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Research paper

Cultivation study of the marine microalga *Picochlorum oculatum* and outdoor deployment in a novel bioreactor for high-density production of algal cell mass

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ABSTRACT

Microalgae are considered a promising source of renewable diesel and jet fuel. Currently, large-scale microalgae cultivations are performed in open ponds because of their low capital and operating costs, but they generally suffer from low cell mass yield and high risk of contamination. A novel, cost-effective, and modular horizontal bioreactor (HBR) for algae cultivation was developed, as described in the present study. The HBR was designed to keep costs low and was engineered to minimize water and energy use while enhancing CO_2 and nutrient uptake. The selected marine microalgal strain, *Picochlorum oculatum (Nannochloris oculata)*, has shown potential for biofuel production. A series of controlled indoor growth experiments was first performed to identify the appropriate *P. oculatum* growth conditions before demonstrating the HBR performance. Supplying CO_2 continuously or by pH-control (pulsed) did not affect culture progression. Growth on urea and nitrate yielded comparable results, while ammonium was less effective. Varying inoculum size from 10% to 15% or 20% had no significant effect on lag time and final cell concentration and comparable growth was measured in the 7–8 pH range. The 150-L HBR's performance was successfully demonstrated outdoors by growing *P. oculatum* at the identified growth conditions selected to reduce operating costs (pH-controlled CO_2, pH 7.5, 10% inoculum, and nitrate). High-density growth was achieved without any contamination issues in outdoor HBR cultivations over 68 days in central Florida during two consecutive growth cycles.

1. Introduction

Renewable transportation fuels have been receiving increasing attention in recent years, as the transportation sector, including the aviation industry, seeks ways to reduce carbon emissions and dependence on imported fossil fuels [1]. Algae have the potential to become a significant source of renewable jet and diesel fuels and to help mitigate climate change due to their projected lower carbon footprint [2,3]. Many promising microalgal species can grow readily in culture, double their cell mass within a few days, and be cultivated in a sustainable way by using low quality water sources, like wastewaters, and CO_2 from flue gas of industrial plants [4–6]. After cultivation, algal lipids (or whole algae cells) can be converted to fuel, proteins in algal cells may be used as animal feed and fish meal, while carbohydrates may be valuable in nutraceutical applications, thus improving the life-cycle sustainability and profitability of algae [7,8]. Presently, most microalgae cultivation systems at industrial scale are open ponds, due to their low capital and operating costs, but they often suffer from low cell mass yields and culture crashes due to contamination problems [9]. On the other hand, closed photobioreactor systems can support higher algal cell mass concentrations and productivities, but usually at the expense of higher capital and operating costs. Hybrid systems attempt to combine characteristics of both systems as they generally try to approximate open ponds to reduce cost [10].

We describe the development of an inexpensive modular horizontal cultivation system for algal biofuel production developed by an academia-private sector partnership. The horizontal bioreactor (HBR) was designed to keep manufacturing costs low and provide good mixing for efficient CO_2 and nutrient uptake by algae cells. The capital cost (materials and manufacturing) of the HBR at full

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Abbreviations

HBR	horizontal bioreactor
LED	light-emitting diode
MH	metal halide lamp
PE	polyethylene
OD	optical density
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
DW	dry weight
NO_3–N	nitrate nitrogen
PO_4–P	phosphate phosphorus
NH_4–N	ammonium nitrogen
DO	dissolved oxygen
T	PE transparency percentage
I_0	light intensity without the PE piece in front of the light sensor
I_{PE}	light intensity with the PE piece in front of the light sensor
N	cell concentration (cm^{-3})
μ_{max}	maximum specific growth rate (d^{-1})
R_N	average NO_3–N consumption rate (mg L^{-1} d^{-1})
R_P	average PO_4–P consumption rate (mg L^{-1} d^{-1})
P_v	average volumetric productivity (g L^{-1} d^{-1})
P_a	average areal productivity (g m^{-2} d^{-1})
Y_N	cell mass yield on nitrogen (g of cell mass per g of NO_3–N)
N_{total}	total consumed g of nitrate nitrogen
Y_P	cell mass yield on phosphorus (g of cell mass per g of PO_4–P)
P_{total}	total consumed g of phosphate phosphorus
PAR	photosynthetically active radiation
Y_I	cell mass yield on light energy (g of cell mass per mol of photons)
I_{total}	total incident mol of photons
TAG	triacylglyceride
SD	standard deviation

production is estimated to be $25,000 per hectare using scaled-up units (each with a surface area of 1000 m^2) which is comparable to open ponds and many times lower than reported for closed photobioreactors according to a previous analysis [11]. The HBR is readily scalable due to its modular design, can operate both on the ground and on water, and utilizes a fraction of cultivation water compared to traditional open systems. Scale-up is envisioned in the form of multiple HBR units connected in series or in parallel to provide the required surface area for a commercial operation. Its enclosed design acts as a barrier that reduces considerably the chances of culture contamination. A first HBR prototype was previously developed by our team and successfully demonstrated for algal cultivations [11]. That unit was equipped with airlift systems for culture mixing and gas diffusion and operated in a body of water. A larger 150-L prototype with different mixing and CO_2 diffusion systems was subsequently developed and demonstrated for algal cultivation as presented in the current study. The new HBR design is equipped with an 8-blade paddlewheel that enables culture mixing with very low energy consumption compared to the air compressor required for the original airlift system. Power requirements of paddlewheels have been reported to be almost half that for airlifts, while their efficiency is double that of airlift circulation systems [8]. With the removal of the airlifts in the newly designed HBR, we introduced a ceramic gas micro-diffuser for high-efficiency CO_2 supply directly into the culture.

For demonstration of the HBR cultivation performance, the green microalgal species *Nannochloris oculata* (Chlorophyceae), reclassified as *Picochlorum oculatum* [12], was selected. *P. oculatum (N. oculata)* is a marine alga widely used as feed by zooplanktons and corals [13]. It has good potential for biofuel production due to its high growth rate, ability to grow readily in culture, and high lipid production [5,13,14]. Moreover, it grows under a wide range of environmental conditions, such as temperature and salinity, thus rendering this microalga a promising candidate for outdoor cultivations that are subject to highly variable conditions [15]. *P. oculatum* has been reported to grow at temperatures as low as 1 °C, it is common in temperate estuaries, and often dominates in open cultures that reach temperatures above 25 °C [16]. Furthermore, it can tolerate salinities from 2 to 300% of seawater [16], hence enabling its cultivation using various non-potable water sources that are locally available, such as seawater, brackish or wastewater.

First, a series of controlled indoor growth experiments was performed to identify appropriate *P. oculatum* growth conditions that would serve as the basis for the subsequent outdoor HBR tests. We started by investigating light sources for indoor inoculum preparation and by examining different CO_2 supply modes for inoculation and HBR operation. More specifically, illumination by different light sources, metal halide lamps (MH) and light-emitting diodes (LED), was assessed and the difference between continuous CO_2 delivery and a pulsed pH-controlled CO_2 supply on *P. oculatum* growth was investigated. LED illumination has been reported to foster high-density cultivation of microalgae [17] and may provide a more efficient source of illumination than the traditionally used MH lights. Following these experiments, we investigated the effect of inoculum size and culture pH on cell growth. Subsequently, we examined the effect of nitrogen source on the growth of *P. oculatum* by comparing urea and ammonia (compounds often found in wastewaters) to potassium nitrate (control) in order to assess the suitability of wastewater as a potential inexpensive and abundant nitrogen source; if wastewater could be utilized as a nitrogen source, the production costs of algal cell mass and biofuels would be reduced [18,19]. Finally, after selecting those conditions that yielded in the aforementioned experiments the best growth performance at the lowest apparent cost, *P. oculatum* was cultivated in the developed HBR under real-world conditions in central Florida. The bioreactor's performance was documented and is presented in the current study.

2. Materials and methods

2.1. The horizontal bioreactor (HBR)

The HBR unit is a fully enclosed algae cultivation system (Fig. 1) constructed from greenhouse low-density UV-stabilized polyethylene (PE) sheet of 150 μm thickness (International Greenhouse Company, Danville, IL, USA), as described previously [20]. The top and the bottom PE sheets were welded together to form a closed bioreactor, similar to a covered raceway, but with the advantage of a protected cultivation environment that reduces water evaporation and minimizes the risk of outside contaminations. The HBR was equipped with a centrally positioned plastic tube that acted as

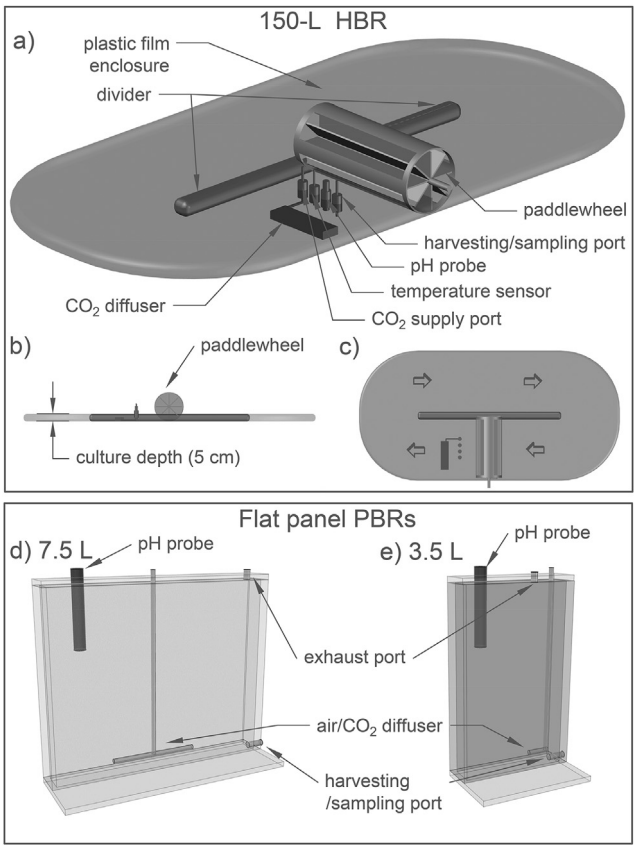

Fig. 1. Schematic of the pilot-scale 150-L horizontal bioreactor for the production of algal cell mass [(a) overview; (b) side view; (c) top view] and the bench-scale flat panel photobioreactors for algal physiology studies and inoculum production [(d) 7.5-L; (e) 3.5-L].

divider forming a closed-loop channel (Fig. 1c). Ground preparation entailed only leveling of the ground. During operation, the culture depth was just 5 cm, which enhanced exposure of the algal cells to light even at high densities, while at the same time minimizing water use and reducing downstream processing. Mixing of the culture was achieved by an 8-blade aluminum paddlewheel placed between the top and bottom PE sheets at the center of the HBR, as shown in Fig. 1. The HBR used in the present study was manufactured with a working volume of 150 L and an effective surface area of 3 m^2. Due to its modular design with each unit comprising a PE sheet and a paddlewheel, it can be readily scaled up by connecting multiple units in parallel and in series to cover the industrial scale area available for mass production of algal biomass. The culture velocity inside the HBR was measured at various paddlewheel rotation speeds using neutral-buoyancy (density of 1.0 g cm^{-3}) polyethylene 1.0–1.2 mm microbeads (Cospheric, Santa Barbara, CA, USA). Six spots were selected around the bioreactor, including the two channel turns, and the time required for each of five beads to travel a set distance was recorded and used to calculate the average local and overall speeds at 0.33, 0.5, and 0.66 Hz.

The unit's monitoring systems included real-time measurements of culture pH, culture temperature, and solar irradiance. Culture pH was measured by a submersible self-cleaning pH electrode with automatic temperature compensation (Cole-Parmer, USA) connected to a digital controller (Alpha pH200, Eutech Instruments, USA). The temperature sensor (12-Bit Temperature Smart Sensor, Onset, USA) was submerged in the culture. Sunlight levels on the HBR surface were measured with a silicon pyranometer sensor (Solar Radiation Smart Sensor, Onset, USA). The pH controller, temperature probe, and light sensor were connected to an automated logging system (HOBO U30, Onset, USA). The data sampling rate of the logger was set to 1 min for all the sensors, and the averaged value over 10 min was stored on the logger.

Pure industrial grade CO_2 (Airgas, USA) was diffused through a high-efficiency ceramic micro-diffuser (PMBD 75, Point Four Systems, USA). A pH-stat system controlled the delivery of CO_2 as a means of maintaining the pH of the medium fixed at the desired set point. The system included a direct-acting solenoid valve (Burkert, USA) connected to a pH controller (Alpha pH200, Eutech Instruments, USA) set to open the valve at the set point and shut off at a pH value 0.05 lower than the set point (0.05 hysteresis).

2.2. Microorganism and growth conditions

The microalgal strain *P. oculatum* UTEX LB 1998 was used to demonstrate the cultivation performance of the HBR. The strain was obtained as *N. oculata* LB 1998 from the UTEX Culture Collection of Algae at the University of Texas at Austin, but was reclassified as *P. oculatum* UTEX LB 1998 by Henley et al. [12] and verified as chlorophyte species using 18S rRNA partial sequences in NCBI GenBank (accession numbers AY422075 and GQ122335) and chloroplast partial gene sequence (GenBank accession number EF113455) [21]. *P. oculatum* was grown in marine medium, as defined previously [11]. Phosphorus was added in the form of KH_2PO_4. The initial P concentration was adjusted to 29.3 mg L^{-1} ("full strength"), except for the nutrient toxicity flask tests (section 3.1.3), where it was additionally set at 14.7 mg L^{-1} ("half strength") and 6.2 mg L^{-1} ("original medium strength"). The nitrogen source was KNO_3 and the initial N concentration was 316 mg L^{-1} ("full strength") with the exception of the nitrogen source experiments (section 3.1.3), where additional concentrations of nitrogen, 158 mg L^{-1} ("half strength") and 70 mg L^{-1} ("original medium strength"), were also used in the form of nitrate (KNO_3), urea, or ammonium (NH_4Cl). Overall, nitrogen and phosphorus were supplemented when needed during the cultivations to prevent any nutrient limitation phenomena.

During the cultivation experiments a sequential culturing scheme was applied, as described previously [11]. Briefly, the initial inoculum was prepared in flask cultures maintained at 23 °C in a rotary shaker operating at 2.5 Hz under continuous LED illumination. A volume of 10% inoculum was transferred from the flask cultures to bleach-sterilized vertical flat panel photobioreactors with a working volume of 7.5 L for high-density inoculum production (Fig. 1d). When those cultures reached optical density at 680 nm (OD_{680}) around 8, a volume of 10% inoculum (unless stated otherwise) was transferred from the 7.5-L bioreactors to several 3.5-L flat panel photobioreactors for the indoor growth study experiments or to the 150-L HBR for outdoor cultivation. The flat panel photobioreactors were bubbled with CO_2-enriched air for culture mixing and carbon source delivery. The pH in the cultures was controlled by automatically varying the CO_2-air mix via a pH-stat system as described earlier (section 2.1). The controller was set at 7.50 ± 0.05 with the exception of the pH experiments (section 3.1.5), where the pH values of 7.0 and 8.0 were also studied. Samples were taken daily for algal growth measurement and nutrient consumption monitoring. The flat panel bioreactors were exposed to artificial light by either LED panels (BloomBoss Panel by NEH, Southampton, MA, USA) or a grow light system comprising a 14,000 K Aqualite^{tm} MH lamp (Ushio America, Cypress, CA, USA) and an Xtrasun reflector-ballast system (Hydrofarm, Petaluma, CA, USA). The illumination systems were positioned to provide approximately 10 *k*lux, as measured at six locations on the

bioreactor's active surface by a portable light meter (Model CA813, AEMC Instruments, Dover, NH, USA), operating on a 16:8 h light:-dark cycle in an air-conditioned lab at 23 °C. The media were sterilized in situ using sodium hypochlorite (common household bleach) overnight and neutralized for 1 h with sodium thiosulfate prior to inoculation, as described in Ref. [11].

The *P. oculatum* growth study experiments were conducted indoors and the various conditions studied are presented in Table 1. First, the effect of light source (section 3.1.2) was studied in two 7.5-L flat panel photobioreactors, which were inoculated with flask cultures as described above. The LED or MH panels were positioned at an appropriate distance from the reactors to provide 3 *k*lux of light to the cultures upon inoculation and were repositioned to provide about 10 *k*lux of light once the cultures grew to an optical density greater than 1.0. The effects on algal growth of CO_2 delivery method (section 3.1.1), nitrogen source (section 3.1.3), inoculum level (section 3.1.4), and pH (section 3.1.5) were investigated in duplicate 3.5-L flat panel photobioreactors (Fig. 1e) with pH and temperature recording as described above. The pulsed CO_2 setup involved the pH-stat system, as described earlier, while during the continuous CO_2 delivery the valve was omitted and CO_2 was constantly supplied directly into the mixing air stream at 2–3% CO_2-to-air ratio by manually adjusting, when needed, the CO_2 flow to keep the pH at 7.5. During the nitrogen source experiments, the 3 sources (nitrate, urea, and ammonium) were added to the growth medium at the same elemental nitrogen level, as described above. In order to buffer any pH changes from the utilization of NH_4Cl and urea by the algae, 1.9 g L^{-1} of HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) buffer was added by filter sterilization. The effect of inoculum size was studied by varying the inoculum concentration from 10% to 15% and 20% of culture volume, using inoculum from *P. oculatum* cultivations in the 7.5-L photobioreactors, as described above. The three pH levels, 7.0, 7.5, and 8.0, were achieved by adjusting the pH controller setup.

Outdoor growth experiments were performed in a 150-L HBR prototype unit operated in central Florida. A 10% inoculum was transferred to the HBR from the indoor flat panel photobioreactors after the culture reached high density (OD_{680} 8.6). Samples were taken regularly for growth measurement and nutrient monitoring. During inoculation, usually completed around noon, a sample was drawn after 30 min to allow sufficient time for cells and nutrient mixing. The rest of the samples were drawn consistently in the morning, around 9 am. Sample analysis was done immediately after sampling and culture samples were kept in a cold bath until analysis was completed.

2.3. Analytical procedures

The OD of the samples was measured in duplicate at 680 nm using a spectrophotometer (DU 730, Beckman Coulter, USA). The cell concentration *N* was measured in duplicate using an automated cell counter (Auto X4, Nexcelom, USA). Dry weight (DW) was determined by filtering 5 cm^{-3} of culture volume through pre-dried and pre-weighed 0.47 μm Whatman nylon filters, followed by rinsing with 50 cm^{-3} of deionized water and by drying to a constant weight in a moisture analyzer (MB25, Ohaus, USA) set at 100 °C. The nutrient (N and P source) concentrations were measured in the sample filtrate. Nitrate nitrogen (NO_3–N) concentrations were determined using UV spectroscopy at 220 nm, as described elsewhere [22]. Total phosphate test kits (Hach, USA) were used for PO_3–P estimation. The concentrations of urea nitrogen and ammonium nitrogen (NH_4–N) were measured using Total Nitrogen test kits (Hach, USA), after appropriate standard curves were prepared for each N source (data not shown).

Dissolved Oxygen (DO) content in the outdoor HBR samples was measured immediately after each sampling, using a portable Dissolved Oxygen Meter HI 9147 (HANNA Instruments, Romania) calibrated at 30‰ salinity following the manufacturer's instructions. The salinity of the culture medium was measured in each sample with a portable salinity meter SALT6+ (OAKTON, USA), as described in the manufacturer's instructions.

2.4. Measurement of bleached PE transparency

To ensure that the chemical sterilization (bleaching) did not affect the transparency of the HBR's PE film, the light transmittance of three 10 cm × 10 cm pieces of the PE sheet was measured before (control) and after bleaching using the same conditions as described above. The PE pieces were exposed to 5 different light intensities from a metal halide bulb and light intensity (*k*lux) was recorded by a portable light meter (Model CA813, AEMC Instruments, USA). The transparency (T) was calculated using Eq. (1).

$$T = \frac{I_0 - I_{PE}}{I_0} \times 100 \quad (1)$$

where T is the transparency percentage, I_0 is the light intensity without the PE piece and I_{PE} is the intensity with the PE piece in front of the light sensor. Furthermore, the absorbance of the PE

Table 1
Summary of all *P. oculatum* cultivation experiments conducted in the present study for indoor small-scale growth optimization. Growth parameters (cultivation time, cell mass and cell concentrations), growth and nutrient consumption rates, productivities, and yields are shown for each test. All experiments were performed in flat panel vertical photobioreactors with working volume 3.5 or 7.5 L under artificial light.

Effect of	CO_2 delivery	Light source	N source	Inoculum level (%)	pH	Time	DW_{max}	N_m	μ_{max}	R_N	R_P	P_v	P_a	Y_N	Y_P
						days	g L^{-1}	cm^{-3}	d^{-1}	mg L^{-1} d^{-1}	mg L^{-1} d^{-1}	g L^{-1} d^{-1}	g m^{-2} d^{-1}	g g^{-1} N	g g^{-1} P
CO_2 delivery	Continuous	MH	KNO_3	10	7.5	14	2.17	$0.89 \cdot 10^9$	1.27	17.5	1.8	0.15	7.7	8.8	88.9
	pH-stat	MH	KNO_3	10	7.5	14	1.86	$0.94 \cdot 10^9$	1.27	17.8	1.7	0.13	6.8	7.5	79.4
Light source	pH-stat	LED	KNO_3	10	7.5	16	2.06	$1.40 \cdot 10^9$	2.06	18.1	1.4	0.10	5.4	7.4	70.2
	pH-stat	MH	KNO_3	10	7.5	16	2.04	$1.41 \cdot 10^9$	1.40	18.5	1.4	0.11	5.5	7.0	69.5
N source	pH-stat	LED	KNO_3	10	7.5	18	2.94	$1.36 \cdot 10^9$	1.62	23.6	1.8	0.19	10.0	7.9	104.9
	pH-stat	LED	Urea	10	7.5	18	3.20	$1.45 \cdot 10^9$	1.40	51.6	1.8	0.26	13.6	7.0	123.5
	pH-stat	LED	NH_4Cl	10	7.5	14	1.56	$0.59 \cdot 10^9$	1.17	45.2[a]	2.2	0.22	11.6	1.2	78.1
Inoculum level	pH-stat	LED	KNO_3	10	7.5	14	2.05	$1.18 \cdot 10^9$	1.39	18.6	1.8	0.14	7.2	7.5	69.8
	pH-stat	LED	KNO_3	15	7.5	14	1.63	$1.21 \cdot 10^9$	1.22	21.3	1.9	0.13	6.7	6.4	58.4
	pH-stat	LED	KNO_3	20	7.5	14	1.91	$1.35 \cdot 10^9$	0.75	18.9	1.9	0.12	6.2	7.5	67.4
pH	pH-stat	LED	KNO_3	10	7.0	21	2.66	$1.20 \cdot 10^9$	1.34	19.9	1.9	0.21	10.7	7.2	80.7
	pH-stat	LED	KNO_3	10	7.5	21	2.36	$1.15 \cdot 10^9$	1.26	20.4	1.9	0.14	7.0	6.2	72.0
	pH-stat	LED	KNO_3	10	8.0	21	2.56	$1.21 \cdot 10^9$	1.32	19.4	1.5	0.19	7.3	6.4	69.3

[a] Estimated after the 8-day daily feeding period.

pieces at 680 and 420 nm was measured in a spectrophotometer (DU 730, Beckman Coulter, USA).

To ensure that bleaching of the HBR's PE did not affect cell growth, the presence of bleached UV-stabilized PE during algal growth was tested in *P. oculatum* flask cultures in duplicate. Same-size pieces of bleached PE were added to the culture medium and inoculated with algal cells in 250-cm^{-3} Erlenmeyer flasks (100 cm^{-3} culture liquid) to simulate the contact of the culture with the PE surface in the HBR. Control flasks without PE pieces were also included in duplicate. Samples were taken after 7 and 14 days for growth measurement.

2.5. Calculations and statistical analysis

The average values of two growth metrics, maximum cell concentration N_{max} and maximum dry cell weight DW_{max}, between the duplicate photobioreactors (where applicable) during the cultivations of *P. oculatum* were calculated and reported in Tables 1 and 2 for indoor and outdoor cultivations, respectively. Growth and nutrient consumption rates, cell mass productivity, as well as cell mass yields on light energy (outdoors only), nitrogen, and phosphorus, were calculated based on growth and nutrient consumption (Tables 1 and 2). More specifically, the maximum specific growth rate μ_{max} (d^{-1}) of algae during the indoor and outdoor cultivations of *P. oculatum* was calculated during the exponential phase from the slope of the linear regression curves of the natural logarithm of cell density (ln$N$) versus cultivation time (t). Because the exponential phase was relatively brief and the cultures subsequently grew linearly, only 2–4 growth data points were used for the estimation of μ_{max}. Those points were also used to calculate the slope and R^2 of the linear regression (data not shown).

The nutrient consumption rates for nitrogen R_N and phosphorus R_P (mg L^{-1} d^{-1}) were calculated from the change in the residual N or P concentration C (mg L^{-1}) over time within a certain period of cultivation t (d), as described by the following equation:

$$R = \frac{C_1 - C_0}{t_1 - t_0} \tag{2}$$

The volumetric productivity P_v (g L^{-1} d^{-1}) was calculated from the change in cell mass concentration X (g L^{-1}) over time within a certain period of cultivation t (d) according to the following equation:

$$P_v = \frac{X_1 - X_0}{t_1 - t_0} \tag{3}$$

The average areal productivity P_a (g m^{-2} d^{-1}) was calculated from the volumetric productivity based on the HBR volume $V = 150$ L and surface area $S = 3.0$ m^2 according to the following equation:

$$P_a = P_v \times \frac{V}{S} \tag{4}$$

The total cell mass yields on nitrogen Y_N (5) and phosphorus Y_P (6) were calculated by dividing the final algae cell mass concentration by the consumed grams of nitrate nitrogen N_{total} and the consumed grams of phosphate phosphorus P_{total}, respectively, according to the following equations:

$$Y_N = \frac{DW_{max}}{N_{total}} \tag{5}$$

$$Y_P = \frac{DW_{max}}{P_{total}} \tag{6}$$

The solar radiation data from the pyranometer were converted to photosynthetically active radiation (PAR) at 400–700 nm and expressed in photon flux density (μmol m^{-2} s^{-1}) by using the approximate conversion factor 4.57 [23]. The total cell mass yield on light Y_I was calculated by dividing the final algae cell mass concentration by the total incident mol of photons I_{total} according to the following equation:

$$Y_I = \frac{DW_{max}}{I_{total}} \tag{7}$$

Calculations and statistical analysis were performed using Microsoft Excel 2013.

3. Results

3.1. P. oculatum growth optimization

In order to determine key growth parameters that will guide proper outdoor cultivation, a set of indoor lab experiments was first conducted using vertical flat-panel photobioreactors with working volume of 3.5 or 7.5 L, as summarized in Table 1. The concentrations of residual nitrogen (N) and phosphorus (P) in the medium were regularly monitored throughout the cultivations and appropriate amounts of N and P sources were supplemented, when needed, to prevent nutrient limitation.

3.1.1. Effect of CO_2 delivery method

The continuous vs. pulsed CO_2 (pH-stat) delivery comparison was studied in four 3.5-L vertical flat panel photobioreactors (each supply mode in duplicate) running in parallel with pH and temperature being recorded. Less than 1 day of lag phase was observed and the culture grew exponentially up to day 12, where the stationary phase started (Fig. 2a–c). The CO_2 delivery method did not affect the culture progression, as OD_{680} (Fig. 2a) and cell concentration (Fig. 2b) patterns were essentially identical. Moreover, similar nutrient (N and P) consumption curves were observed between the two CO_2 supply conditions (Fig. 2d). Final OD was 11.8 and 11.7 after 14 days for cultures supplied with CO_2 continuously and pulsed, respectively. Maximum cell concentration was also similar between cultures with continuous, $0.89 \cdot 10^9$ cm^{-3}, and pulsed CO_2 delivery, $0.94 \cdot 10^9$ cm^{-3}. Some differences in the cell mass production curve shape were observed after day 10 between the two delivery conditions. Overall, the pulsed CO_2 provided a smoother growth curve, while the continuous supply resulted in incremental cell mass increases and a 17% higher final DW (Fig. 2c). Same maximum growth rates and comparable nutrient consumption rates were calculated for both conditions, although cell mass

Table 2
Algae cultivation performance in a 150-L HBR system during two consecutive outdoor operation cycles. Growth parameters, average nutrient consumption rates (n = 7–30), and daily productivities (n = 19–27) and their standard deviations (±) are reported for each cycle.

Parameter	1st cycle	2nd cycle	Unit
Cultivation Period	34	34	days
DW_{max}	2.70	3.22	g L^{-1}
N_m	1.61×10^9	1.75×10^9	cm^{-3}
μ_{max}	0.55	0.27	d^{-1}
R_N	9 ± 7	11 ± 2	mg L^{-1} d^{-1}
R_P	1 ± 1	0.6 ± 0.3	mg L^{-1} d^{-1}
P_v	81 ± 41	101 ± 86	mg L^{-1} d^{-1}
P_a	4 ± 2	5 ± 4	g m^{-2} d^{-1}
Y_I	0.074	0.079	g per mol of photons
Y_N	8.9	10.9	g per g of NO_3–N
Y_P	125.4	119.1	g per g of PO_4–P

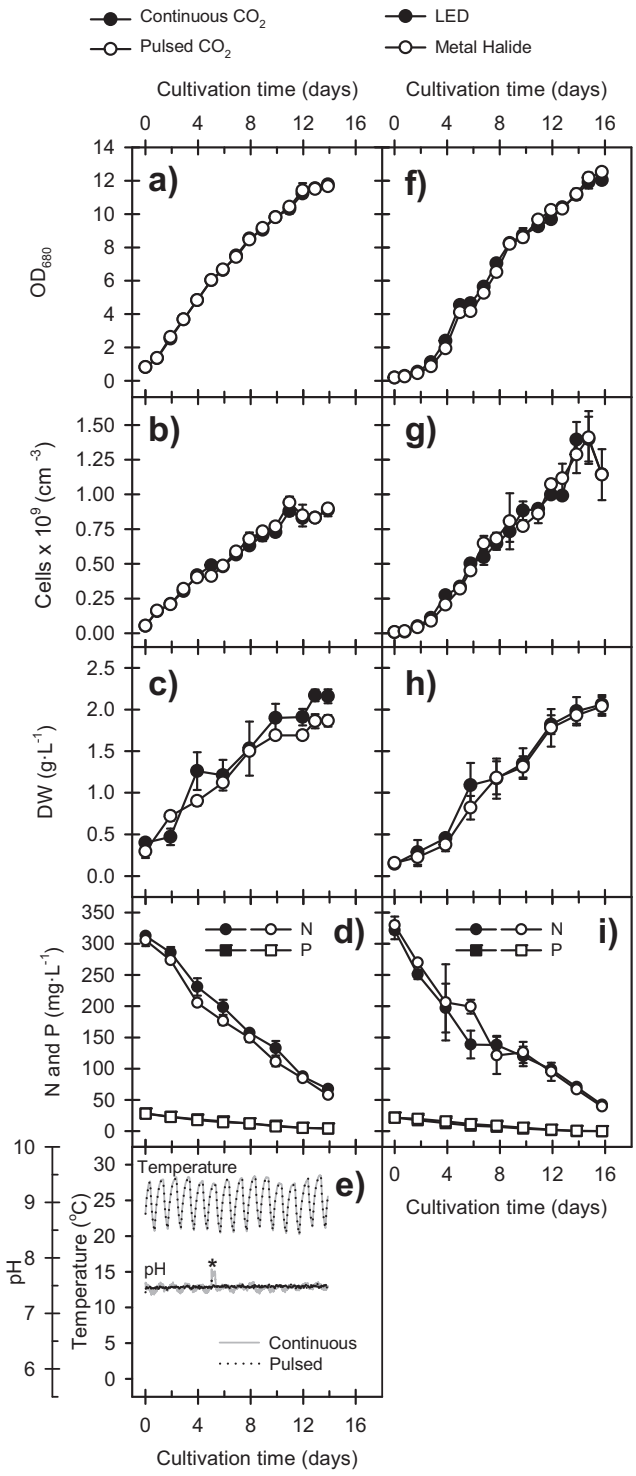

Fig. 2. Effect of CO_2 delivery method (a–e) and light source type (f–i) on *P. oculatum* growth (OD_{680}, cell concentration, DW, N and P consumption). The continuous and pulsed CO_2 delivery was studied in four 3.5-L vertical flat panel photobioreactors (each supply mode in duplicate) running in parallel with pH and temperature being recorded (e). The effect of LED and metal halide lamps was studied in two 7.5-L vertical flat panel photobioreactors running in parallel. The symbol * marks a spike in pH measurement due to CO_2 cylinder switching.

productivity was 12% lower in the pH stat system and the yields per consumed N and P were lower by 15% and 11%, respectively (Table 1). The recorded culture temperatures were the same in all four reactors (Fig. 2e). Temperature rose to 28.5 °C during the light phase and dropped to 20.2 °C during the dark phase of cultivation. The average culture pH of the pH-stat bioreactors was 7.48 ± 0.02, whereas the pH of the continuous supply showed higher fluctuation, 7.47 ± 0.05, and followed the pattern of culture temperature and light:dark phases.

3.1.2. Effect of light source (LED vs. MH)

LED panels and MH lamps were used to illuminate two 7.5-L vertical flat panel photobioreactors running in parallel. A 400-W ballast provided power to the MH lamps, while each LED panel required 32 W, according to the manufacturer. A single MH lamp was used to illuminate the 7.5-L flat panel PBRs while three LED panels were used to cover the same surface. There was no significant difference in growth as estimated by OD_{680}, cell concentration, and DW of cultures exposed to LED and MH lights (Fig. 2f–i). Maximum OD_{680} was 12.0 and 12.5 after 16 days for cultures exposed to LED and MH lights, respectively. Cell concentration and DW reached similar maxima on Day 15 and 16, respectively (Table 1). Maximum dry cell weight was 2.06 g L^{-1} for cultures subjected to LED lights and 2.04 g L^{-1} for cultures under MH illumination. Furthermore, similar nutrient consumption rates, cell mass productivities, and yields were calculated for both light sources (Table 1). However, the μ_{max} of *P. oculatum* cells was 47% higher under LED illumination compared to the MH lights.

3.1.3. Effect of nitrogen source

Based on preliminary indoor cultures in flat panel photobioreactors (data not shown), *P. oculatum* consumed about 316 mg of nitrate nitrogen and 29.3 mg of phosphate phosphorus to support cell mass growth of 2 g L^{-1} per dry basis. To minimize the chances of contamination during repeated nutrient feedings, the option of adding the above nutrient amounts (full strength) at the beginning of each batch cultivation was explored. In order to prevent any nutrient toxicity effects to *P. oculatum* cells, a set of flask experiments was performed at three initial levels of nutrients, full strength, half strength, and original medium strength, as described earlier, and presented in Fig. 3a. All cultures started with the same inoculum, as shown by OD_{680} at day 0, and continued to grow in parallel under the same conditions. The OD_{680} of samples at day 7 and 14 were the same between the 3 nutrient levels and no growth inhibition due to toxicity was observed. Similar flask experiments were conducted for assessing the toxicity of two other nitrogen sources, urea and ammonium, at the same (316 mg L^{-1}) and half (158 mg L^{-1}) the elemental N concentration of the nitrate source (Fig. 3b). Based on OD_{680} of samples at day 4 and 7, changing the N source to urea had no effect on the growth of *P. oculatum*, as it was comparable with nitrate. In contrast, growth on ammonium seemed to be greatly diminished. Ammonium appears to be toxic at the above initial N concentrations, as no increase in OD_{680} was observed over the 7-day cultivation period. The final pH of the cultures varied from 8.0 to 8.8. Lower pH values were observed in flasks with ammonium (8.1), whereas the final pH values in flasks with nitrate and urea were the same, 8.7. In order to identify the maximum initial concentration of ammonium that would not inhibit growth, a number of various initial concentrations was investigated (Fig. 3c). Initial NH_4–N concentrations above 39.5 mg L^{-1} seem to inhibit growth, while growth on 39.5 mg L^{-1} and 19.8 mg L^{-1} of NH_4–N was comparable to growth on 316 mg L^{-1} of nitrate nitrogen. The final pH of the ammonium cultures increased as the initial NH_4–N concentration decreased. The final pH values in NH_4 flasks were 8.1, 8.2, 8.3, 8.5 and 8.6 at 316, 158, 79, 39.5 and 19.8 mg L^{-1} of NH_4–N, respectively.

The effect of the nitrogen source type on *P. oculatum* growth was studied in six 3.5-L vertical flat panel photobioreactors (each N

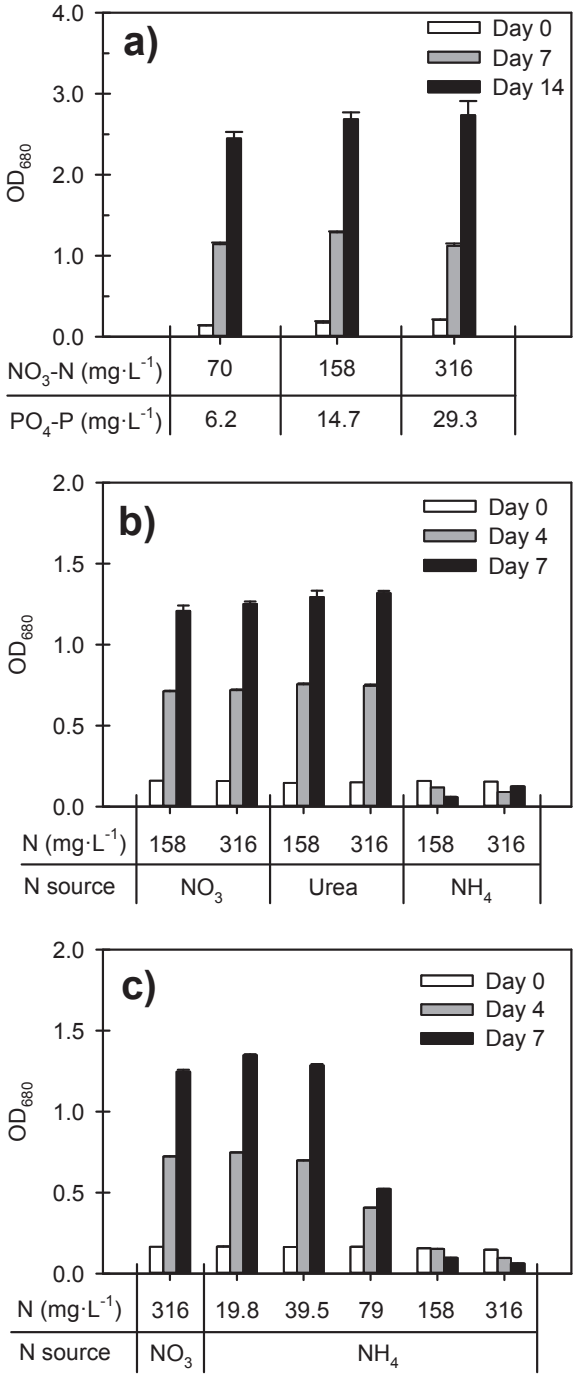

Fig. 3. *P. oculatum* nutrient (nitrogen and phosphorus) source toxicity. Effect of (a) Three levels of initial NO_3–N and PO_4–P concentrations; (b) Two levels of each of the nitrogen sources nitrate, urea and ammonium; and (c) Five levels of NH_4–N on *P. oculatum* growth in flask cultures. The OD_{680} at day 0 and after 14 (a) or 7 (b and c) days is compared. Initial PO_4–P concentration (29.3 mg L^{-1}) was kept the same in all (b) and (c) flasks. HEPES buffer was added to (b) and (c) flasks.

source in duplicate) running in parallel with pH and temperature being recorded. Despite being initially supplied with the same elemental nitrogen concentration, substantial differences became apparent when NH_4Cl was used as nitrogen source compared to KNO_3 or urea (Fig. 4a–e). The cultures supplied with NH_4Cl reached stationary phase earlier, at about half the cultivation time compared to the cultures provided with the other two nitrogen sources, and no further growth was observed even after 14 days (Fig. 4a–c). The maximum OD_{680} was 14.9 ± 0.6 on Day 19 for NO_3, 15.6 ± 0.7 on Day 19 for urea, and only 7.7 ± 0.3 on Day 13 for NH_4. The growth as estimated by maximum OD_{680}, cell concentration, and DW was comparable between the nitrate and urea sources, but was about half of that in ammonium cultures (Table 1). The maximum growth rate in ammonium was 28% and 16% lower than in nitrate and urea cultures, respectively. It should be noted that because of the ammonium toxicity identified in the flask study outlined earlier, we elected to supply NH_4Cl in the photobioreactor study in 8 equal aliquots to keep the ammonium concentration below the inhibition levels reported earlier, whereas the total nitrate and urea amounts were added from the beginning of the cultivations and their consumption curves are shown in Fig. 4d.

The average nitrogen source consumption rates were comparable in ammonium and urea, while nitrate consumption rate was about half. The average ammonium consumption rate was estimated between the time of the final aliquot and the experiment's shutdown. The phosphorus consumption rate was similar in cultures with NO_3 and urea and 22% higher than in ammonium. The highest cell mass productivity was observed in urea cultures, 13.6 g m^{-2} d^{-1}, followed by growth on ammonium, 11.6 g m^{-2} d^{-1} and on nitrate, 10.0 g m^{-2} d^{-1}. The cell mass yield was higher with nitrate and urea compared to ammonium as nitrogen source (Table 1). The recorded bioreactor temperatures were similar in all six cultures and ranged from 21.3 °C (dark phase) to 32.3 °C (light phase) (Fig. 4e). The pH of the nitrate and urea cultures was maintained by the pH-stat system at 7.5 as shown in Fig. 4e, except for some artificial pH spikes recorded in one of the pH controllers (nitrate source) that was replaced twice. On the other hand, a significant drop in pH was recorded after day 4 in ammonium cultures reaching pH 4.9 at shutdown.

3.1.4. Effect of inoculum size

No significant difference was observed in OD_{680} (Fig. 4f) and cell concentration (Fig. 4g), when inoculum size was varied (10%, 15%, and 20% of culture volume). No significant lag phase was observed in any of the runs and all cultures grew exponentially up to day 12, where stationary phase was reached. Maximum OD_{680} after 14 days was 12.6 ± 0.4, 10.8 ± 0.3, and 12.0 ± 0.2 for cultures provided with 10%, 15% and 20% inoculum, respectively. There was some deviation in OD after the 9th day between the two bioreactors that started with 15% inoculum (Fig. 4f). This was most likely due to a malfunction of the light source of one of these two bioreactors, which resulted in half the normal light output after the 9th day. Still, there were only small differences between maximum OD_{680} and final cell concentration among inoculum sizes (Table 1), although the final DW at 10% inoculum was 26% higher than at 15% and 7% higher than at 20% inoculum. The highest DW, 2.05 g L^{-1}, as well as highest maximum growth rate, 1.39 d^{-1}, and cell mass productivity 0.14 g L^{-1} d^{-1}, were achieved after starting the culture with 10% inoculum. Interestingly, the average nitrogen consumption rate at 15% inoculum was 13–14% higher than at 10% and at 20% inoculum levels, while the average phosphorus consumption was the same in all runs. The cell mass yields on supplied N and P were lower at 10% and 20% inoculum compared to the 15% inoculum level (Table 1). Culture temperature variations during the light:dark photoperiods were the same in all 6 photobioreactors (Fig. 4j). The pH-stat system maintained the cultures' pH at 7.5 during the experiment as shown in Fig. 4j, except for some minor artificial pH spikes recorded in one of the pH controllers (one of the 10% inoculum duplicate bioreactors) that had to be replaced.

3.1.5. Effect of pH

Three pH levels, 7.0, 7.5, and 8.0, were tested in duplicate in 3.5-L bioreactors to determine their effect on *P. oculatum* growth. All

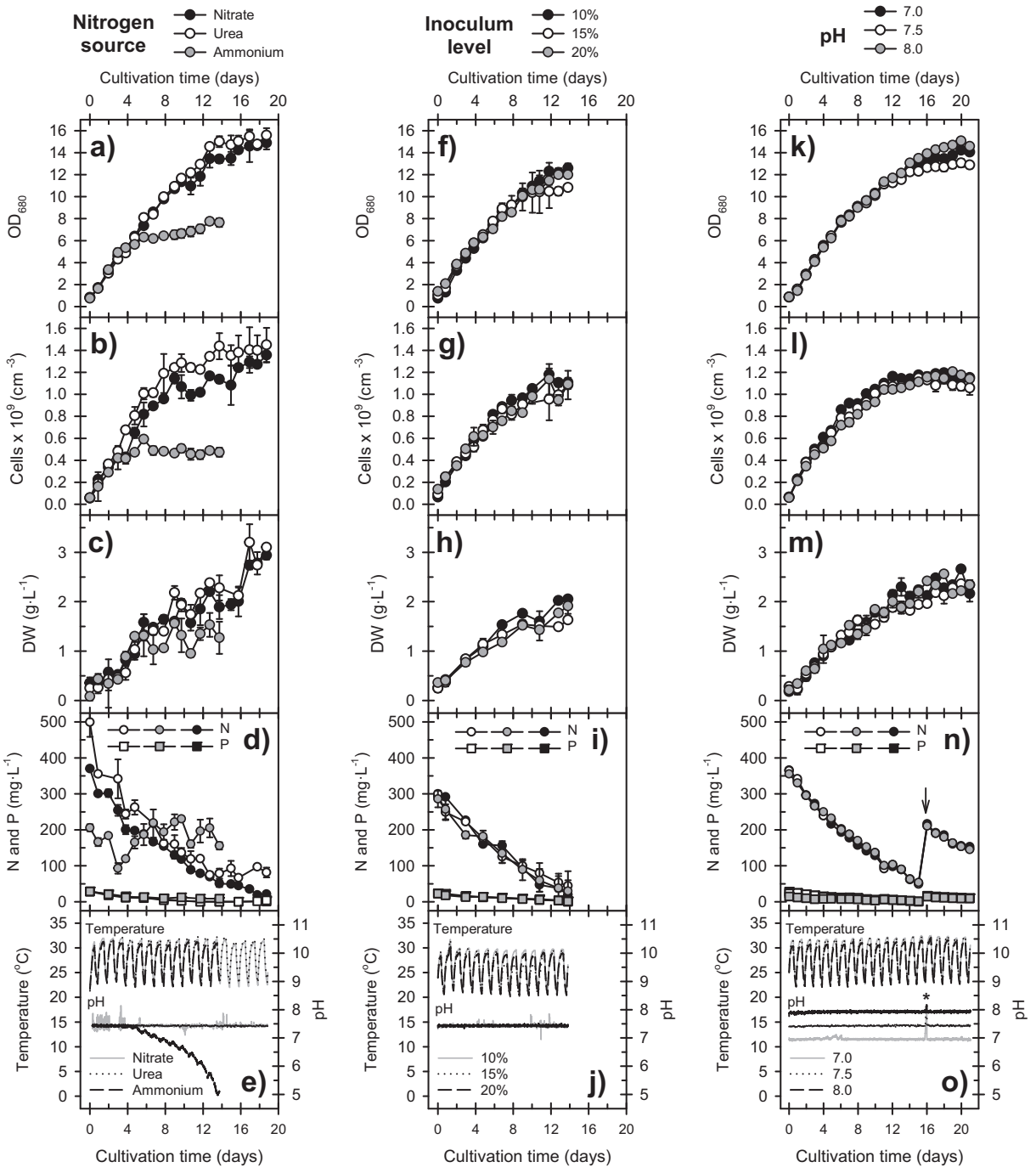

Fig. 4. Effect of nitrogen source (a–e), inoculum level (f–j), and culture pH (k–o) on *P. oculatum* growth (OD_{680}, cell concentration, DW, N and P consumption). The cultivations were performed in six 3.5-L vertical flat panel photobioreactors (each condition in duplicate) running in parallel with pH and temperature being recorded (e, j and o). The symbol * marks a spike in pH measurement due to CO_2 cylinder switching. The arrow marks nutrient (N and P) feeding.

cultures grew exponentially after a very brief lag phase of about 1 day and reached stationary phase around days 12–13 based on cell concentration change (Fig. 4l). However, OD continued to rise in cultures maintained at pH 7.0 and 8.0 (Fig. 4k). Maximum OD observed after 20 days was 14.2 ± 0.1, 13.1 ± 0.4, and 15.1 ± 0.1 for cultures at pH 7.0, 7.5, and 8.0 respectively. While there was a deviation in OD after the 11th day, overall the cultures followed a similar growth pattern based on the measured OD_{680} (Fig. 4k), cell concentration (Fig. 4l), and DW (Fig. 4m). Comparable maximum cell concentrations and DW were estimated for all cultures regardless of set pH, as summarized in Table 1. Moreover, no significant difference on maximum growth rates and nitrate and phosphorus consumption rates was observed between the different pH setups. Nitrate and phosphate were provided on day 16 to prevent nutrient limitations from affecting growth (Fig. 4n). Cell mass productivity was 45% and 51% higher in pH 7.0 than in 8.0 and 7.5, respectively (Table 1). Furthermore, cell mass yields on nutrients (nitrate and phosphate) were slightly higher in pH 7.0 than in 7.5 and 8.0. No difference in the recorded culture temperature daily variation was observed in the six reactors (Fig. 4o). During the light

period temperature rose to 32.5 °C and dropped to 22.1 °C during the dark period. The pH-stat system maintained the cultures' pH generally constant at 7.0, 7.5 and 8.0 during the experiment.

3.2. HBR operation

3.2.1. Material assessment and general operation

The HBR and the growth medium were sterilized in situ using sodium hypochlorite (household bleach) and the possible effects of bleach sterilization of the HBR plastic material on its light transmittance was assessed. The mean light transmittance of the HBR PE material (control) was 95.0% (standard error = 0.7), while the bleached PE sheet allowed 92.9% (standard error = 0.4) of light to pass through. Their absorbance at 680 nm (red light) and 420 nm (blue light) was also measured as the red and blue lights are considered preferable among the green algae species for the purposes of photosynthesis [24]. At 680 nm no difference in the absorbance between the control and the bleached sample was measured, whereas the absorbance at 420 nm increased by 25% after bleaching (data not shown). The possible effect of bleached PE on algal growth was tested with *P. oculatum* cultures, which were inoculated in the absence or presence of bleached PE pieces and the OD_{680} of the cultures was recorded. The OD_{680} of the unbleached PE (control) cultures on day 0, 7, and 14 was 0.174, 0.736 and 1.750, respectively. The bleached PE cultures showed a very similar growth pattern, where the OD_{680} of the on day 0, 7, and 14 was 0.178, 0.860 and 1.790, respectively.

The 150-L HBR prototype was successfully subjected to hydraulic testing, leak test, and wind and rain resistance tests. The enclosed reactor was designed with an 8-blade paddlewheel that was rotated by a low-speed motor. The average ($n = 30$) fluid velocity inside the HBR was 15.1, 20.4, and 21.1 cm s^{-1} when the paddlewheel rotation speed was set at 0.33, 0.5, and 0.66 Hz, respectively (data not shown). The rotation speed of 0.5 Hz was deemed satisfactory and energy-efficient and was selected for HBR operation. The paddlewheel was able to ensure adequate mixing with a very low energy consumption of just 4.7 W to circulate the 150 L of culture. The motor operated continuously without any overheating or other issues.

3.2.2. Algae cultivation performance

The algae cultivation performance of the 150-L HBR was demonstrated outdoors in central Florida by growing *P. oculatum* over 68 days, spanning from February until April. A volume of 10% (15 L) inoculum was transferred from the indoor flat-panel bioreactors after reaching an OD_{680} of 8.6 and was supplemented with 90% (135 L) fresh medium as described earlier. The HBR was operated outdoors for two consecutive cycles of 34 days each. After the 1st cultivation cycle reached stationary phase, 90% (135 L) of the culture volume was harvested and the remaining 10% was used as inoculum for the 2nd cultivation cycle. As growth progressed, the culture medium was supplemented with macro-nutrients (nitrate and phosphate) in order to prevent any nutrient limitations and thus a reduction in algal cell mass productivity. The growth parameters (OD_{680}, DW, and cell concentration) and nutrient (NO_3–N and PO_4–P) concentrations were measured in samples drawn from the HBR samples. They are presented, along with the HBR operating conditions (DO, pH, temperature, and solar radiation), in Fig. 5.

A maximum culture OD_{680} of 16.8 was observed at the end of 2nd cycle, after 34 days of cultivation, which is 11% higher than the maximum observed in the 1st cycle (Fig. 5a). High cell concentrations were achieved in the 1st and 2nd cycles, $1.54 \cdot 10^9$ cm^{-3} and $1.75 \cdot 10^9$ cm^{-3}, respectively. An increase of 13% was observed in final cell concentration during the 2nd cultivation. Consequently, the final dry cell mass concentration reached high levels, 2.70 and 3.22 g L^{-1} (Fig. 5a, Table 2). Strong linear correlations were identified between OD_{680}, DW, and cell concentration in the HBR for both cultivation cycles. As a result, cell concentration (N, in cm^{-3}) could be used to expediently calculate cell mass concentration (DW, in g L^{-1}) using the correlations $DW = 1.66 \cdot 10^{-9} \times N$ (Eq. (8), $R^2 = 0.999$, $P < 0.0001$) and $DW = 1.88 \cdot 10^{-9} \times N$ (Eq. (9), $R^2 = 0.985$, $P < 0.0001$) for the 1st and 2nd operation cycle, respectively. Furthermore, the OD_{680} of an algal sample could also be used to estimate the algal cell mass concentration (DW, in g L^{-1}) via the correlations $DW = 0.180 \times OD_{680}$ (Eq. (10), $R^2 = 0.997$, $P < 0.0001$) and $DW = 0.189 \times OD_{680}$ (Eq. (11), $R^2 = 0.995$, $P < 0.0001$) for the 1st and 2nd operation cycle, respectively.

The correlation between DW with OD_{680}, which allows for faster and less labor-intensive (if manually counted) or less costly (if automatically counted) measurements than cell counting, showed less variation between the two HBR operation cycles. OD_{680} was selected as a proxy for rapidly estimating the daily algal cell mass concentration and productivity during the 1st and 2nd outdoor HBR operation cycles using Eqs. (10) and (11), respectively.

The cell mass concentration, growth and nutrient consumption rates, productivity, and yields achieved in the 150-L HBR system during the outdoor operation cycles are summarized in Table 2. Highest average productivity was achieved during the 2nd cycle, 5 g m^{-2} d^{-1} or 101 mg L^{-1} d^{-1}, which is 25% higher than in the 1st cycle. However the productivity varied considerably from the mean values in both cultivations as depicted by their high standard deviations (SD) (Table 2), as the reactor was operated under realistic conditions. A limited lag phase was observed in both cycles, 1–2 days, followed by a brief exponential phase and then the cultures continued to grow linearly until stationary phase (Fig. 5a). The maximum specific growth rate, μ_{max}, was lower in the 2nd run (Table 2) and was observed during the first 3–4 days in both cycles (Fig. 5a). Nitrate concentration decreased linearly in both cycles (Fig. 5b), and the average NO_3–N consumption rate, R_N, was higher in the 2nd run, but within one SD of the average in the 1st run. The cell mass yield on the total consumed nitrogen, Y_N, was 22% higher in the 2nd cycle than the 1st (Table 2). On the other hand, the phosphate levels in the medium dropped by 42% in the first 5 days of cultivation in the 1st run and continued at a lower consumption rate (Fig. 5b). A more linear consumption rate of phosphate was observed during the 2nd cycle, where the average PO_4–P consumption rate, R_P, was about half compared to the 1st cycle. Nevertheless, the cell mass yield on the total consumed phosphate, Y_P, was about the same in both cycles (Table 2).

The average salinity of the medium was 2.8% and varied slightly (SD 0.1) during both cycles of the outdoor HBR cultivation. The DO levels in the culture varied between the 2 cultivation cycles. During the first cycle, a range of 115–311% was measured during each morning sampling with an average of 215% (Fig. 5b). On the other hand, a range of 121–201% and an average of 160% were observed during the second cultivation. The culture temperature ranged from a low of 3.7 °C during one cold night up to 38.0 °C in daytime, with an overall mean of 22.6 °C and a SD of 6.3 °C (Fig. 5c). The measured solar flux on the HBR surface ranged from 600 mW m^{-2} (night time) to 1.0994 kW m^{-2} (daytime) (Fig. 5c). No direct linear correlation was found between the daily productivity and (a) daily maximum temperature ($R^2 = 0.041$), (b) daily average temperature ($R^2 = 0.028$) or (c) daily solar energy ($R^2 = 0.038$).

4. Discussion

The marine microalgal strain *P. oculatum* was employed to demonstrate the algae cultivation potential of the novel HBR. First, we ran a series of controlled indoor growth experiments to identify key cultivation parameters, such as CO_2 supply mode, type of

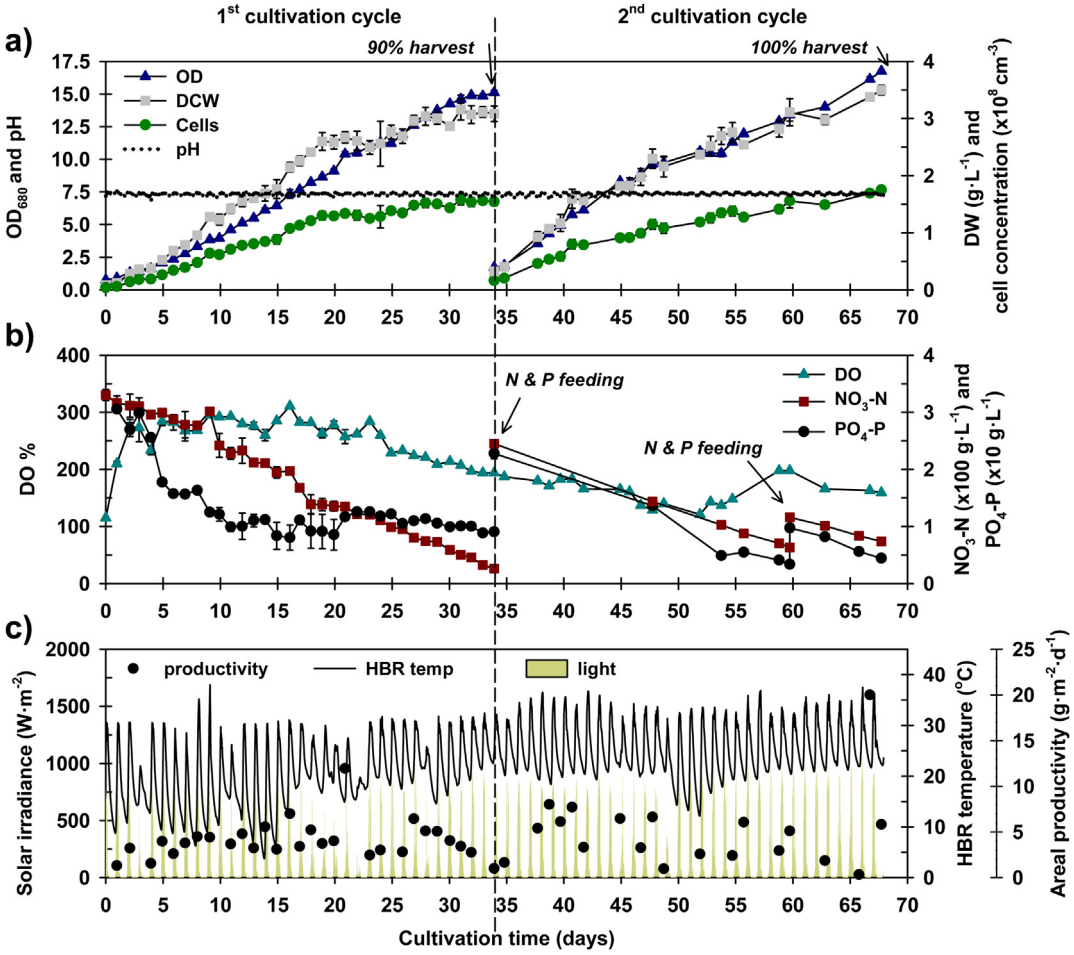

Fig. 5. Outdoor cultivation of the marine microalga *P. oculatum* in the 150-L HBR. At the end of the 1st cultivation cycle, 90% of the culture volume was harvested and the residual 10% was used as inoculum to initiate the 2nd cycle. (a) Growth parameters (OD_{680}, DW, and cell concentration) measured in HBR samples; (b) culture DO levels (in-situ sampling) and nutrient (NO_3—N and PO_4—P) concentrations measured in HBR samples; and (c) continuous recording of solar irradiance on HBR surface, culture pH, and temperature. Error bars show the standard deviation.

nitrogen source, inoculum size, and pH, that could boost growth, while reducing the operating cost of algae cultivation, a consideration of paramount importance to the technology commercial success. The *P. oculatum* growth study experiments were conducted in flat panel vertical photobioreactors at bench scale. The CO_2 delivery method did not affect the culture progression, and similar maximum growth and nutrient consumption rates were recorded in both modes. However, from an operating and economic standpoint, pulsed supply of CO_2 offered certain advantages: (1) It resulted in lower pH fluctuations; (2) provided a better means of controlling culture pH; and (3) generated a smoother growth curve thus making cell mass productivity more predictable, all of which are important for industrial applications. Furthermore, the pulsed system prevents oversupply or undersupply of CO_2 in the event of a pressure rise or drop at the CO_2 source. Even if CO_2 is available at a low or no cost from industrial sources, such as flue gas, it still requires energy for pumping [8].

The effect of the light source was investigated in order to select the best and more economical source for indoor algae cultivation needs, such as inoculum preparation. Given that light has been found to be limiting in *Chlorella vulgaris* cultures grown in photobioreactors [25], and that a closely related *Picochlorum* strain exhibited different physiological response to different light intensities [26], it was necessary to address whether different types of illumination (LED versus MH lights) would affect *P. oculatum* yields. In addition, capital cost, lifespan, and operating cost (in terms of energy use) of light panels should be given serious consideration to ensure high culture productivity at the lowest cost possible [17]. In our study we found that algae growth using LED lights was comparable to using more traditional MH lights at similar illumination levels. The LED panels were specifically designed by the manufacturer for growing plants and thus were equipped with more red and blue LEDs than white ones, since the red and blue lights are preferable for green algae photosynthesis [24]. Therefore, the higher maximum growth rate of *P. oculatum* under LED illumination compared to the MH lights could be attributed to more appropriate light wavelengths (red and blue) being available from the LED panels. Red light LEDs have been reported to yield high cell densities for *C. vulgaris* [17]. Furthermore, the combined power requirement of the three LED panels, 96 W, was four times lower than what one MH lamp required to operate. Since LEDs were more energy-efficient and more cost- and space-effective than MH lamps and their ballasts, all subsequent indoor experiments were conducted using LED lights exclusively.

Given that previous studies have indicated that inoculum size could have a substantial effect on algae cultivation performance, it was important to determine whether similar considerations applied to our reactor system and target organism. For example,

Hallenbeck et al. [27] found that final dry weight of *Nannochloropsis gaditana* increased with inoculum size at low and moderate light. Chen et al. [28] found that cell mass increased with inoculum size, but that lipid content and triacylglyceride (TAG, a biodiesel precursor) percentage of total lipids decreased with inoculum size in cultures of *Nannochloropsis* sp. Wang et al. [29] found that *Nannochloropsis oculata* cultures with the lowest initial OD_{682} had the highest growth rates at all light levels. Thus, optimal inoculum size may depend on culture goals (cell mass or lipid production) and culture conditions and may be species- or strain-specific [27–29]. Based on the results from our inoculum size experiment, we determined that increasing inoculum size from 10% to 15% or 20% did not affect growth significantly (Fig. 4f–h). Therefore, to reduce the cost associated with larger inoculum size preparation, a 10% inoculum level was adopted for subsequent indoor and outdoor *P. oculatum* cultivations.

High production costs have been an obstacle to commercial-scale algal biofuel production, so considering wastewater as a potential source of nitrogen is an attractive proposition [18,19]. Three popular nitrogen sources were tested: potassium nitrate, which is commonly used in artificial growth media, ammonium chloride, which is a common constituent of wastewaters and therefore readily available and inexpensive, and urea, which is a widely used inexpensive industrial feedstock and is also found in wastewaters. Response to wastewater-derived nitrogen sources is reported to be species-specific, as numerous species are able to grow as well or better using urea as a nitrogen source as compared to using nitrate [18,30,31]. Studies using ammonium as a nitrogen source have been less straight forward: Lourenco et al. [32] in a study of 10 species of marine microalgae found that some grew well and had larger cell volumes (i.e., *Isochrysis galbana* and *Synechococcus subsalsus*), while others failed to grow when provided ammonium as a nitrogen source (i.e., *Hillea* sp. and *Prorocentrum minimum*). Other species of green algae were able to grow on ammonium as a N source, but the use of synthetic buffers (PIPES and HEPES) or KOH dosing was required, when CO_2 was also supplemented [33]. The use of synthetic buffers or KOH, however, could become cost-prohibitive or even toxic. It may also be fruitful to investigate the possibility of co-culturing with ammonia-oxidizing bacteria [34]. Overall, urea from wastewater may be a viable and sustainable source of nitrogen for algal cultures [9,35], but the use of NH_4Cl may be problematic [5,33,36,37]. While response is largely species-specific, chlorophytes in general tend to be the most tolerant [38], so there is the potential to determine optimal culture conditions using urea and ammonium species from wastewater as a nitrogen source for *P. oculatum*.

No significant differences between *P. oculatum* cultures grown on nitrate and urea as nitrogen source were observed in our study; thus, industrial urea could serve as a low-cost source of nitrogen for algae cultivation. On the other hand, ammonium in the form of NH_4Cl, demonstrated significant toxicity towards *P. oculatum* growth necessitating the use of lower concentrations to prevent growth inhibition based on flask culture tests. To prevent such inhibition, ammonium was not added at full strength to the photobioreactors at the beginning of the batch cultivations, but rather in 8 daily doses (aliquots). Even then, ammonium addition resulted in about half the growth compared to the other N sources and dramatic decreases in culture pH were observed (Fig. 4e) that had a negative effect on algal growth even though HEPES was supplied as a buffer [33]. The toxicity of ammonium is probably due to the translocation of protons out of the algae cells during uptake of ammonium ions to maintain charge balance, causing a detrimental decrease in the pH of the growth medium [33,36]. Using NH_4Cl as a nitrogen source from wastewater streams is economically attractive [5,33,37], but additional research is necessary to reduce toxicity to the algal cells and buffer the pH variations adequately and cost-effectively. Prior acclimation of the algal cells to ammonium could improve its tolerance and pH regulation and thus enhance algal growth performance [38].

Three pH levels, 7.0, 7.5, and 8.0, were tested to determine the effect of pH on *P. oculatum* growth. CO_2 was used to control the pH by the pH-stat system at the selected pH set points. Since the relative proportion of the inorganic carbon species in the medium, CO_2, HCO_3^-, and CO_3^{2-}, depends on the pH of the culture, pH and carbon availability could have a compounding effect on algae growth. At high pH the HCO_3^- form is prevalent compared to CO_2, which is the preferred C source for algae [8]. Based on the results from this experiment, *P. oculatum* growth did not vary significantly in the 7.0–8.0 pH range. While a deviation in OD started after the 11th day (Fig. 4k), overall cultures followed a similar growth pattern. Cell mass productivity was a bit higher at pH 7.0 than at 7.5 and 8.0, which could be attributed to CO_2 prevalence at the lower pH. Furthermore, cell mass yields on nutrients were slightly higher at pH 7.0. However, maintaining pH at 7.0 required double the flow rate of each CO_2 pulse in the pH-stat system, which can lead to increased amounts of undissolved CO_2 that escapes the system and thus lowers CO_2 utilization efficiency. Previous research work reports that the growth rate does not depend on pH levels within these ranges for closely related *Nannochloris* species [39]. Additionally, Negoro et al. [40] reported that the tolerance of *N. oculata* to pH changes between 7.0 and 8.0 allows for culturing without strict pH control, a major advantage from a commercial standpoint. Based on the above, the midpoint pH of 7.5 was selected for outdoor *P. oculatum* cultivation in the HBR.

Our original 65-L HBR prototype used airlift systems for culture mixing and gas diffusion [11]. Although that unit was successful in cultivating a similar marine algal strain, *Nannochloris atomus* (reclassified as *Picochlorum atomus* [12]), its energy consumption was significant, as it required compressed air for culture mixing. Even at optimum mixing velocities, the efficiency of air-lift systems is usually less than 50% and the power consumption can be twice as high compared to paddlewheels [8]. In the present improved HBR design we introduced an eight-blade paddlewheel to reduce energy demand for mixing. The removal of the air-lifts created a need for efficient CO_2 supply, which was addressed by employing a ceramic micro-diffuser positioned at the bottom of the bioreactor directly downstream from the paddlewheel. The paddlewheel rotation speed of 0.5 Hz was selected for HBR operation for algae cultivations as no significant increase in flow velocity was observed above that speed. Borowitzka and Moheimani [8] suggested a culture circulation speed of 20–30 cm s^{-1} to prevent algae cells from settling, provide uniform light, and avoid thermal stratification phenomena in raceway ponds. Therefore, the attained average velocity in the HBR of 20.4 cm s^{-1} at 30 rpm should provide sufficient culture mixing to operate the unit efficiently. The energy utilization for culture mixing at 30 rpm of the 150-L improved prototype was only 4.7 W, which is considerably lower than air compressor demand in general. The 150-L HBR ran continuously for about 70 days without any leaks or mechanical issues caused by sun, wind or rain. Furthermore, the paddlewheel operated continuously without any motor overheating. The salinity of the culture remained fairly stable throughout the outdoor cultivation suggesting minimal water evaporation from the HBR and validating its enclosed design.

The HBR and the growth medium were sterilized in situ using sodium hypochlorite (household bleach) and the possible effects of bleach sterilization of the HBR plastic material on its light transmittance were assessed before the cultivations. Our previous tests on the transparency and algal compatibility of bleached PE showed less than 1% reduction in transparency and no effect on algal

growth [11]. This study confirmed that indeed there are no negative effects of the chemical sterilization on the light transmittance of the PE material. The visible light in general and the more relevant (to photosynthesis) red and blue light can be transmitted efficiently through the bleached PE of the HBR, which does not limit the available sunlight. Furthermore, the algal compatibility results verified that the addition of bleached PE did not cause any growth inhibition to *P. oculatum*, thus chemical sterilization of the HBR is considered harmless for algal growth.

Using the most promising cultivation conditions from an operating and cost standpoint, namely CO_2 supply controlled by a pH-stat system, nitrate as N source, inoculum 10% of culture volume, and pH 7.5, we cultivated *P. oculatum* in the HBR outdoors at a central Florida location and documented the bioreactor's performance. During the two outdoor growth cycles, the HBR resulted in highly reproducible algal growth (Fig. 5) with the added benefited of culture adaptation. The cell mass concentration reached 3.22 g L^{-1} in the 2nd cycle, which was 19% higher than in the 1st cycle. This cell mass density is comparable to that achieved in our previous HBR prototype with *P. atomus (N. atomus)* [11] and is typical of costly PBRs and considerably higher than in open raceway ponds [5]. It is critical for process economics to achieve high final cell mass concentration to reduce the cost of downstream processing (algae dewatering and recovery) [41]. The 2nd growth cycle resulted in slightly higher algae productivity and final cell mass concentration probably due to algae adaptation to the outdoor environmental conditions, such as light and temperature, and to slightly more sun insolation. The HBR operated under conditions of light intensity and ambient temperature that were realistic and hence highly variable. Several days were cloudy or rainy, but most were sunny. The total light recorded by the pyranometer on the surface of the HBR was 12% more in the 2nd cycle, during March–April, compared to the 1st cycle in February–March. Furthermore, the average temperature in the 2nd cycle, 25.2 °C, was higher than in the 1st cycle, 20.0 °C, during which a low of 3.7 °C was also briefly observed during one cold night.

Another operating factor that could affect algae growth is the level of dissolved oxygen in the HBR culture. Oxygen (O_2) is generated during photosynthesis and its accumulation can negatively affect algal productivity [8]. The average DO levels were 36% lower in the 2nd HBR cycle compared to the 1st cycle possibly in part due to acclimation of the culture to the outdoor conditions. DO concentrations above 300% of air saturation could be detrimental to algal cells and therefore could reduce productivity [42]. The average DO levels were 251% and 160% of air saturation in the 1st and 2nd cycle, respectively, with apparently no loss of productivity. However, the cell mass productivity was 25% higher in the 2nd cycle, where lower DO levels were observed, but other factors could also have attributed to that increase, such as higher light intensity, more favorable temperatures, and culture acclimation in the 2nd cycle. The airlift units in our previous HBR prototype, in addition to culture mixing, provided a means of reducing the O_2 concentration in the culture below 100% of air saturation [11]. The current HBR unit was able to operate without the need for additional oxygen removal.

The average productivity achieved during the 2nd cycle was 5 g m^{-2} d^{-1} or 101 mg L^{-1} d^{-1}, which is 25% higher than in the 1st cycle, but high daily variations were observed in both cultivation cycles. During indoor *P. oculatum* cultivations in the flat panel vertical photobioreactors, the overall average productivity was higher, 8 ± 2 g m^{-2} d^{-1}, probably due to well controlled and more favorable conditions, such as temperature and light, or the lower O_2 build-up due to air bubbling. The productivity in the outdoor HBR operation was lower than the one achieved in our previous HBR cultivations of *P. atomus (N. atomus)* [11], but the latter was operated during the sunnier months of the year (March–September) in Florida. Operating the HBR in more favorable weather and reducing O_2 accumulation could potentially increase cell mass productivity.

It should be noted that the high-density algal growth in the HBR over an extended period of time was achieved without any contamination issues, although the system was exposed to outdoor conditions. The enclosed design of the HBR system reduces considerably the chances of contamination compared to open systems. Contaminations with unwanted algae and/or other organisms is a common problem in open systems and can lead to costly culture crashes, which are a significant barrier to commercialization of algal biofuels [43].

5. Conclusions

A novel low-cost modular horizontal bioreactor (HBR) was developed for microalgae cultivation intended for biofuel and bioproducts manufacture. High-density growth of the marine microalgal strain *P. oculatum* was achieved in the HBR without any contamination issues over long periods of time under real-world outdoor conditions. Growth was reproducible in consecutive cultivation cycles and resulted in high cell mass productivity and final concentration. Possible acclimation of *P. oculatum* after the first cycle in addition to more favorable environmental conditions may have also contributed to the higher cell mass concentration and productivity in the subsequent growth cycle. By design, the HBR achieves significant cost savings in water and energy use compared to conventional bioreactor systems.

Indoor studies of *P. oculatum* growth preceded the HBR runs in order to optimize key growth parameters, such as nitrogen source, inoculum size, and pH, which were subsequently adopted outdoors. *P. oculatum* was able to grow well in the entire pH range of 7.0–8.0. Increasing the inoculum size above 10% did not yield better growth performance, so using a small inoculum is recommended as it allows higher throughput at commercial scale. Growth of *P. oculatum* on urea and nitrate yielded comparable results. This means that urea, a rather inexpensive bulk source of nitrogen, could serve as nitrogen source in commercial HBR cultivations. In contrast, ammonium was found to be inhibitory to *P. oculatum* cells and therefore its usefulness as a nitrogen source (e.g. from wastewaters) requires additional research.

We continue our research on HBR design and algal growth optimization. The design improvements implemented since our original prototype, which was equipped with airlifts, led to lower energy consumption and design robustness during lengthy outdoor operation. Our next step is the scale-up and techno-economic evaluation of a commercial-size HBR to assess the scalability of the technology. Through algal strain selection and adaptation, as well as with longer operating experience, we expect the HBR to achieve even higher sustained productivity. The reactor shows promise for large-scale algal cultivation and could thus help advance the potential of algae as a cost-effective and sustainable feedstock for renewable transportation fuels and bioproducts.

Disclosure

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