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Study of landfill leachate as a sustainable source of water and nutrients for algal biofuels and bioproducts using the microalga *Picochlorum oculatum* in a novel scalable bioreactor

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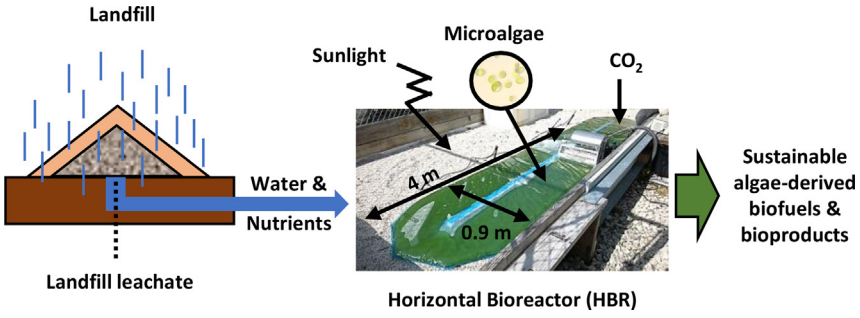
GRAPHICAL ABSTRACT



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ABSTRACT

High water demand is a major challenge for the algae industry, so cultivating algae in wastewater can have the double benefit of biomass production and water remediation. The use of landfill leachate (LL), which is wastewater generated in landfills, was investigated to grow the microalga *Picochlorum oculatum* in a novel horizontal bioreactor (HBR), a low-cost modular cultivation system that reduces water evaporation and contamination risk thanks to its enclosed design. Pilot-scale (150 L) and commercial-scale (2000 L) HBRs that were operated outdoors in Florida using LL in batch and semi-continuous modes generated high cell density cultures ($1.7{\cdot}10^{9}$ cells mL^{-1}) and reached up to 1.9 g L^{-1} of dry biomass suitable for biofuel production. Demonstrating the ability of ample non-potable water sources, such as LL, to support algae cultivation is essential for improving the sustainability and cost-effectiveness of commercial algal biofuels and bioproducts, as freshwater resources become increasingly scarce.

1. Introduction

Microalgae are considered a promising renewable fuel feedstock based on the lipids that they synthesize (Nhat et al., 2018; Wijffels and Barbosa, 2010). However, the algae industry is confronted with major commercialization and sustainability challenges due to the high water and energy demands involved in large-scale cultivations (Lam and Lee, 2012; Pate et al., 2011). These high demands, in turn, increase the production cost of algal products and therefore limit their competitiveness as fossil fuel alternatives. Recent projections for fresh water use during large-scale algae cultivations raise significant sustainability issues (Pate et al., 2011), whereas evaporative water losses can range

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from 35 to 3400 gallons per gallon of produced algal biofuel, depending on the location, cultivation system, downstream post-harvest processing, and degree of water capture/recycling (Cooney et al., 2011; Pate et al., 2011; Yang et al., 2011). Therefore, efforts for improving water and nutrient cost-efficiency and sustainability in the algae industry should target both the source (low-cost, renewable) and utilization (efficiency, loss reduction) of water and nutrients. Strategies include reduction of process losses (e.g. water evaporation, process energy, nutrient feed), recovery and recycling of water and nutrients in the process, and utilizing wastewater and nutrient sources from existing industrial operations (co-location).

Cultivating algae in wastewater can accomplish two goals simultaneously: produce algal biomass for energy (fuels) and bioproducts and remediate low-quality water for recycling and reuse (Cheah et al., 2016; Edmundson and Wilkie, 2013; Nhat et al., 2018; Pittman et al., 2011; Salama et al., 2017). Landfill leachate (LL), an untapped source of wastewater, is generated in large amounts annually in landfill operations by rainwater percolating through the deposited waste materials. Just in the State of Florida, LL generated in active landfills is estimated at 7000 gallons per day per acre (about 24 million liters per hectare per year), which must be treated effectively to be safely discharged back into the environment (Meeroff et al., 2016). However, LL water may contain pollutants and toxic substances that can inhibit algal growth, such as dissolved organic matter, inorganic macro-components (including some that can serve as algal nutrients), heavy metals, and anthropogenic 'xenobiotic' organic compounds (Cheah et al., 2016; Kjeldsen et al., 2002). Microalgae have been previously reported to grow in LL usually after some treatment or dilution of the leachate in lab environments (Cheung et al., 1993; Edmundson and Wilkie, 2013; Lin et al., 2007), while some algal species have been explored for biological treatment of LL effluents (Cheah et al., 2016). Hence, landfill leachate can potentially become an asset as a sustainable source of water for cultivating microalgae for biofuel production (Cheah et al., 2016; Cho et al., 2011; Ji et al., 2013; Salama et al., 2017). Furthermore, landfills tend to have unutilized land that would allow co-location of algae operations, eliminating the competition with agriculture for arable land and thus reducing land use change (Pate et al., 2011).

Microalgae are currently cultivated at large scale mostly in open raceway ponds due to their low capital and operating costs. Photobioreactor systems are also used because of higher productivity and lower contamination compared to open systems, but they suffer from high capital cost and energy use (Wijffels and Barbosa, 2010; Zittelli et al., 2013). A novel horizontal bioreactor (HBR) has been designed and previously demonstrated for outdoor algal cultivation that combines the positive features of raceways and photobioreactors to reach high productivity, while keeping both capital and operating costs low and reducing water and energy use (Dogaris et al., 2016, 2015). Based on the HBR's modularity, projected low cost, and enhanced sustainability profile, the feasibility of an integrated algal process is envisioned that uses wastewater, reclaimed nutrients, and recycling loops to produce a portfolio of renewable fuels (jet fuel and diesel), bioproducts (nutraceuticals and chemicals), and animal and fish meal in a more sustainable way, as outlined in the block diagram of Fig. 1.

In the present study, the potential of LL to serve as an abundant and inexpensive source of water was assessed in outdoor HBR cultivations that can render algae processes more sustainable and cost effective. LL from a Florida landfill operation was collected and analyzed for nutrients and growth-inhibitory components. Algal biomass production utilizing LL as the sole water source was demonstrated using the microalgal strain *Picochlorum oculatum* in a 150-L pilot-scale HBR unit, followed by scaling up and successful cultivation in a 2000-L commercial-scale unit under real-world conditions in batch and semi-continuous modes over extended periods of time. Furthermore, algal biofuel production projections were performed based on the actual productivity and compositional analysis of the produced algal biomass.

2. Materials and methods

2.1. Wastewater collection and chemical analysis

LL wastewater was collected from the Charlotte County Zemel Road Municipal Solid Waste Landfill (Punta Gorda, Florida). The Zemel Road Landfill, which is owned and operated by the Charlotte County government since 1975, covers 44 ha and is encircled with an underground bentonite slurry wall that serves as a liner for ground water protection (Charlotte County, 2017). Like other properly managed landfills, the Zemel Road Landfill has an on-site leachate collection and treatment facility, which includes biophysical treatment (mixing with powdered activated carbon and aeration in activated sludge), sand filtration (polishing and removal of suspended solids), chlorination, and a deep injection well for final LL disposal (Rogoff et al., 1999). The LL used in the present study for cultivating algae was collected from the landfill's confined deep well.

Wastewater analysis was conducted at Advanced Environmental Laboratories, Inc. (Tampa, Florida) according to water analysis protocols established by the U.S. Environmental Protection Agency (EPA) and the National Environmental Methods Index (NEMI). The analysis included the metals Ag, As, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Pb, Sb, Se, Zn, and Hg (method: EPA SW-846); ammonia (method: EPA 350.1); semivolatile organic compounds (method: EPA 8270C); chloride (method: EPA 300.0); total phosphorus (method: EPA 365.4 after copper sulfate digestion); chemical oxygen demand (COD) (method: EPA 410.4); alkalinity (method: NEMI SM 2320B); conductivity (method: NEMI SM 2510B); total solids (method: NEMI SM 2540B); sulfide (method: NEMI SM 4500-S D); nitrate and nitrite (method: NEMI SM 4500NO3-F); total organic carbon (TOC) (method: NEMI SM 5310B).

2.2. Strain selection

The marine microalgal strain *P. oculatum* UTEX LB 1998, previously classified as *Nannochloris oculata* (Henley et al., 2004), was obtained from the Culture Collection of Algae at the University of Texas at Austin and used in the present study. Flask stock cultures of *P. oculatum* were maintained at 23 °C in a shaker (150 rpm) under continuous LED illumination (approximately 2 klux) and diluted (90% volumetrically) with fresh medium every 30 days. The marine growth medium was prepared as described previously (Dogaris et al., 2015).

Preliminary water toxicity experiments were conducted to assess the suitability of LL as a water source for growing microalgae. A 10% (v/v) inoculum of *P. oculatum* from the stock cultures was transferred into sterilized (autoclaved at 120 °C for 20 min) 250-mL flasks containing 90 mL of: (a) clean water (sterile deionized water), (b) sterile LL (autoclaved at 120 °C for 20 min), and (c) raw LL (non-sterile). All media were supplemented with artificial salts and nutrients as described in Section 2.2. The toxicity assay was conducted in duplicate in a temperature-controlled shaker rotating at 2.5 Hz (150 rpm) at 23 °C. The cultures were aseptically sampled regularly, and the algal growth and culture pH were measured and recorded.

2.3. HBR cultivation procedures

Pilot scale (150 L) and commercial scale (2000 L) HBR units were operated using the marine alga *P. oculatum* outdoors in central Florida. The commercial unit had a 13-fold larger surface area and volume than the pilot unit (40.5 m^2 and 2000 L), but the water depth was kept at 5 cm as in the pilot-scale HBR (Dogaris et al., 2016). The body and paddlewheel of all HBRs were constructed of polyethylene (PE) thin film and aluminum, respectively. The commercial HBR was both deployed on the ground and floated on water using a buoyant platform in an artificial pond. Growth conditions were similar to those optimized in previous smaller-scale runs of the HBR with regards to inoculum size,

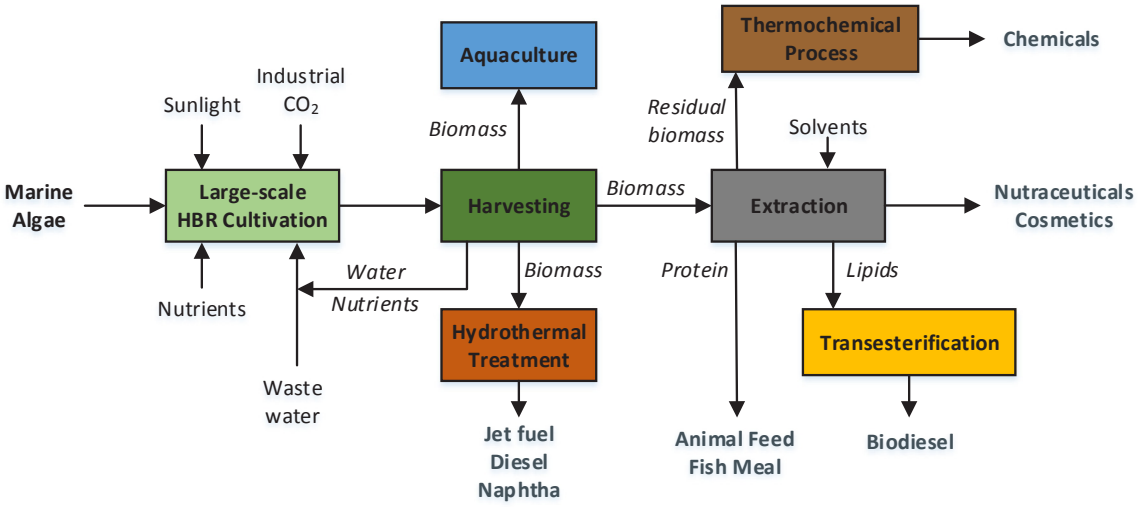

Fig. 1. Block diagram of an envisioned potentially more sustainable and cost-effective process for manufacturing a portfolio of algal biofuels and bioproducts via a low-cost modular bioreactor (HBR), wastewater use, nutrient reclaim, and recycling loops in an industrial setting.

pH regulation, and media composition (Dogaris et al., 2016). The inoculum of 150 L for the commercial units was grown in the pilot HBR over the course of 2 weeks using municipal water. In turn, the pilot unit's inoculum of 15 L with $5.6{\cdot}10^8$ cells mL^{-1} was prepared in the lab with municipal water. Before each cultivation, the commercial HBR units were tested for integrity, leaks, and rain resistance for prolonged periods (2–4 weeks) with just water to minimize the chances of culture loss due to technical issues. Culture temperature was regulated automatically using external water sprinklers that delivered LL water droplets to the surface of the HBR, but only when the culture temperature exceeded 30 °C.

All HBR units were equipped with monitoring systems for real-time pH, temperature, and solar irradiance measurements (Dogaris et al., 2016). The bioreactors and LL water were sterilized in situ before each inoculation using sodium hypochlorite and neutralized with sodium thiosulfate, while supplemental nutrients were autoclaved and added aseptically, as described elsewhere (Dogaris et al., 2015). Pure CO_2 was provided to all bioreactor cultures automatically using a pH-stat at a pH set point of 7.5 and nutrients (nitrate and phosphate) were supplemented as needed to ensure no macronutrient limitations.

Batch cultivations in the commercial HBR lasted 23 and 19 days in the floating and ground mode, respectively. Semi-continuous operation in the pilot HBR was achieved with the use of three consecutive harvest cycles (50%, 50%, and 100%, v/v), each followed by addition of LL supplemented with nutrients as needed, spanning from January to April, for a total of 74 days. Specifically, after the 1st cultivation cycle of 37 days, 75 L of the culture were harvested and replaced with fresh LL and nutrients to initiate the 2nd cycle of 18 days and then the 3rd cycle of 18 days. Culture samples were taken in the morning daily and 2–3 times per week in the 150-L and 2000-L HBRs, respectively, to monitor algal growth, macronutrient consumption (nitrate and phosphate), salinity, and dissolved oxygen level.

2.4. Analytical methods

Optical density (OD) at 680 nm, cell concentration (N) and dry cell weight (DW) were measured in duplicate, as described previously (Dogaris et al., 2016). Chlorophyll fluorescence (CF) was measured in duplicate after proper dilution using a microplate reader (Infinite M200Pro, TECAN, Switzerland) set at 435 nm excitation and 683 nm emission wavelengths, based on fluorescence spectra analysis of *P. oculatum* cell suspensions (data not shown). Initial and residual amounts of NO_3-N and PO_4-P were calculated from nitrate (Collos et al., 1999) and phosphate (Condori et al., 2010) concentrations in the filtrate (0.45 µm) in duplicate. Dissolved oxygen (DO) and salinity levels were measured immediately after each sampling, as described previously (Dogaris et al., 2016).

Proximate analysis of the algal biomass on a dry basis was conducted at Eurofins Scientific, Inc. (Eurofins Nutrition Analysis Center, Des Moines, IA). Samples from the algal cultures were centrifuged to about 20% solids content and the solid portion was sent frozen to Eurofins for analysis. Proximate analysis, based on established methods by the Association of Official Analytical Chemists (AOAC), comprised moisture content by forced draft oven (AOAC 930.15), protein by the Kjeltec method (AOAC 2001.11), crude fiber by acid digestion (AOAC 962.09; AOCS Ba 6–84), ash by heating at 600 °C (AOAC 942.05), crude fat by acid hydrolysis (AOAC 954.02), and fatty acid profile using gas chromatography after transesterification with boron-trifluoride/methanol (AOCS Ce 2–66 AOCS Ce 1–62). Carbohydrate content was calculated by subtracting the sum of ash content, crude fat content, and protein content from 100%.

2.5. Calculations

DW data in HBR cultivations were calculated using linear correlation ($R^2 = 0.98$, $P < 0.0001$) with OD_{680}. The maximum specific growth rate μ_m (d^{-1}) of algal cultures was calculated during each exponential phase from the slope of the linear regression curves of the natural logarithm of cell density (ln$N$) versus cultivation time (t). The rates of nitrogen R_N and phosphorus R_P consumption (mg L^{-1} d^{-1}) were calculated from the change in the concentration C (mg L^{-1}) of NO_3-N and PO_4-P, respectively, within a certain period of cultivation t (d), per Eq. (1).

$$R = \frac{C_1 - C_0}{t_1 - t_0} \quad (1)$$

The volumetric biomass productivity P_V (g L^{-1} d^{-1}) was calculated from the change in algal DW (g L^{-1}) within a certain period of cultivation t (d), per Eq. (2), while the areal productivity P_A (g m^{-2} d^{-1}) was derived from the volumetric productivity, per Eq. (3) (HBR volume $V = 150$ L, surface area $S = 3.0$ m^2).

$$P_V = \frac{DW_1 - DW_0}{t_1 - t_0} \quad (2)$$

$$P_A = P_V \times \frac{V}{S} \quad (3)$$

The algal biomass yields based on nitrogen (Y_N), phosphorus (Y_P), and light intensity (Y_I) were calculated by dividing the final biomass concentration DW_{max} by the consumed NO_3-N (N_{total}), PO_4-P (P_{total}), and total incident mol of photons I_{total}, respectively, per Eqs. (4)–(6):

$$Y_N = \frac{DW_{max}}{N_{total}} \quad (4)$$

$$Y_P = \frac{DW_{max}}{P_{total}} \quad (5)$$

$$Y_I = \frac{DW_{max}}{I_{total}} \quad (6)$$

The potential biofuel yield ($L\,ha^{-1}\,y^{-1}$) was calculated from the average daily areal algal biomass productivity (P_A, $g\,m^{-2}\,d^{-1}$) of *P. oculatum* in the commercial-scale HBR, extrapolated to biomass productivity per hectare per year ($kg\,ha^{-1}\,y^{-1}$) assuming 330 days of HBR operation per year. The resulting annual areal productivity was multiplied by a conversion factor of 0.41 kg biofuel per kg algal biomass using a combination of hydrothermal liquefaction (HTL) and hydrotreatment (HT), and finally divided by the density of the final diesel product, $0.8\,kg\,L^{-1}$, as described by Elliott et al. (2013), who used *Nannochloropsis* sp. algae paste for HTL-HT conversion to renewable diesel (biofuel). The overall biofuel yield calculation is summarized in Eq. (7):

$$Biofuel\ yield\ (L\ ha^{-1}\ y^{-1}) = P_A \times \frac{330 \times 10,000}{1000} \times \frac{0.41}{0.8} \approx P_A \times 1700 \quad (7)$$

3. Results and discussion

3.1. Leachate composition and growth inhibition test

The pH of LL wastewater (treated effluent) used in the present study was found to be compatible with microalgae cultivation, matching the optimum value of 7.5 reported previously for *P. oculatum* cultivations (Dogaris et al., 2016). In general, the pH of untreated LL depends on the nature of the waste contained in the landfill and can change with the conditions and natural processes occurring at the landfill. Adjustment of the pH is implemented at the landfill to remove chemical pollutants during the wastewater treatment processes, including metals precipitation and biological treatment (Browner et al., 2000). The salinity of LL was low, 0.19% (conductivity $380\,\mu S\,cm^{-1}$), in the range of freshwater salinity values, while the $110\,mg\,L^{-1}$ of alkalinity provided beneficial buffering capacity to LL against rapid changes in the pH. The COD and TOC values were found to be low, 32 and $1.0\,mg\,L^{-1}$ respectively, representing low levels of organic chemical pollutants. On the other hand, total solids (including floating, settleable, colloidal, and suspended matter) were high at $2.6\,g\,L^{-1}$, which could potentially inhibit algal growth as: (a) solid particles can serve as carriers of pollutants, like heavy metals, which may be released into the culture with changes in its pH; and (b) suspended particles can absorb or scatter incident sunlight, making less light available to the algae cells for growth and lipid production via photosynthesis (Salama et al., 2017).

Table 1 lists the nutrient, heavy metals, and EPA-regulated organic pollutant (Browner et al., 2000) content of LL. In general, macronutrients such nitrate, nitrite, phosphate (as total phosphorus), and iron were below the detection limit of the analysis, while only $0.39\,mg\,L^{-1}$ ammonia were present. Low concentrations of other nutrients, such as K, Mg, Ca and S (as sulfide), were detected in the leachate, ranging from 0.99 up to $140\,mg\,L^{-1}$, while Na and Cl amounts were significantly higher, 500 and $1000\,mg\,L^{-1}$, respectively. Micronutrient metals, such as Mn, Ni, Zn and Co, were not detected, while copper was very low at $0.023\,mg\,L^{-1}$. Finally, neither toxic heavy metals, such as Cr, Pb, and Hg, nor EPA-regulated organic pollutants, such as α-terpineol, benzoic acid, *p*-cresol, and phenol were present in the LL. The above profile of LL is the result of the biophysical treatment done on site at the landfill facility before the effluent is injected back into the ground reservoir and is intended to reduce chemicals to non-polluting levels.

Although the collected LL wastewater constitutes a sustainable abundant source of non-potable water, it contains insufficient nutrients to support algal growth at high productivities and should therefore be supplemented externally with macronutrients, trace metals and vitamins, along with artificial sea salts (Instant Ocean) to provide the desirable growth conditions for algae (Section 2.2). LL water toxicity experiments in flasks, as outlined in Section 2.2, verified the ability of *P. oculatum* to grow in the collected LL without apparent inhibition or toxicity compared to clean water (Fig. 2). Time progression of OD_{680}, cell concentration, and pH during the 31 days of the experiment was almost identical for growth in raw (non-sterile) LL, sterilized LL, and DI (control) water sources. Maximum OD ranged between 6.8 and 7.2, cell counts reached $5.3–5.4{\cdot}10^8$ cells mL^{-1} in all cases, while pH increased to 9.0 within 3 days and fluctuated only slightly afterwards. The non-sterile nature of raw LL did not affect algal growth, which can be explained by the use of chlorination at the landfill treatment facility.

Untreated LL has been previously reported to inhibit to variable extent the growth of *Chlorella pyrenoidosa*, *C. vulgaris*, *Scenedesmus* sp. and *Dunalliela tertiolecta* (Cheung et al., 1993), while algae isolated from a landfill operation in Florida identified as *Chlorella* cf. *ellipsoidea* and *Scenedesmus* cf. *rubescens* were found to grow well in LL with minimum to no chemical pretreatment (Edmundson and Wilkie, 2013). Overall, the raw LL used in the present study supported growth of *P. oculatum* under controlled laboratory conditions. Despite its poor nutritional content, raw LL represents a promising plentiful water source for producing algal biomass and manufacturing biofuels and bioproducts more sustainably by alleviating the need for increasingly scarce potable water.

3.2. Outdoor cultivation of microalgae using landfill leachate

3.2.1. Demonstration in pilot-scale 150-L HBR

The cultivation of the microalga *P. oculatum* in LL was demonstrated outdoors in Florida under real-world ambient conditions initially using a pilot-scale 150-L HBR system. The first cycle lasted for 37 days with a 5-day lag phase and a subsequent sigmoid growth progression until day 12. Subsequently, growth conformed to a linear pattern until slowing down on day 28 (Fig. 3a). The lag phase was much shorter after each harvest-dilution step, possibly due to acclimation of the algal cells to the landfill leachate and/or gradual improvement of the ambient conditions (temperature and sunlight, as seen in Fig. 3c and d). OD_{680} during the first cycle reached 13.0, which represented a high-density cell population of $1.67{\cdot}10^9\,mL^{-1}$ and $1.9\,g\,L^{-1}$ of algal biomass (Table 2). Areal productivity, P_A, in that cycle averaged at $2.8\,g\,m^2\,d^{-1}$, but peaked at 7.8, translating to 55 and $157\,mg\,L^{-1}\,d^{-1}$ of volumetric productivity, P_V, respectively. The following two cycles lasted half the time of the first one, as half of the bioreactor's volume was harvested in each one. The growth in these two cycles was slower, as observed by a 5-fold decrease in the maximum specific growth rate (μ_m) and a 14% (2nd cycle) and 32% (3rd cycle) reduction in biomass productivity. Furthermore, maximum biomass and cell concentration in the 2nd and 3rd cultivation cycles were 25–28% lower than in the 1st one. The progressive drop in growth rate during subsequent cultivation cycles may be indicative of growth inhibition caused by accumulating water constituents and can be practically addressed by periodically harvesting the entire HBR culture and starting with fresh LL to flush out accumulated compounds and particulates.

The rate of nitrogen consumption by *P. oculatum* in LL was at similar levels during all three cultivation cycles, with the average NO_3-N consumption ranging from 8.3 to $10.5\,mg\,L^{-1}\,d^{-1}$. In contrast, significant phosphorus consumption (causing a steep drop in PO_4-P levels) was observed during the first 2–3 days after each dilution or feeding event, but P consumption subsequently slowed down (Fig. 3b) to about 1/10

Table 1
Nutrient, toxic metal, and organic pollutant content of raw LL used in present study.

Macronutrients (mg L^{-1})		Micronutrients (mg L^{-1})		Toxic metals (mg L^{-1})		Toxic organic compounds (μg L^{-1})[b]	
Ammonia	0.39	Sulfur (sulfide)	0.99	Arsenic	< 0.0080[a]	α-terpineol	< 1.6[a]
Nitrate	< 0.18[a]	Manganese	< 0.0014[a]	Antimony	< 0.043[a]	benzoic acid	< 0.98[a]
Nitrite	< 0.18[a]	Copper	0.023	Cadmium	< 0.0012[a]	p-cresol	< 0.88[a]
Phosphorus	< 0.046[a]	Zinc	< 0.0098[a]	Chromium	< 0.010[a]	phenol	< 0.68[a]
Potassium	17	Cobalt	< 0.0012[a]	Lead	< 0.016[a]		
Magnesium	110	Nickel	< 0.0062[a]	Mercury	< 0.000050[a]		
Calcium	140			Selenium	< 0.020[a]		
Iron	< 0.11[a]			Silver	< 0.0032[a]		
Sodium	500						
Chloride	1000						

n.d. not detected.
[a] Minimum detection limit.
[b] EPA-regulated compounds in non-hazardous category of landfills (Browner et al., 2000). All other semi-volatile organic compounds described in EPA 8270C method were below detection limit (not shown).

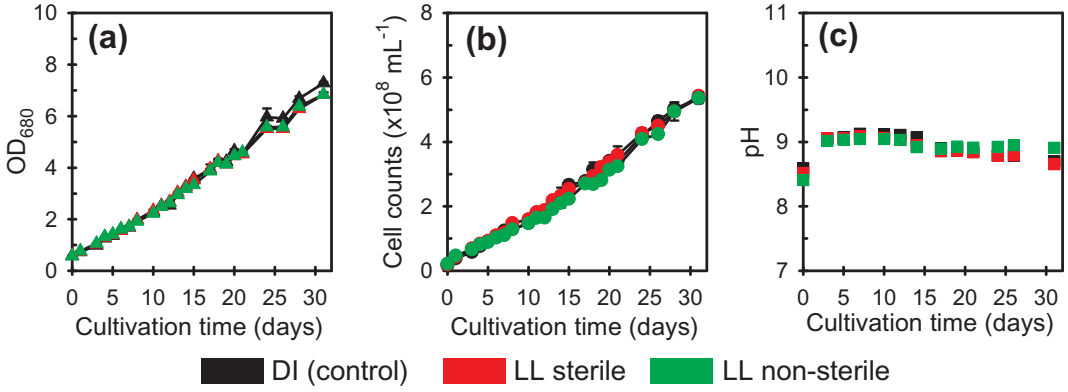

Fig. 2. Landfill leachate toxicity assessment via cultivation of *P. oculatum* cells in flask cultures. Time progression of (A) OD_{680}, (B) cell concentration, and (C) pH during 31 days of growth in DI (control), sterile LL, and non-sterile (raw) LL water sources.

of the nitrate rates (Table 2). Similar rates of nutrient (N and P) consumption have been reported in previous cultivations of *P. oculatum* in the same bioreactor (HBR), but using municipal (clean) water (Dogaris et al., 2016). However, the 50% (v/v) dilution of the initial culture (to initiate the 2nd cycle) with LL led to a 45–56% reduction in biomass yield on consumed nutrients, Y_N and Y_P (Table 2). Similarly, biomass yield on received light (photons) dropped by 41–50% after each dilution with LL potentially due to accumulation of light-scattering particulates.

The ambient temperature (recorded under shade) during the outdoor operation of the pilot-scale 150-L HBR ranged between 3.2 °C and 34.2 °C, often dropping below 10 °C during winter time (Fig. 3d). There were some days with high precipitation (100% relative humidity), as well as days with humidity as low as 21.5% (data not shown). However, there was plentiful sunlight for the microalgae to grow during the entire cultivation period with peak PAR intensity of 2276 and average 415 μmol photons m^{-2} sec^{-1} (Fig. 3d). As the sunlight heated up the HBR, the installed cooling system (sprinklers) was automatically activated during some cultivation days, keeping successfully the temperature inside the HBR below 30 °C (Fig. 3c) to prevent culture overheating and subsequent cell death. On the other hand, the culture experienced periods of low temperature during night time and early in the morning, which led to lower productivities during daytime, as also reported for cultures of *Spirulina* and *Chlorella* (Borowitzka and Moheimani, 2013). Heating large-scale outdoor cultivation systems is energy intensive and rather unsustainable, unless waste heat from co-located operations or renewable energy sources, such as landfill gas, are available on site. Florida's climate on average is warm enough to achieve satisfactory overall algal productivity.

Dissolved oxygen, as recorded by the in-line probe, peaked above 200% air saturation (data not shown) exceeding the sensor's upper limit, so manual measurements of DO were conducted right after each culture sampling. DO levels averaged at 196, 180, and 161% during the 1st, 2nd, and 3rd cultivation cycles, respectively, but reached higher levels during daytime (Fig. 3b). It has been reported that DO levels above 300% may reduce algal productivity as high oxygen concentrations can be harmful to algal cells (Molina Grima et al., 2001) and therefore could have contributed to the reduced observed productivities compared to previous HBR operations (Dogaris et al., 2016, 2015). On the other hand, this high production of dissolved oxygen from microalgae growing in wastewater could have beneficial applications in environmental remediation, where low oxygen levels threaten aquatic ecosystems (lakes and rivers) or could even be applicable to space missions removing exhaled CO_2 from the air and adding oxygen, recycling spent water sources, and generating algal biomass as plant fertilizer or protein supplement for humans.

In contrast, salinity remained almost constant at about 2.8% throughout the cultivations (data not shown), confirming that the enclosed design of the HBR prevents water evaporation and hence improves water sustainability. The pH of the culture medium fluctuated around 7.5 during daytime and dropped to 6.9–7.0 at night. An automatic valve turned on the CO_2 injection to prevent the pH from increasing above 7.5, which was optimum for *P. oculatum* (Dogaris et al., 2016), and shut off the supply below that pH value to conserve CO_2 and minimize its emission into the environment.

When comparing the present performance of *P. oculatum* in raw LL to the previously reported in municipal (clean) water using the same 150-L HBR unit during the same season and at the same location (Dogaris et al., 2016), lower biomass concentration and lower productivity and growth rate were observed in LL, as summarized in

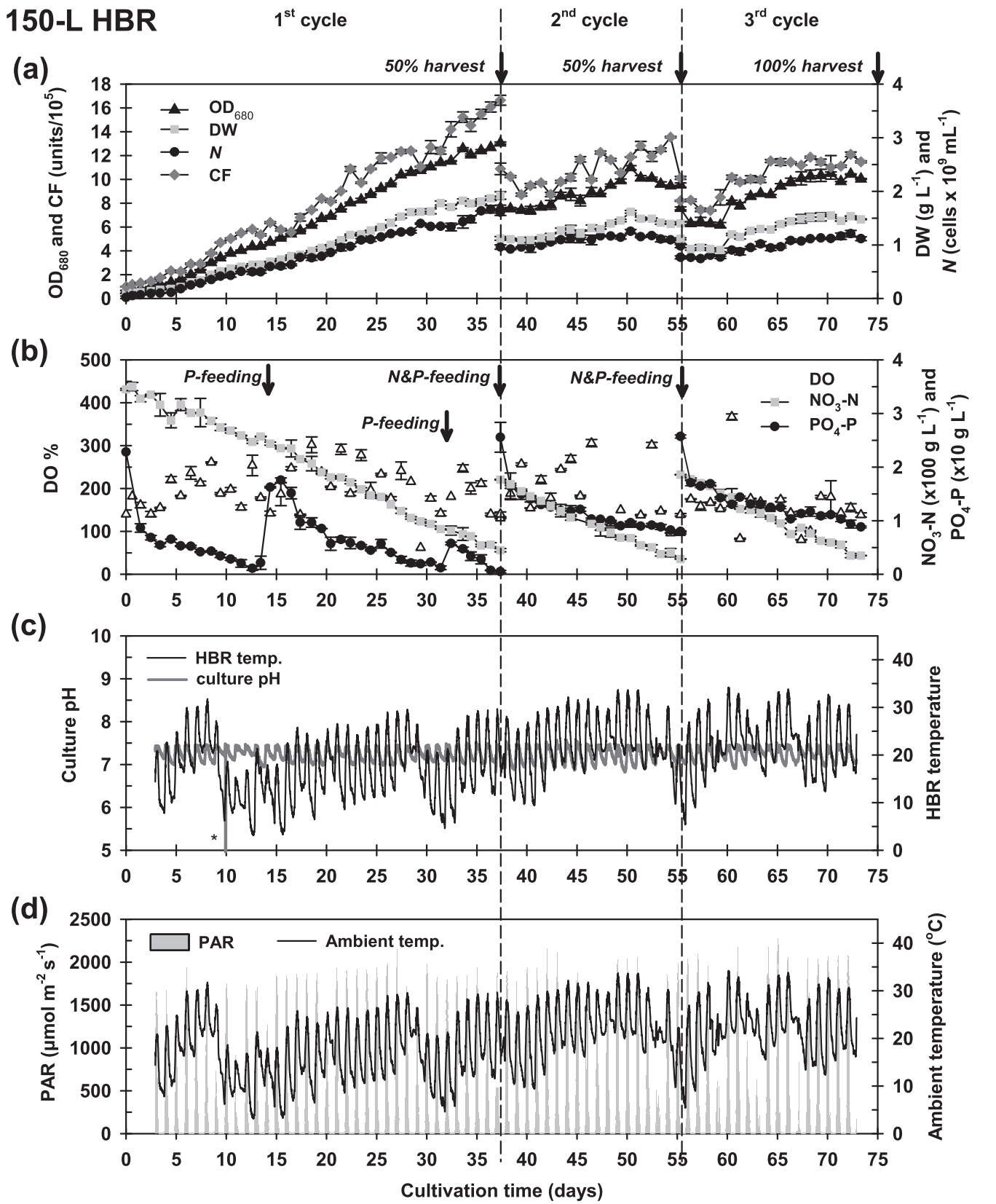

Fig. 3. Extensive outdoor cultivation of the marine microalga *P. oculatum* in the pilot-scale (150-L) HBR unit using raw LL as water source. (a) Growth metrics: OD_{680}, Chlorophyll Fluorescence (CF), cell concentration and dry cell weight (DW); (b) dissolved oxygen and nutrient residual concentration; (c) HBR culture conditions: pH and temperature; (d) ambient conditions: light intensity (PAR) and temperature. Arrows in (a) and (b) mark harvest and nutrient (nitrate-N and/or phosphate-P) addition events, respectively. The brief pH drop marked with * in (c) was due to CO_2 tank replacement.

Table 2
Summary of algae cultivation performance in the 150-L and 2000-L HBRs using raw LL and comparison with our previous study in municipal (clean) water.

HBR volume (L)	150		150			2000	
Deployment	Ground		Ground			Floating	Ground
Water source	Municipal water		LL			LL	
Operation mode	Semi-continuous		Semi-continuous			Fed-Batch	Fed-Batch
Cycle/month (s)	1st/Feb–Mar	2nd/Mar–Apr	1st/Jan–Mar	2nd/Mar	3rd/Mar–Apr	1st/Apr–May	1st/Apr
Cultivation time (days)	34	34	37	18	18	23	19
P_A ($g\,m^2\,d^{-1}$)	average 4.0 (peak 11.9)	average 5.0 (peak 20.0)	average 2.8 (peak 7.8)	average 2.4 (peak 8.2)	average 1.9 (peak 5.3)	average 3.7 (peak 12.8)	average 4.0 (peak 10.5)
P_V ($mg\,L^{-1}\,d^{-1}$)	average 81 (peak 239)	average 101 (peak 400)	average 55 (peak 157)	average 47 (peak 164)	average 37 (peak 106)	average 75 (peak 256)	average 80 (peak 211)
DW ($g\,L^{-1}$)	2.7	3.2	1.9	1.6	1.5	1.9	1.9
N_m (cells mL^{-1})	$1.6{\cdot}10^9$	$1.75{\cdot}10^9$	$1.67{\cdot}10^9$	$1.25{\cdot}10^9$	$1.21{\cdot}10^9$	$0.73{\cdot}10^9$	$0.95{\cdot}10^9$
μ_m (d^{-1})	0.55	0.27	0.24	0.05	0.04	0.68	0.66
R_N ($mg\,L^{-1}\,d^{-1}$)	9	11	8.3	10.5	8.9	18.9	13.3
R_P ($mg\,L^{-1}\,d^{-1}$)	1.0	0.6	0.8	1.2	1.1	1.3	2.2
Y_I (mg per mol photons)	74	79	68	40	34	82	89
Y_N (g per g NO_3-N)	8.9	10.9	6.1	2.7	2.9	5.5	6.3
Y_P (g per g PO_4-P)	125	119	40	22	21	45	67
Reference	(Dogaris et al., 2016)	(Dogaris et al., 2016)	This study	This study	This study	This study	This study

Table 2. These differences in growth metrics depict a reduced ability of the microalga to assimilate light and nutrients into biomass when growing in LL compared to clean water, regardless of the adequate supply of nutrients, carbon, and light. The elevated suspended solids ($2.6\,g\,L^{-1}$) in the LL may have contributed to the lower productivities because of lower light penetration (Salama et al., 2017) and accumulation of particulates in subsequent cultivation cycles. Prolonged lag phase, lower growth rate, and decreased final cell counts have also been reported in cultures of *Chlorella*, *Dunaliella,* and *Scenedesmus* species, when fed with untreated LL (Cheung et al., 1993). Nevertheless, the data in the present study show that algal biomass was still able to reach high densities ($1.5–1.9\,g\,L^{-1}$) in the HBR unit using raw LL as water source, a promising finding for using LL as a more sustainable alternative to clean water sources for algae production.

3.2.2. Demonstration in commercial-scale 2000-L HBR

Cultivations of *P. oculatum* using LL in commercial-scale 2000-L HBR units were conducted in two deployment modes: on the ground and floating in a small artificial pond. The multiple modes are intended to assess HBR performance in a variety of real- world outdoor settings. The deployment mode did not seem to significantly affect the growth of *P. oculatum*, as in both cases OD_{680} reached 8 and DW reached $1.9\,g\,L^{-1}$ (Fig. 4a and e) with a maximum specific growth rate μ_m of $0.66–0.68\,d^{-1}$ (Table 2). Similar areal and volumetric average productivities were also achieved, $3.7–4.0\,g\,m^2\,d^{-1}$ and $75–80\,mg\,L^{-1}\,d^{-1}$, respectively (Table 2).

On the other hand, when comparing the performance of the commercial HBRs to that of the pilot HBR, biomass concentration, productivity, and all yields were higher in the commercial-scale HBRs than in the pilot-scale 150-L units (Table 2). These differences could be attributed at least partially to the different environmental conditions that the cultures experienced. The commercial-scale HBRs were operated in April-May, when higher light intensities and temperatures, which favor cell productivity, are typically recorded in Florida (Fig. 4d and h) compared to January–March, when the pilot-scale HBRs were operated (Fig. 3d). As a result, while the commercial-unit runs were not subjected to temperatures lower than 17.5 °C, the pilot-scale runs often experienced night temperatures well below 10 °C. Higher day temperatures and light intensities during the commercial HBR runs (Fig. 4) could have boosted productivity, since growth close to the optimum temperature of a strain enables algae cells to utilize more efficiently the available light (Borowitzka and Moheimani, 2013).

Interestingly, one growth parameter, the maximum cell concentration N_m, was lower in the commercial HBR than in the pilot HBR, namely $0.73–0.95{\cdot}10^9$ vs. $1.21–1.67{\cdot}10^9$ (Table 2). Observations under the microscope of the collected culture samples revealed a minor presence of motile protozoa in the commercial HBRs cultures. It has been reported that bacteria and protozoa in landfill effluent water have a negative impact on the growth of *Chlorella* (Cho et al., 2011). The presence of protozoa in the commercial-scale operations could potentially justify the lower cell counts.

A similar large-scale photobioreactor system made of polyurethane plastic film, designed by Algae Systems LLC (Daphne, AL), was successfully deployed to treat municipal wastewater using a polyculture of microalgae and heterotrophs (Novoveská et al., 2016). The municipal wastewater source, although pretreated, had a higher pollution level than the LL wastewater used in the present study, in terms of nutrients composition (ammonia, nitrogen, and phosphorus) and chemical/biological oxygen demand, which were removed to high extent by the Algae Systems cultivation process. The HBR system used in the present study has operated so far with clean water and LL that is not as heavily polluted (Table 1), while future testing with more heavily polluted wastewaters is planned to expand its applicability. Since the HBR is made of polyethylene, which is a widely used polymer with high chemical resistance (Lohse and Graessley, 2000), it is expected that it will be able to withstand more heavily polluted water and maintain structural integrity and reusability.

3.3. Algal biomass analysis and biofuel production potential

The composition of algal biomass is essential in assessing the potential of microalgae to serve as a sustainable source for biofuels. The biomass composition of *P. oculatum* harvested from outdoor HBR operations using raw LL water was analyzed and summarized in Table 3 along with the composition of the same strain cultivated on municipal (clean) water. Lipid content (crude fat), which is key to biofuel production, was higher at the end of the 3rd cultivation cycle in LL, reaching 15.3% of dry cell mass. Interestingly, the *P. oculatum* cells synthesized more fat when grown in LL compared to municipal water. Further breakdown of the lipids shows that triacylglycerides (TAG) account for 7.0–8.2% of dry cell mass, which is close to the 9.8–10.8% previously reported for the same alga grown in sterilized natural seawater supplemented with f/2 medium (Park et al., 2012). Higher lipid levels, 30–40%, have been reported for *P. oculatum* UTEX LB1998, but

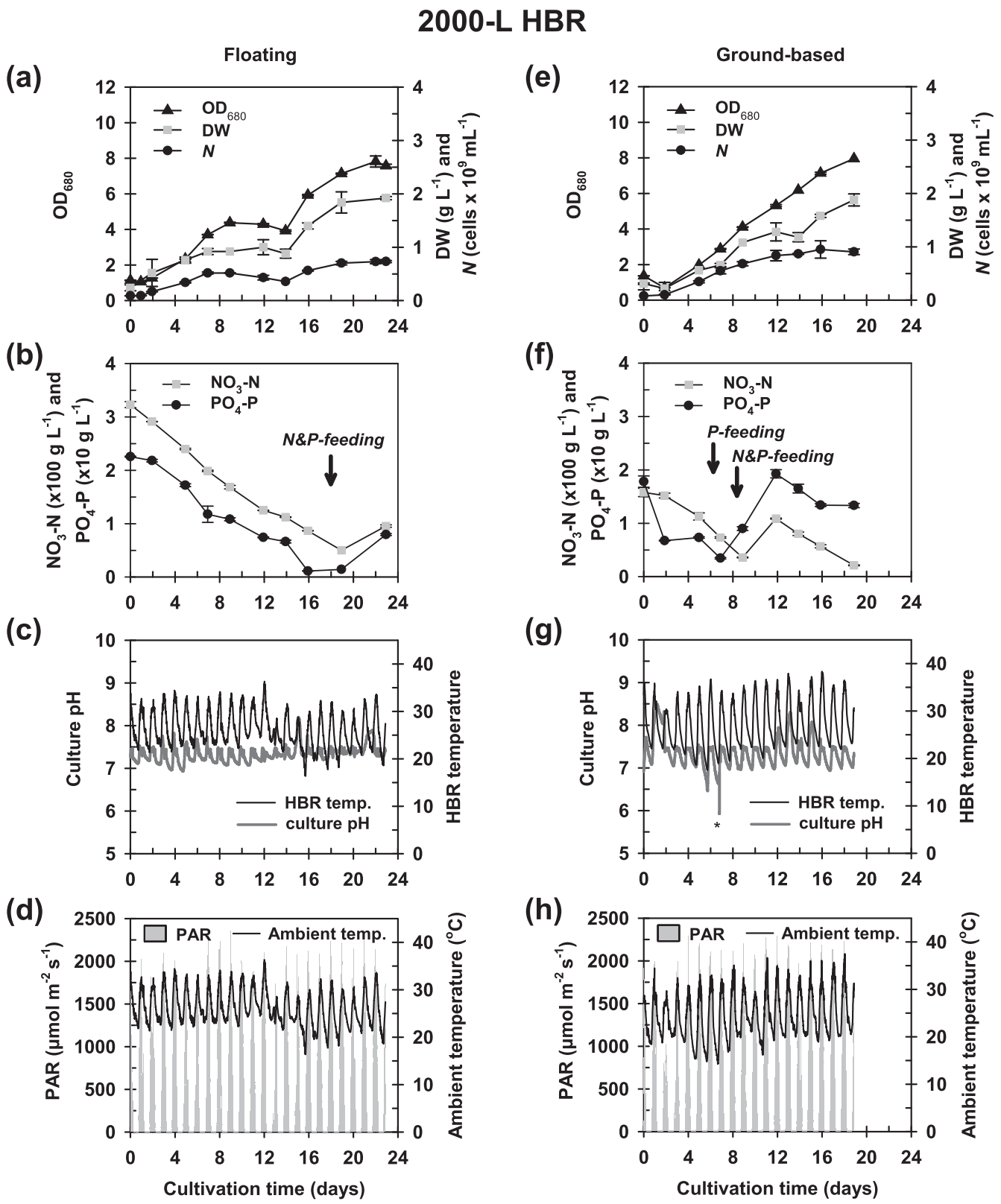

Fig. 4. Cultivation of the marine microalga *P. oculatum* in the commercial-scale (2000-L) HBR unit floating in a pond (a–d) or placed on the ground (e–h) using raw LL as water source. (a, e) Growth metrics: OD_{680}, cell concentration, and dry cell weight (DW); (b, f) nutrient residual concentration; (c, g) HBR culture conditions: pH and temperature; (d, h) ambient conditions: light intensity (PAR) and temperature. Arrows mark nutrient (nitrate-N and/or phosphate-P) feeding events. The brief pH drop, marked with * in (g), was due to CO_2 tank replacement.

Table 3
Biomass composition and fatty acid profile of *P. oculatum* cultivated in the HBR using raw LL water and comparison with the same strain grown in municipal (clean) water.

Component	In municipal water (pilot scale – 2nd cycle)[a]	In LL water (pilot scale – 1st cycle)	In LL water (pilot scale – 3rd cycle)	In LL water (commercial scale[c])
	% dry weight			
Proximate analysis				
Ash	21.5	14.5	15.8	15.7
Protein	50.7	57.9	57.6	57.1
Carbohydrates	17.2	15.4	12.1	15.2
Crude fat	10.6	12.2	15.3	6.9
TAG and fatty acid profile				
Total TAGs	8.2	8.0	7.0	0.13
C14:0 Tetradecanoic (Myristic)	0.07	0.09	0.09	2.56
C16:0 Hexadecanoic (Palmitic)	2.10	2.06	1.91	0.72
C16:1 Hexadecenoic (Palmitoleic)	0.77	0.75	0.56	< 0.01[b]
C17:1 Heptadecanoic (Margaroleic)	< 0.01[b]	< 0.01[b]	0.70	0.01
C18:0 Octadecanoic (Stearic)	0.14	0.09	0.09	0.26
C18:1 Octadecenoic (Oleic)	0.49	0.40	0.30	0.79
C18:2 Octadecadienoic (Linoleic)	1.19	1.27	0.87	2.24
C18:3 Octadecatrienoic (Linolenic)	2.73	2.85	2.00	0.07
C20:1 Eicosenoic (Gondoic)	0.14	0.04	< 0.01[b]	0.13
C24:0 Tetracosenoic (Lignoceric)	< 0.01[b]	< 0.01[b]	0.09	0.13

[a] Analysis of frozen biomass collected in previous cultivation described in (Dogaris et al., 2016).
[b] Minimum detection limit.
[c] Biomass sample collected at the end of the ground-based HBR operation (Fig. 4e–f).

under nitrate-limiting conditions that led to low algal cell concentrations (Takagi et al., 2000).

If biodiesel is the desirable product, then lipids need to be extracted from the cell mass and subjected to transesterification (Fig. 1). In this case, analysis of the lipid (fatty acid) content of the algal biomass helps project the quality of biodiesel to be produced (Ramos et al., 2009). The fatty acid composition of *P. oculatum* biomass in the HBR cultivations using LL and municipal water sources is summarized in Table 3. Switching the water source from municipal (clean) water to LL did not seem to affect the fatty acid profile of *P. oculatum* biomass, although some differences were observed after the culture was diluted with additional LL for the 3rd cycle. The main fatty acids found in all algae samples were palmitic (C16:0), 1.91–2.10%, linoleic (C18:2), 0.87–1.27%, and linolenic (C18:3) 2.00–2.85%. Similarly, other *Nannochloris* species (re-classified as *Picochlorum*) that are closely related to *P. oculatum* have been reported to accumulate high amounts of palmitic (C16:0), linoleic (C18:2) and linolenic (C18:3) acids (Mourente et al., 1990; Park et al., 2012). Interestingly, in the 3rd cycle of the HBR cultivation in LL margaroleic (C17:1) and lignoceric (C24:0) acids started to accumulate, while the levels of C18 fatty acids decreased, which indicates a change in the *P. oculatum* TAG metabolism due perhaps to the effect of accumulating LL components in the culture.

Typically, high quality biodiesel production requires the presence of C16:0, C16:1, C18:1 and to a lesser extent C18:3 fatty acids (Mathimani and Nair, 2016), which were all identified in the present study (Table 3). Furthermore, the high C16:0 content of *P. oculatum* TAGs, 27–29%, can contribute to biodiesel with a high cetane number and hence a good compression ignition in the engine (Mathimani et al., 2015), while the presence of monounsaturated fatty acids (e.g. C16:1 and C18:1) helps prevent auto-oxidation of the fatty acid methyl esters and leads to a fuel with good cold flow characteristics (Ramos et al., 2009).

On the other hand, if hydrothermal treatment is used for renewable fuel manufacturing (such as jet fuel, naphtha, and diesel), then the cell mass recovered during harvesting can serve as feedstock without the need for lipid extraction (Nhat et al., 2018; Olivares et al., 2014) as shown in Fig. 1, while culture water is recycled back to the HBR. Based on the HBR's biomass productivity, estimates of potential biofuel yield were derived by employing conversion factors for direct conversion of whole algae biomass as reported before (Elliott et al., 2013) and summarized in Eq. (7). The potential biofuel yield using biomass produced in the HBR at a 3.0 $g\,m^{-2}\,d^{-1}$ average productivity with raw LL and subjecting it to hydrothermal treatment is projected to be 5100 $L\,ha^{-1}\,y^{-1}$. Further increase in biomass productivity, and thus biofuel yield, is expected through optimization of the HBR design using computational fluid dynamics (CFD) modeling (Pirasaci et al., 2017).

4. Conclusions

Based on the cultivation performance and biomass analysis results, the microalga *P. oculatum* LB 1998 could be produced outdoors in commercial-scale HBR units for renewable diesel and bioproduct manufacturing using abundantly available and inexpensive landfill leachate as its main water source. Demonstrating the use of non-potable low-cost water sources, such as LL, for outdoor mass cultivation of algae and practicing process water and nutrient recycling are crucial for the sustainability and cost effectiveness of commercial algal operations, as freshwater resources become increasingly scarce in the United States and around the world.

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