA content of *N. oceanica* was moderate (Fig. 10), however, if successfully scaled up, at the current AFDW productivity (25.2 g m^{-2} d^{-1}, Fig. 8), we estimate 9198 tons km^{-2} yr^{-1} of algal biomass productivity (AFDW) and 164.6 tons km^{-2} yr^{-1} of EPA (C20:5n-3) productivity. It is estimated that about 0.8 million tons of EPA+DHA per year is currently produced for human consumption [49, 50], falling short of the estimated nutritional demand of 1.4 million tons yr^{-1} required to supply the global population a recommended daily dosage of 500 mg EPA + DHA at an EPA:DHA ratio of 2:1 [50,51]. At our current productivity, if EPA is produced as a byproduct, about 2430 km^2 land would be needed for *Nannochloropsis* culture to fill the gap between the recommended EPA dosage and the current market. Currently, major carbon capture, utilization, and storage (CCUS) applications in the industry include enhanced oil recovery (EOR) and enhanced gas recovery (EGR), which use concentrated CO_2 to recovery oil and gas from mature fields [12]. CO_2 leakage from these short-term partial solutions may provide opportunities for permanent removal of CO_2 through the MadCAP process if successfully scaled up.

In nature and in most outdoor algae culture facilities, microalgae and bacteria often grow in non-axenic cocultures, but their molecular interactions remain poorly understood [52]. MICP is mostly studied with bacteria-induced $CaCO_3$ precipitation directed by urea hydrolysis [20, 21], while microalgae-bacteria interaction during $CaCO_3$ precipitation in polyculture is not understood. As such, we characterized the bacterial communities associated with *N. oceanica* IMET1. Among the bacterial groups found to be associated with *N. oceanica* IMET1, *Maricaulis maris*

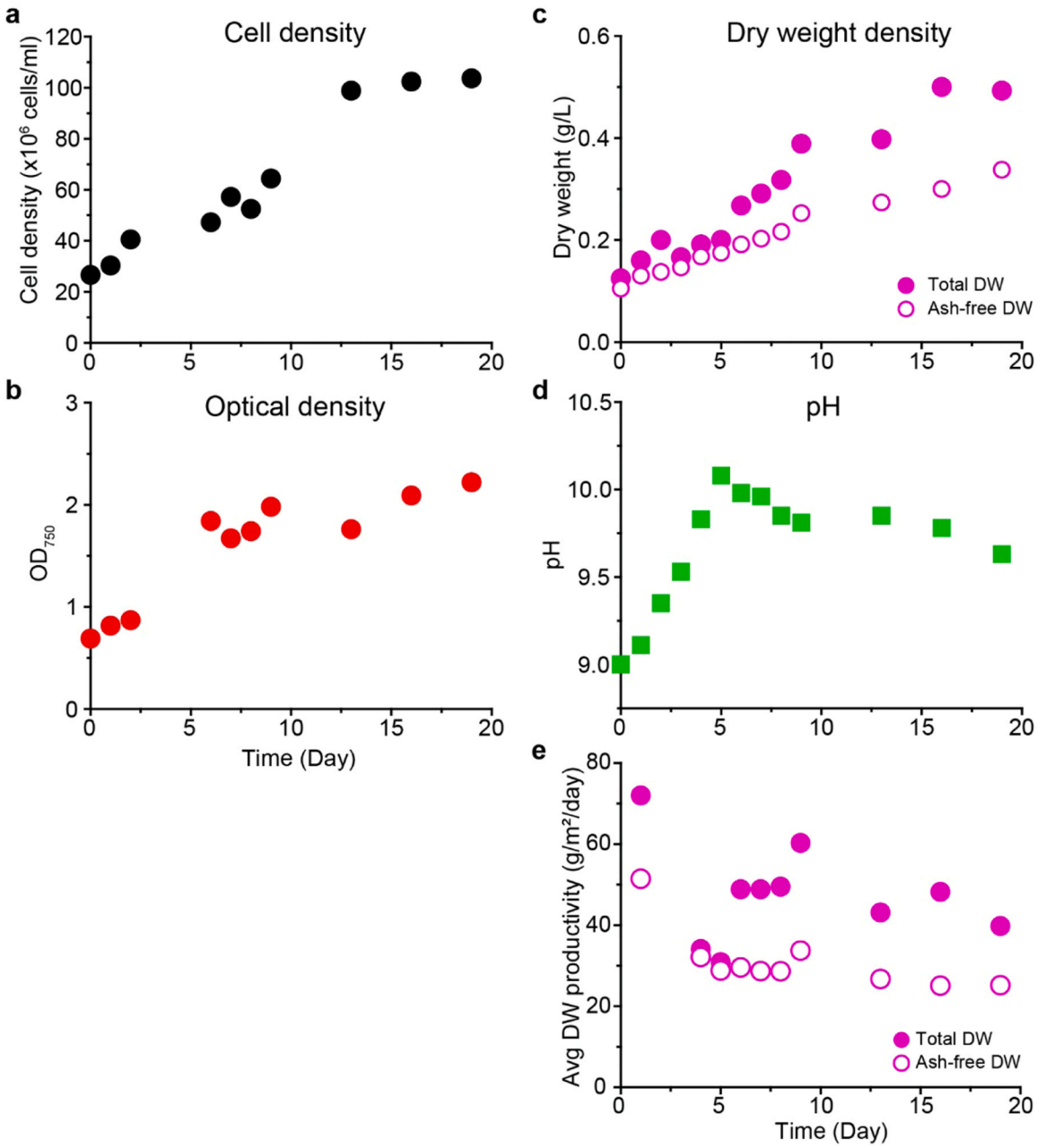

Fig. 8. Growth indexes of a *Nannochloropsis oceanica* IMET1 culture in the second 340 L-bioreactor experiment under the improved lighting conditions. (a) Cell density (10^6 cells ml^{-1}), (b) optical density at 750 nm (OD_{750}), (c) dry weight (g L^{-1}), (d) pH, and (e) dry weight productivity (g m^{-2} d^{-1}) were measured to indicate the growth.

Table 1
Physiological and precipitate analysis of *Nannochloropsis* grown in 340-L bioreactor under improved lighting conditions.*

	Day 0	Day 2	Day 3
pH	8.8	10.0	10.6
OD_{750}	1.0	1.2	1.1
Cell density ($\times 10^6$ cells/ml)	37.0	48.8	48.2
Precipitate yield (g/L)	0.012	0.20	0.03

* *N. oceanica* culture was collected from the second 340-L bioreactor experiment under improved lighting conditions (as described in Table S1).

(previously described in the Caulobacter genera) is a common aquatic and halophilic species that has been described as scavenger of carbon in oligotrophic habitats (JGI Genome Portal; [53]). *Gracilimonas* sp. have been found associated with the photosynthetic bacterium *Synechococcus* [54]. Previously, a community of *Gracilimonas* containing microorganisms collected from sediment slurries collected at Soap Lake (WA) was shown to have a comparable or higher $CaCO_3$ precipitation rate when compared with the standard MICP strain *Sporosarcina pasteurii* [55], suggesting this bacteria may contribute to $CaCO_3$ precipitation through MICP in our culture. Previous work with *Nannochloropsis oculata* observed autoflocculation of algal cells in the culture started at pH 9.5 [56];this was not observed in this study. One possible explanation is the difference between the bacterial community in the algal culture, with *Flavobacteriales* being the dominant bacteria order in their *N. oculata* culture [56], while we could not rule out the possibility that algal cells

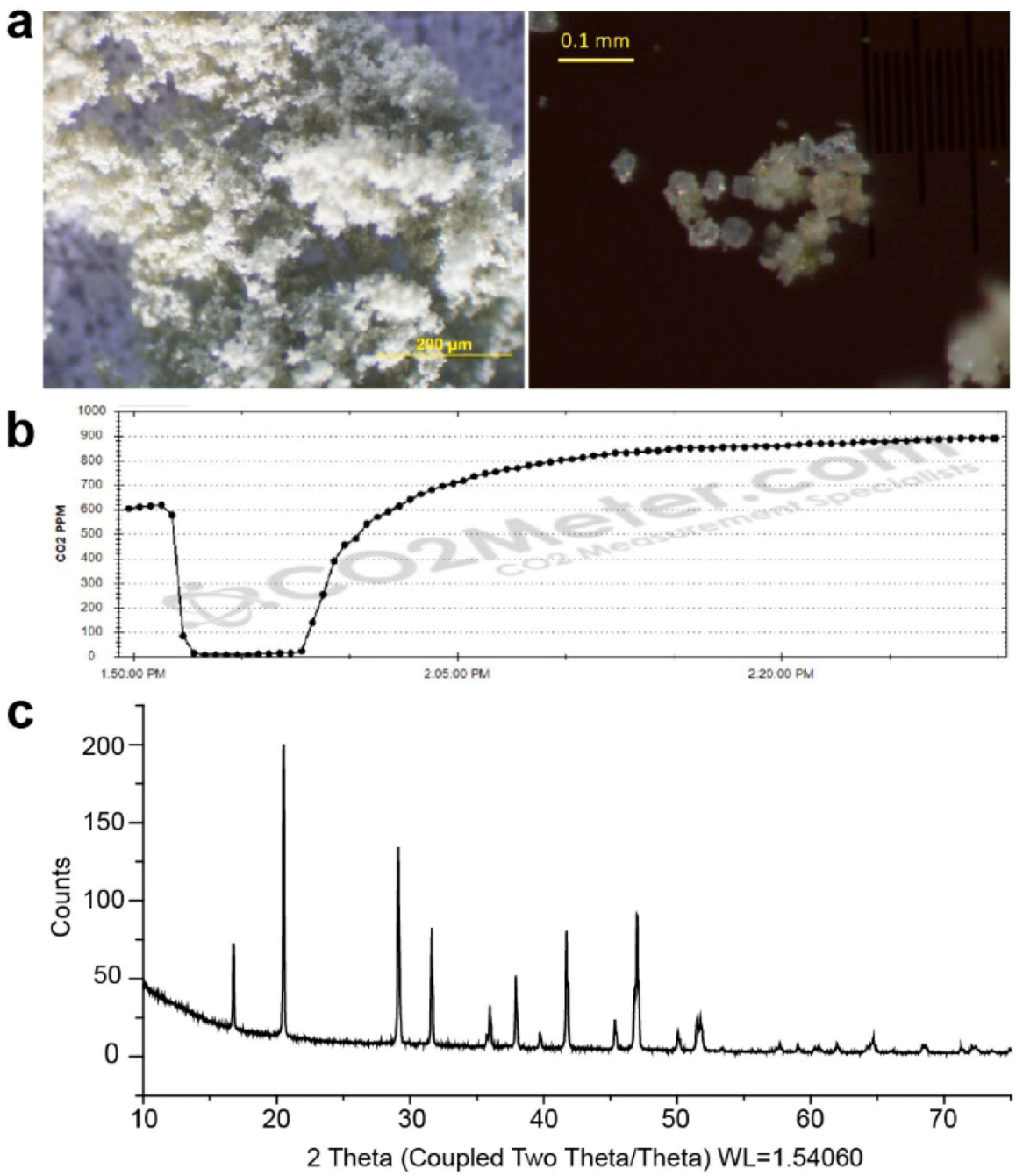

Fig. 9. Examination of properties of the precipitate collected from the 340 L-bioreactor. (a) Images of the precipitate crystalline were taken under a dissection microscope. (b) The release of CO_2 over time when a sample of the precipitate was immersed in 90 % phosphoric acid. Results indicated that > 60 % of the mass of the precipitates was carbonate (c) X-ray diffraction (XRD) analysis showed that the crystalline component of the precipitate was monohydrocalcite.

may provide nucleation sites for $CaCO_3$ precipitation. Interestingly, after days 0 and 4, all our samples became heavily dominated by an uncultured bacterium clone related to the novel and enigmatic uncultured OD1 phylum. Our previous findings suggest that some bacteria promote algal growth in co-cultures through producing growth hormones such as auxin [17,57]. Therefore, this is an intriguing finding that warrants further investigation because it is possible that this bacterium plays a role in stimulating growth of algal culture and in the process of MICP.

CRediT authorship contribution statement

Yi-Ying Lee: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. **Scott Hunsicker:** Data curation, Methodology, Supervision, Writing – review & editing. **Elizabeth North:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing. **Yantao Li:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Lauren Jonas:** Data curation, Methodology, Writing – review & editing. **Russell Hill:** Supervision, Writing – review & editing. **Allen Place:** Supervision, Writing – review & editing. **Greg Silsbe:** Data curation, Methodology, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial

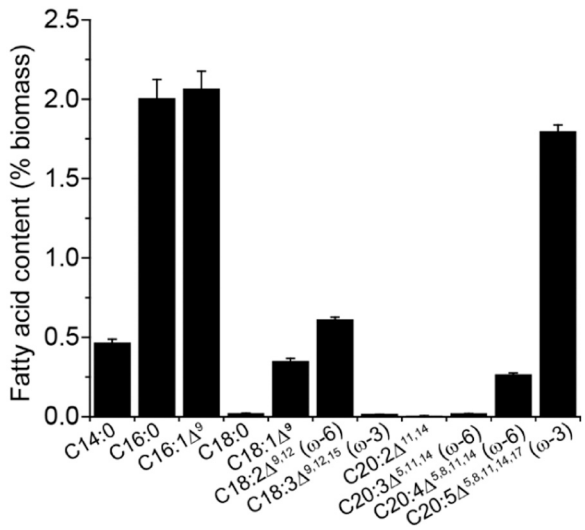

Fig. 10. Fatty acid profile of total lipids in *Nannochloropsis* biomass. Total lipids were extracted from three independent biomass samples that had been cultured in the second experiment of the 340 L-bioreactor. Data represents mean ± standard deviation (SD) from the three independent measurements.

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jcou.2024.102669.

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