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HIGHLIGHTS

- Bicarbonate added to enhance algal biofilm growth and nutrient uptake.
- Bicarbonate added to trigger lipid production in nitrogen stressed algal biofilms.
- Nutrient removal influenced by growth phase of algal biofilms.
- Bicarbonate addition enhanced photosynthesis in algal biofilms.

Keywords

Microalgae
Biofilms
Wastewater
Dissolved inorganic carbon Biofuels

ABSTRACT

Microalgal biofilms grown to evaluate potential nutrient removal options for wastewaters and feedstock for biofuels production were studied to determine the influence of bicarbonate amendment on their growth, nutrient uptake capacity, and lipid accumulation after nitrogen starvation. No significant differences in growth rates, nutrient removal, or lipid accumulation were observed in the algal biofilms with or without bicarbonate amendment. The biofilms possibly did not experience carbon-limited conditions because of the large reservoir of dissolved inorganic carbon in the medium. However, an increase in photosynthetic rates was observed in algal biofilms amended with bicarbonate. The influence of bicarbonate on photosynthetic and respiration rates was especially noticeable in biofilms that experienced nitrogen stress. Medium nitrogen depletion was not a suitable stimulant for lipid production in the algal biofilms and as such, focus should be directed toward optimizing growth and biomass productivities to compensate for the low lipid yields and increase nutrient uptake.

1. Introduction

Cultivation of microalgae in wastewater streams has been pro-posed as a means of reducing competition for freshwater sources, as an inexpensive source of nutrients, and as a biological wastewa-ter treatment alternative (Cai et al., 2013; Sturm and Lamer, 2011). Microalgae can utilize nutrients in wastewater for growth to gen-erate considerable amounts of biomass. However, recovery of microalgae from the liquid medium is difficult and represents a substantial capital cost in suspended cultivation systems (Greenwell et al., 2010; Hoffmann, 1998), consequently there is a growing interest in attached algal growth platforms. Algal biofilm based systems such as the rotating algal biofilm reactor (RABR), algal turf scrubber (ATS™), revolving algal bioreactor (RAB), and Algaewheel have been developed, and algal biofilm growth dem-onstrated in bench and pilot scale operations (Christenson and Sims, 2011, 2012; Gross et al., 2013; Kesaano and Sims, 2014; Pizarro et al., 2006). However, there is still limited fundamental information on algal biofilm physiological processes and growth especially in wastewater remediation.

Widespread application of algal biofilm-based systems is also limited but can be promoted through integration of wastewater treatment with the production of valuable bioproducts from the harvested algal biomass. Algal biomass composition (i.e., lipid,

carbohydrate, and protein content) is influenced by the chemical composition of the medium and the environmental growth conditions (*e.g.*, temperature, pH, and light), which subsequently determines the by-products that can be synthesized. Conventionally, microalgae grown as feedstock for biofuels require a two stage process where biomass accumulation occurs under nutrient-rich conditions followed by an environmental challenge to induce secondary byproduct accumulation (*e.g.*, tri-acylglycerols as energy storage compounds) (Su et al., 2011). Nutrient starvation is typically employed as an environmental stress to stimulate lipid biosynthesis in microalgae cultures (Devi et al., 2012; Rodolfi et al., 2009; Sharma et al., 2012). However, stimulation of lipid production in algal biofilms as a result of nutrient starvation has not been as successful as in suspended cultures (Bernstein et al., 2014; Schnurr et al., 2013).

Furthermore, information on the use of other lipid inducing techniques such as chemical addition, pH stress, and temperature either independently evaluated or in combination with nutrient starvation is limited in algal biofilm studies. For example, addition of bicarbonate salts (HCO_3^-) was reported as an effective trigger for lipid production in nutrient limited suspended microalgae cultures (Gardner et al., 2012, 2013; Peng et al., 2014; White et al., 2013). The bicarbonate salts not only induce lipid production, but also provide a stable and readily available source of inorganic carbon essential for photosynthesis and microalgae growth (Chi et al., 2013; Mus et al., 2013; Wensel et al., 2014). In addition, Glud et al. (1992) observed an increase in photosynthetic rates and a simultaneous reduction in respiration rates (17%) in a diatom-dominated biofilm community amended with bicarbonate.

The potential use of bicarbonate in minimizing photorespiration is especially of interest in algal biofilms because of the high O_2/CO_2 ratios due to localized supersaturated oxygen concentration from active oxygen photosynthesis (Bernstein et al., 2014; Glud et al., 1992). Photorespiration is a competing process to carboxylation, where ribulose-1,5-biphosphate carboxylase oxygenase (RuBisCO) acts as an oxygenase, thereby inhibiting carbon dioxide fixation and subsequently reducing photosynthetic efficiency. The study presented here evaluated the effects of adding dissolved inorganic carbon in the form of 2 mM HCO_3^- to synthetic wastewater medium to grow algal biofilms in order to:

(1) Enhance algal biofilm growth, nutrient uptake, and lipid accumulation during nutrient deplete culturing.
(2) Increase photosynthetic rates with biofilm depth within the photic zone.

2. Methods

2.1. Microalgal biofilm culturing and sampling

The chlorophyte isolate *Botryococcus* sp. strain WC-2B, previously described in Bernstein et al. (2014), was cultured in 8 L laboratory scale rotating algal biofilm reactors (RABRs) operated at 12 rpm and 25 °C. Each reactor was comprised of two plastic cylindrical wheels (10 cm diameter) onto which 3/16 inch (diameter) untreated cotton cord was attached as the biofilm substratum. Synthetic wastewater was made to simulate typical medium strength domestic wastewater for total nitrogen (TN) and total phosphorus (TP) concentrations without a carbon source (Metcalf and Eddy, 2003). The medium consisted of 60 mg L^{-1} NH_4Cl, 150 mg L^{-1} $NaNO_3$, 16 mg L^{-1} Na_2HPO_4, 15 mg L^{-1} K_2HPO_4, 4 mg L^{-1} KH_2PO_4, 75 mg L^{-1} $MgSO_4{\cdot}7H_2O$, 25 mg L^{-1} $CaCl_2{\cdot}H_2O$, and micronutrients (8.82 mg L^{-1} $ZnSO_4{\cdot}7H_2O$, 1.44 mg L^{-1} $MnCl_2{\cdot}4H_2O$, 0.71 mg L^{-1} MoO_3, 1.57 mg L^{-1} $CuSO_4{\cdot}5H_2O$, 0.49 mg L^{-1} $Co(NO_3)_2{\cdot}6H_2O$ and 4.98 mg L^{-1} $FeSO_4$).

The experimental set up consisted of four laboratory RABRs under fluorescent lights with a photosynthetically active radiation (PAR) of 227 ± 65 μmol m^{-2} s^{-1} on a 14:10 L/D cycle. Duplicate reactors were amended with 2 mM HCO_3^- in the form of $NaHCO_3$ and another duplicate set without HCO_3^- amendment was cultured for comparison. The reactors were operated in sequenced batch mode with a 5 day hydraulic retention time (HRT) for a period of 18 days, after which nitrogen stress was induced for an additional 5 days by replacing all liquid medium with synthetic wastewater without a nitrogen source. For each cycle of hydraulic retention time, the reactors were drained, cleaned, and filled with fresh medium. Prior to the start of the experiment, the medium was inoculated with microalgae and the RABRs operated for 3 days (seeding period) to allow the microalgae to attach to the rope strands. As shown in Fig. S1, after the seeding period, the RABRs with the exception of the substratum (rope strands) were covered with black polyethylene sheet to minimize microalgae growth in the liquid medium. Culturing and sampling was performed under non-aseptic conditions (open air).

Rope samples with attached microalgae were excised for oxygen microsensor measurements, microscopy characterization, biomass dry weight measurements, and lipid analysis. Biomass cell dry weights (CDW, gcdw m^{-2}) were obtained by removing the biofilm from a known length of cord into a pre-weighed aluminum weigh boat using a flat end spatula. The biomass was dried at 70 °C for 18 h until the biomass weight was constant. Biomass CDWs were calculated by subtracting the dry weight of the oven dried boat with biomass and normalizing by the total cylindrical surface area for the length of cotton cord substratum excised.

2.2. Water quality monitoring

Nitrate (NO_3^-), nitrite (NO_2^-), and orthophosphate (PO_4^{3-}) concentrations were monitored in the bulk medium and measured by ion chromatography (IC) using a Dionex IonPac AS22 carbonate eluent anion-exchange column set at a flow rate of 1.2 mL min^{-1}. IC data was analyzed by Chromeleon 7 Chromatography Data system (CDS) software. Ammonium (NH_4^+-N) concentrations were determined according to the 2-phenylphenol method (Rhine et al., 1998) with a BioTek PowerWave XS microplate reader (Vermont, USA) at an absorbance of 660 nm. The dissolved inorganic carbon (DIC) was measured on 8 mL filtered (0.2 μm pore size filters) medium samples using a Skalar Formacs$^{HT/TN}$ TOC/TN analyzer (model CA16, Netherlands) and Skalar LAS-160 autosampler. DIC was quantified using peak area correlation against a standard curve from a bicarbonate-carbonate mixture (Sigma Aldrich). Culture pH and optical density (OD) measurements were taken using a standard laboratory Accumet pH electrode (Fisher Scientific) and Genesys 10 UV-Model 10-S spectrophotometer (Thermo Electron Corporation), respectively.

2.3. Oxygen microsensor analysis

Clark-type oxygen microelectrodes (10 μm tip diameter; OX-10 Unisense) and specialized computer controlled hardware (Unisense) were used to analyze the reactive transport of dissolved oxygen with biofilm depth under steady-state diffusive conditions corresponding to light and dark conditions. Photosynthetic rates (coupled with photo-respiration) were estimated using the light/dark shift technique (Kühl et al., 1996; Revsbech and Jørgensen, 1986). The light/dark shift measurements are valid under the following assumptions: (1) initial steady state oxygen distribution is achieved before darkening, (2) oxygen consumption rates before and after dark incubation are identical, and (3) identical diffusive fluxes are maintained during the measurement time at each position. Two point calibrations were performed for the oxic conditions

(medium saturated with air) and anoxic conditions (medium sparged with nitrogen gas).

2.4. Biodiesel analysis

Biodiesel precursors *i.e.*, free fatty acids (FFAs), mono-acylglycerols (MAGs), di-acylglycerols (DAGs), and tri-acylglycerols (TAGs) were extracted from dried biomass by bead beating extraction and the biodiesel potential (total FAMEs) was determined by direct *in situ* transesterification according to protocols published by Lohman et al. (2013). The total FAMEs and the fatty acid compositions of these FAMEs were quantified using gas chromatography-mass spectroscopy (GC–MS; Agilent 6890N and 5973 Network MS). The FFAs, MAGs, DAGs, and TAGs were analyzed using gas chromatography flame ionization detection (GC-FID; Agilent 6890N).

3. Results and discussion

3.1. Microalgae growth rate and productivity

Microalgae successfully attached to the cotton cord and grew as a biofilm for the entire study period (Fig. S1). The lag phase was minimized by the 3-day seeding period. The microalgal biofilms were in exponential growth from days 3 to 10 as determined from linear the portion of the natural log transformed growth data and the stationary phase occurred after 10 days of growth (Fig. 1). Curve fitting of the growth data also showed that the 1st order equation provided a better description of the microalgal growth between days 3 and 10 compared to the zero order equation with R^2 values of 0.946 and 0.999 for biofilm amended with bicarbonate and those without bicarbonate respectively (Fig. S2). The maximum specific growth rates measured during the exponential phase were 0.18|0.07 (mean|range) and 0.20|0.07 day^{-1} for algal biofilms amended with bicarbonate and the unamended control, respectively. The maximum areal biomass density measured during the stationary phase was 20.95 and 25.98 g m^{-2} for biofilms with bicarbonate and biofilm samples without bicarbonate, respectively. Additionally, the biofilm production rates, calculated as the total biomass accumulated per rope surface area divided by the time taken to reach stationary phase, were 1.45 and 1.79 g m^{-2} day^{-1} for biofilms with bicarbonate and biofilm without bicarbonate added, respectively.

Growth curves for the algal biofilms (attached to rope) and microalgae growth in suspension are shown in Fig. 2A. Microalgae growth in the bulk medium was negligible over the study period indicating that covering the reactors with black plastic effectively prevented light penetration and minimized growth in suspension. There was no statistical difference observed in growth characteristics for algal biofilms amended with bicarbonate and biofilms that did not receive bicarbonate (p value of 0.4517 from t test). Although it was hypothesized that the addition of bicarbonate would increase the algal biofilm growth, this was not observed. With the 8 L medium reservoirs, even the unamended algal biofilms were not carbon limited, such that bicarbonate addition did not enhance growth in this reactor system. DIC measurements remained relatively constant for each 5-day retention time with slight differences observed in the medium concentrations, with the exception of the first 3 days, (Fig. 2B).

3.2. Removal of nitrogen and phosphorus from synthetic wastewater using algal biofilms

A basic requirement of wastewater treatment is the removal of nutrients (*i.e.*, nitrogen and phosphorus) to acceptable limits prior to discharge. Microalgae based systems promote nutrient removal through plant uptake and subsequent harvesting of the nutrient-rich biomass from the effluent. In addition, microalgae increase the medium pH via photosynthesis thereby promoting volatilization of ammonia and possible precipitation of phosphate ions (Boelee et al., 2012). It should be noted that all the RABRs were covered in black polyethylene, cleaned, and had the bulk medium replaced every 5 days to minimize algal growth in the bulk medium, which also minimized the pH increase of the medium resulting from photosynthesis. Therefore, at the measured pH of 8.5 ± 0.15 for medium amended with bicarbonate and 7.97 ± 0.22 for medium without bicarbonate respectively, nutrient removal was attributed to the activity of the biofilms.

The synthetic wastewater was prepared with ammonia and nitrate salts as the only nitrogen sources. Initial concentrations of total nitrogen and phosphorus in the medium were approximately 40 mg-N L^{-1} and 7 mg-P L^{-1}, respectively, giving a molar N:P ratio of approximately 13:1. The measured residual total nitrogen

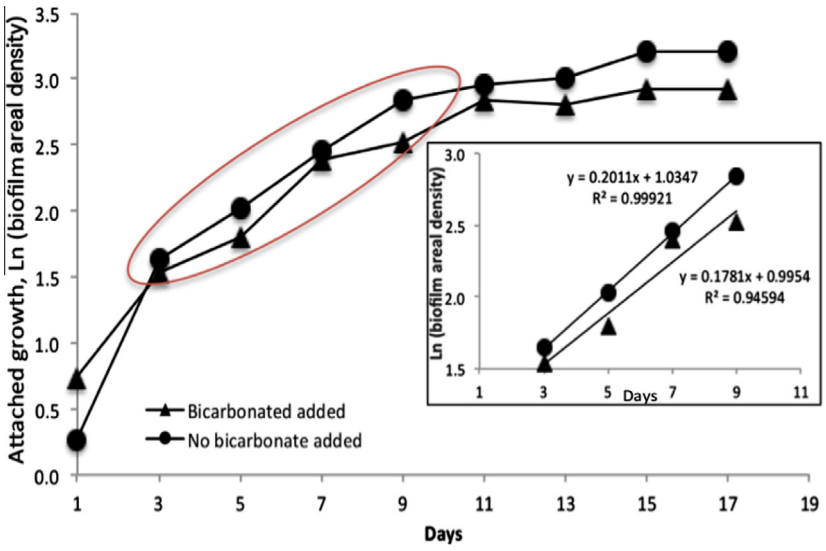

Fig. 1. Algal biofilm growth curves from natural log transformed data showing the exponential phase (days 3–10) and stationary phase (days 11–18). Insert: equations and R^2 values describing the exponential phase of biofilms with and without bicarbonate amendment, respectively.

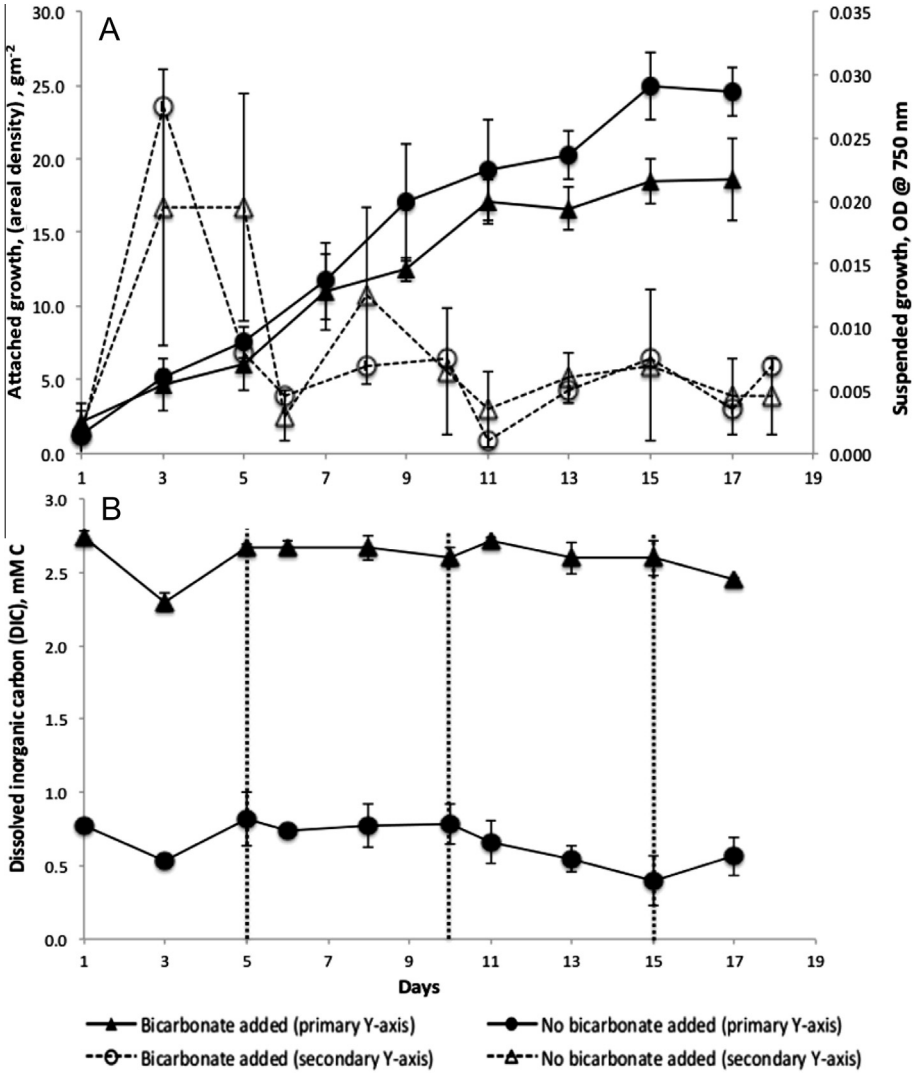

Fig. 2. Growth curves for attached (solid lines) and suspended (dotted lines) microalgae (A) and dissolved inorganic carbon (DIC) concentrations (B) in laboratory-RABRs amended with bicarbonate and without bicarbonate addition. Error bars for algal biofilm areal density and DIC measurements represent the standard deviation ($n = 4$). Error bars for suspended growth represent the range ($n = 2$). Vertical dotted lines represent end of 5 day hydraulic retention time.

concentrations (including NO_2^--N) ranged from 7.95 to 19.66 and 8.20 to 19.72 mg-N L^{-1} for RABRs with and without bicarbonate amendment, respectively. Similarly, final total phosphorus concentrations ranged from 3.39 to 3.57 and 3.35 to 3.55 mg-P L^{-1} for RABRs with and without bicarbonate amendment, respectively. The lowest N and P residual concentrations were obtained during the retention time cycles corresponding to the exponential growth phase of the biofilms (Fig. 3). Therefore, as expected, nutrient removal from the wastewater was closely linked to algal biofilm growth *i.e.*, higher removal efficiencies were obtained during the exponential growth phase of the biofilm compared to the onset of the stationary phase.

The N and P removal efficiency ranged from 27% to 74% (NO_3^--N), 89% to 100% (NH_4^+-N), and 19% to 41% (PO_4^{3-}-P) during the experiments, with no significant difference observed between liquid samples from reactors amended with bicarbonate and those that did not receive additional dissolved inorganic carbon. Similarly, for the entire duration residual N and P concentrations followed the same trend in cultures amended with bicarbonate and those that did not receive bicarbonate (Fig. 3). Complete uptake of ammonium ions was observed unlike nitrate ions in this study, probably due to preferential uptake of ammonia by microalgae compared to nitrate (Eustance et al., 2013). Microalgal cultures supplied with mixed nitrate and ammonium sources may repress NO_3^--N uptake due to feedback inhibition, since ammonium is an end product of assimilatory nitrate reduction (Crofcheck et al., 2012). Similar to the algal biofilm growth results, phosphate and nitrogen removal rates were not influenced by the addition of bicarbonate to the medium. Maximum nutrient removal from wastewater with algal biofilms can be attained via harvesting at the end of the exponential growth phase preferably after 8–10 days of growth using this RABR system.

The nitrite concentrations observed in solution were probably a result of incomplete nitrification of ammonia since the algal biofilms were grown in a non-aseptic oxygenated environment (Fig. 3). An abiotic control was used to verify that the presence of NO_2^--N ions was due to biological processes (Table S1). The chemoautotrophic bacteria involved in nitrification require a carbon source such as CO_2 or HCO_3^-, therefore the reactor with bicarbonate treatment possibly had more favorable initial conditions for the bacteria to grow, thus the higher nitrite concentrations observed (Fig. 3). However, quasi-steady state concentrations of nitrite were eventually attained and the difference ceased to be significant later in the experiment.

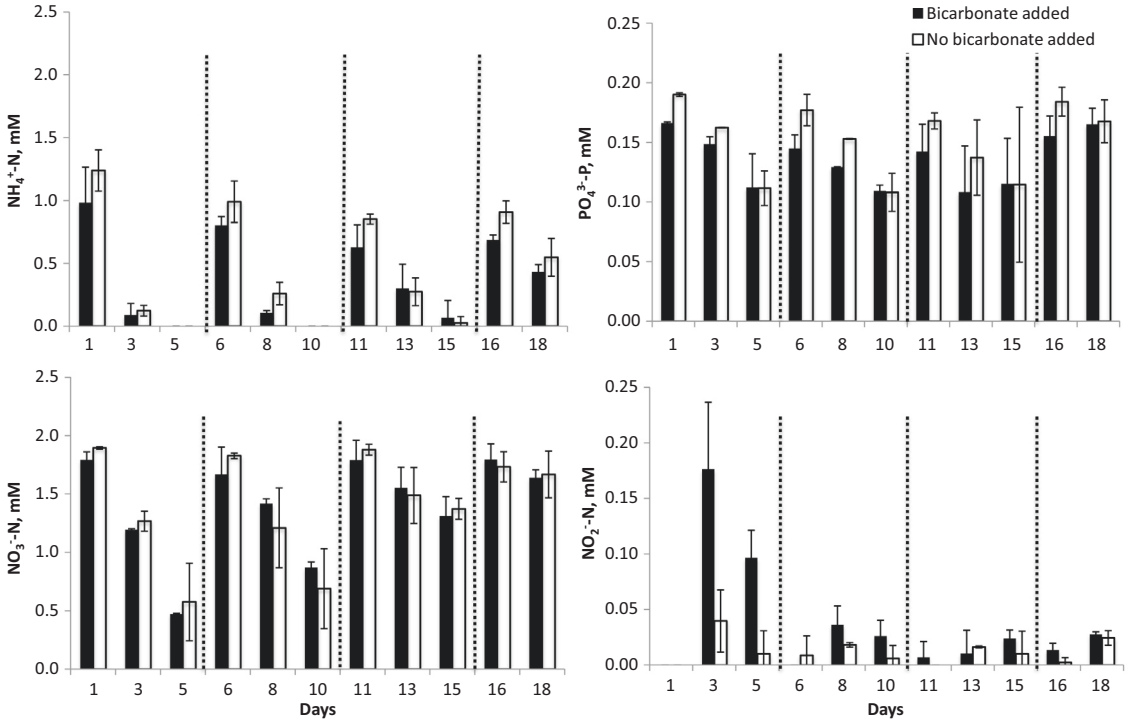

Fig. 3. Ammonium, nitrate, nitrite, and phosphate ion concentrations in medium amended with bicarbonate and without bicarbonate addition. Error bars represent range for (n = 2). Vertical dotted lines represent end of 5 day hydraulic retention time.

3.3. Microalgal biofilm photosynthesis and coupled respiration

3.3.1. Oxygen microprofiles under illumination

Oxygen microprofiles were taken before and after N-deprivation was initiated, at 18 and 23 days of RABR operations. Steady state oxygen microprofiles for biofilm samples under light showed an initial increase in oxygen concentrations (compared to equilibrium with saturated air ≈260 μM oxygen), which peaked at a depth of 200 ± 25 μm from the biofilm surface (biofilm/air interface) for both N-replete and N-deprived biofilms (Fig. 4A and B). Oxygen production in illuminated algal biofilms is a result of photosynthesis, and spatial gradients of light are known to affect the rate of oxygenic photosynthesis and corresponding oxygen concentrations in algal biofilms (Wieland and Kühl, 2000). Photosynthetic activity was highest in the upper layers of the biofilm and decreased with biofilm depth, possibly due to light attenuation and/or substrate diffusion limitations. Biofilms cultured under N-replete conditions had peak oxygen concentrations that were twice that of N-deprived biofilms (Fig. 4A and B). Furthermore, under illumination there were no anoxic zones observed in N-replete biofilms, an indication that the oxic zone (oxygen penetration depth) extended into the cotton cord substratum (Fig. 4A). In nitrogen replete systems, the steady state oxygen microprofiles showed no significant differences under either light or dark conditions for biofilms with or without bicarbonate (Fig. 4A and C).

On the contrary, differences in steady state oxygen microprofiles were revealed between N-deprived algal biofilms with and without bicarbonate amendment (Fig. 4B). For example, bicarbonate amended biofilms had higher oxygen concentrations compared to biofilms that did not receive bicarbonate. This is an indication of either higher photosynthetic rates and/or reduced oxygen consumption rates due to respiration. Indeed, higher photosynthetic rates and lower areal respiration rates (in the light) were calculated for bicarbonate amended biofilm samples under N-stress (Table 1). Additionally, anoxic zones were observed in N-deprived algal biofilms and the depth of oxygen penetration for the bicarbonate amended biofilms was 1500 μm compared to 850 μm for biofilms without bicarbonate addition (Table 1).

3.3.2. Oxygen microprofiles in the dark

Oxygen is consumed by algal biofilms in the dark as a result of respiration. Assuming oxygen diffusivity is constant, the rate at which oxygen decreases (slope) is an indication of the consumption rate *i.e.*, a steeper decline in oxygen concentration indicates greater consumption and a smaller depth of oxygen penetration can be assumed to occur as a result of high heterotrophic activity (Glud, 2008). Steady state oxygen concentrations for biofilms in the dark decreased with depth to anoxic conditions for both N-replete and N-deprived biofilms (Fig. 4C and D). Biofilms under N-replete culturing showed a more gradual decline in oxygen concentration compared to N-deprived biofilms, where steeper slopes and shorter oxygen penetration depths were observed. This was an indication of greater potential for heterotrophic oxygen consumption in N-deprived biofilms compared to N-replete biofilms, an observation that is contrary to what was reported in Bernstein et al. (2014). The current study provided a longer N-starvation period of 120 h compared to 60 h in the study by Bernstein et al. (2014), which may have promoted greater heterotrophic activity in the N-deprived biofilms.

Both before and after N-deprivation, biofilm samples amended with bicarbonate had greater oxygen penetration depths under dark conditions compared to biofilms that did not receive bicarbonate. Oxic zones of 1100 ± 25 μm and 950 ± 25 μm in depth were estimated for biofilms amended with bicarbonate and without added bicarbonate under N-replete culturing. Similarly, oxygen penetration depths of 650 ± 25 μm and 300 ± 25 μm for biofilms amended with bicarbonate and without added bicarbonate during N-deprivation were observed (Table 1). This showed that the bicarbonate amended biofilms had lower oxygen consumption in the

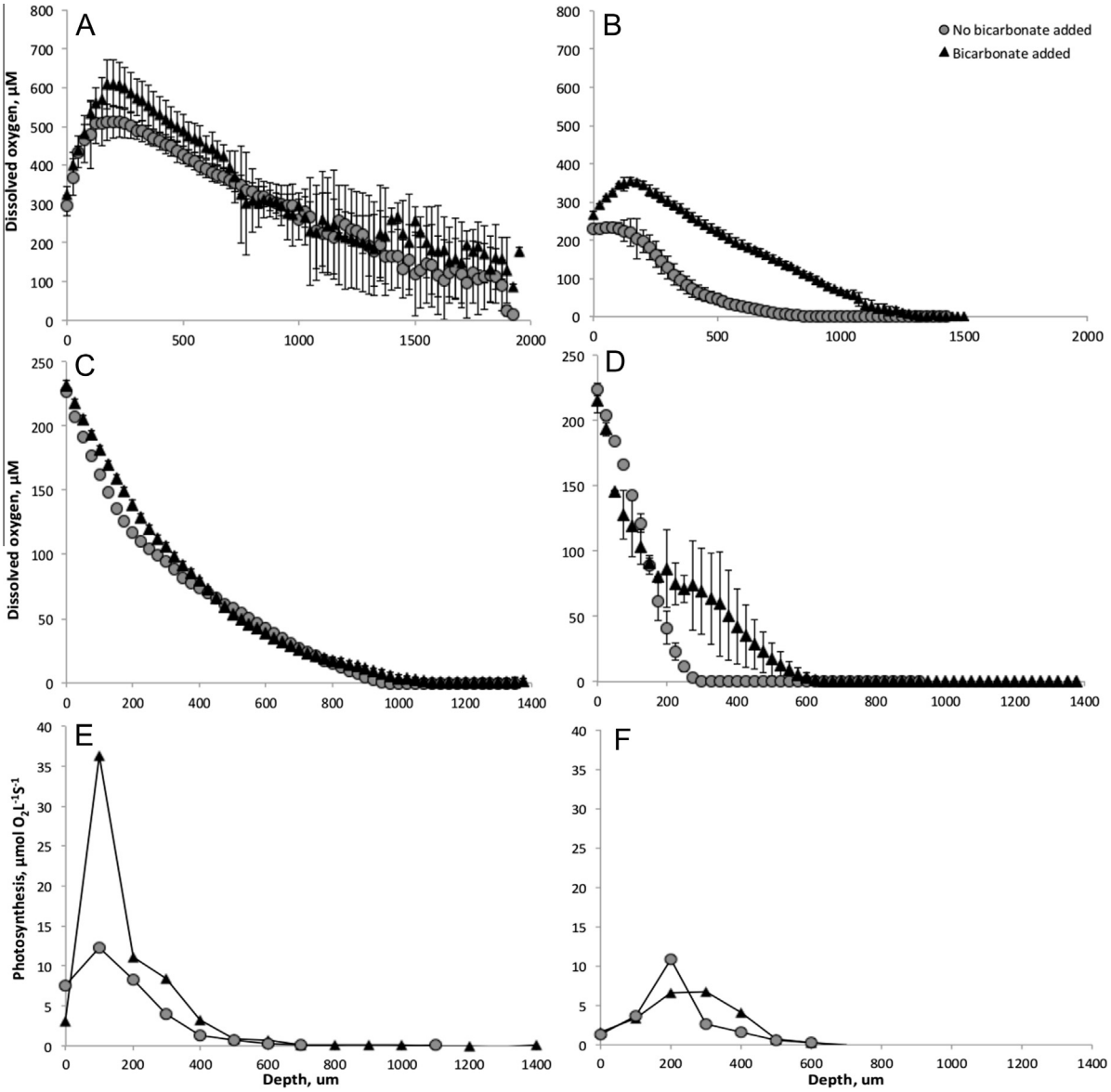

Fig. 4. Steady state oxygen microprofiles for illuminated algal biofilms under nitrogen replete (A) and nitrogen deprived (B) conditions. Error bars represent standard deviation of replicate profiles ($n = 3$); steady state oxygen microprofiles in the dark for algal biofilms under nitrogen replete (C) and nitrogen deprived (D) conditions. Error bars represent the standard deviation of replicate profiles ($n = 3$); and representative photosynthesis profiles for algal biofilms under nitrogen replete (E) and nitrogen deprived (F) conditions. Zero depth (surface) is at the algal biofilm/air interface.

Table 1
Measurements of photosynthetic rates, respiration rates, and relevant depth parameters for laboratory grown microalgal biofilms with and without bicarbonate amendment.

Parameter (µmol O_2 cm^{-2} s^{-1})	Bicarbonate		No bicarbonate	
	N-replete	N-deprived	N-replete	N-deprived
Gross photosynthesis, P_g	6.27E−04	2.26E−04	3.08E−04	2.02E−04
Net areal rate of biofilm photosynthesis, P_n (% P_g)	2.43E−04 (38.74%)	9.2E−05 (40.76%)	2.21E−04 (71.59%)	7.43E−06 (3.68%)
Net areal rate of photic zone photosynthesis $P_{n,phot}$ (% P_g)	2.99E−04 (47.72%)	1.36E−04 (60.26%)	2.61E−04 (84.87%)	5.52E−05 (27.33)
Areal respiration of the biofilm, R_{light} (% P_g)	3.84E−04 (61.26%)	1.34E−04 (59.24%)	8.75E−05 (28.4%)	1.94E−04 (96.32%)
Areal respiration of the photic zone, R_{phot} (% P_g)	3.28E−04 (52.28%)	8.97E−05 (39.74%)	4.66E−05 (15.13%)	1.47E−04 (72.67%)
Respiration in the dark, R_{dark}	0.59E−04	1.49E−04	0.74E−04	0.98E−04
Depth of photic zone, L_{phot} (µm)	1000 ± 100	600 ± 100	700 ± 100	600 ± 100
Depth of oxic zone in light (µm)	>1950	1500	>1950	850
Depth of oxic zone in the dark (µm)	1100 ± 25	650 ± 25	950 ± 25	300 ± 25

dark compared to the biofilms without bicarbonate amendment for both nutrient conditions.

3.3.3. Spatial rates of photosynthesis and respiration

The gross photosynthesis profiles were generated at a spatial resolution of 100 μm vertical depth using the volumetric photosynthetic rates (*i.e.*, the rate of oxygen depletion within 3 s of dark incubation) determined from the light/dark shift technique. Photosynthesis occurred within a depth of 500 μm from the biofilm surface (Fig. 4E and F). Similarly, increasing rates of areal gross photosynthesis (P_g) resulted in higher areal net biofilm photosynthesis (P_n) and photic zone photosynthesis ($P_{n,phot}$), which corresponded to deeper oxic zones (Table 1).

However, photosynthetic rates significantly varied with both nutrient conditions and presence/absence of bicarbonate in medium. Biofilm samples under nutrient replete culturing had higher photosynthetic rates (P_g, P_n, and $P_{n,phot}$) compared to N-deprived algal biofilms indicating a greater potential for photo-productivity when nutrient replete (Fig. 4 and Table 1). Biofilms amended with bicarbonate also had higher photosynthetic rates (P_g, P_n, and $P_{n,phot}$) compared to the biofilms that did not receive bicarbonate for both N-replete and deprived conditions (Table 1). The distribution of P_n and $P_{n,phot}$ as a fraction of the gross photosynthesis in the bicarbonate amended biofilms was different from that of biofilms that did not receive bicarbonate. P_n and $P_{n,phot}$ represented a greater proportion of gross photosynthesis under N-deprived conditions for bicarbonate amended biofilms, whereas for biofilm samples that did not receive bicarbonate the reverse was observed *i.e.*, P_n and $P_{n,phot}$ represented a greater proportion of gross photosynthesis under nutrient replete conditions.

Dark respiration and photorespiration are the two basic types of respiration that occur in photosynthesizing microalgae. Dark respiration is assumed to be constant and occurs both in the light and dark whereas photorespiration is mostly active in the light and a few seconds after dark incubation (Wieland and Kühl, 2000). The dark respiration term (R_{dark}) was obtained as the slope of the initial portion of the O_2 microprofiles (linear part) in the dark. The light respiration terms (R_{light} and R_{phot}) were determined as the difference between P_g, and P_n and $P_{n,phot}$, respectively. Although, there was no clear trend observed for respiration rates (R_{light} and R_{phot}) across nutrient conditions, addition of bicarbonate to the biofilms revealed some differences. For biofilms cultured under N-replete conditions, higher areal respiration rates (R_{light} and R_{phot}) were observed in bicarbonate amended biofilms compared to biofilms that did not receive bicarbonate (Table 1). This may have been due to the higher photosynthetic rates and subsequent increase in oxygen concentration in the biofilms amended with bicarbonate during N-replete culturing (Fig. 4). For algal biofilms cultured under N-deprived conditions, lower R_{light} and R_{phot} were observed with added bicarbonate compared to biofilm samples without bicarbonate (Table 1). This indicated that addition of bicarbonate reduced light respiration in N-deprived biofilms possibly due to an increased DIC supply.

Dark respiration measurements were greater for N-deprived biofilms indicating a higher capacity for heterotrophic (or light independent) respiration. The influence of bicarbonate addition on R_{dark} values varied with nutrient condition. For example, N-replete cultures had higher R_{dark} in biofilms that did not receive bicarbonate, whereas higher R_{dark} were observed in biofilms amended with bicarbonate for N-deprived cultures (Table 1).

3.4. Biofuel precursor production

Extractable biofuel precursor molecules (FFAs, MAGs, DAGs and TAGs) and total biofuel potential (as FAMEs, *i.e.*, extractable and non-extractable molecules) for each biofilm type, both before and after N-starvation, were measured and are presented in Table 2 and Fig. 5. An increase in total extractable precursor concentrations was observed in the biofilms after the 120 h N-starvation period (Table 2). Stressed microalgae have been reported to accumulate TAG as a carbon and energy storage material (Mus et al., 2013). The sum of extractable precursors increased from 5.62% to 7.13% (w/w) for biofilms amended with bicarbonate and 4.84% to 5.18% (w/w) for the biofilms that did not receive bicarbonate, respectively (Table 2). Although the FFA, MAG, and DAG concentrations remained relatively constant, twice as much TAGs accumulated in the biofilms after N-starvation leading to the overall increase in total biofuel precursor molecules (Table 2). Bicarbonate amended algal biofilms had higher weight percentage of extractable molecules.

The total FAME-weight percent and yield for N-replete and N-starved biofilms with or without bicarbonate amendment were similar (Fig. 5A and C). Although, the total FAME potential ranged from 12% to 20% (w/w) of the biomass (Fig. 5B), the total extractable lipids were less than 10% (w/w) (Table 2). As previously reported by Bernstein et al. (2014), the most notable difference regarding lipid production in the RABR-grown algal biofilms was the difference in the total extractable weight percent of lipids between the N-replete and deplete conditions (Table 2). Depletion of nitrogen and addition of dissolved inorganic carbon in the medium were not effective in stimulating substantial lipid production in the microalgal biofilms. Qualitative analysis of lipid profiles

Table 2
Total and percent composition of extractable biofuel precursor weight (%) in laboratory grown microalgal biofilms with and without bicarbonate amendment.

Precursor molecules, weight % (w/w)	Condition			
	Nutrient replete		Nutrient deplete	
	[a]Bicarbonate	[a]No bicarbonate	[a]Bicarbonate	[a]No bicarbonate
C14 FFA	1.44|0.01	0.67|0.14	1.07|0.18	1.23|0.15
C16 FFA	1.11|0.26	1.49|0.03	0.86|0.58	1.45|0.21
C18 FFA	0.73|0.21	1.53|0.05	0.48|0.35	0.79|0.13
C16 MAG	0.11|0.05	0.18|0.01	0.11|0.09	0.13|0.03
C18 MAG	0.11|0.01	0.16|0.01	0.09|0.04	0.13|0.02
C16 DAG	0.09|0.03	0.10|0.02	0.09|0.05	0.10|0.00
C18 DAG	0.21|0.06	0.19|0.06	0.19|0.06	0.17|0.01
C16 TAG	0.49|0.41	0.17|0.08	0.58|0.41	0.35|0.15
C18 TAG	1.32|1.30	0.36|0.37	3.65|1.51	0.84|0.40
Sum of extractables				
Weight % (w/w)	5.62|1.08	4.84|0.71	7.13|0.57	5.18|0.42
Areal concentration (gm^{-2})	1.03	1.22	1.30	1.30

[a] Mean and range (|) for $n = 2$.

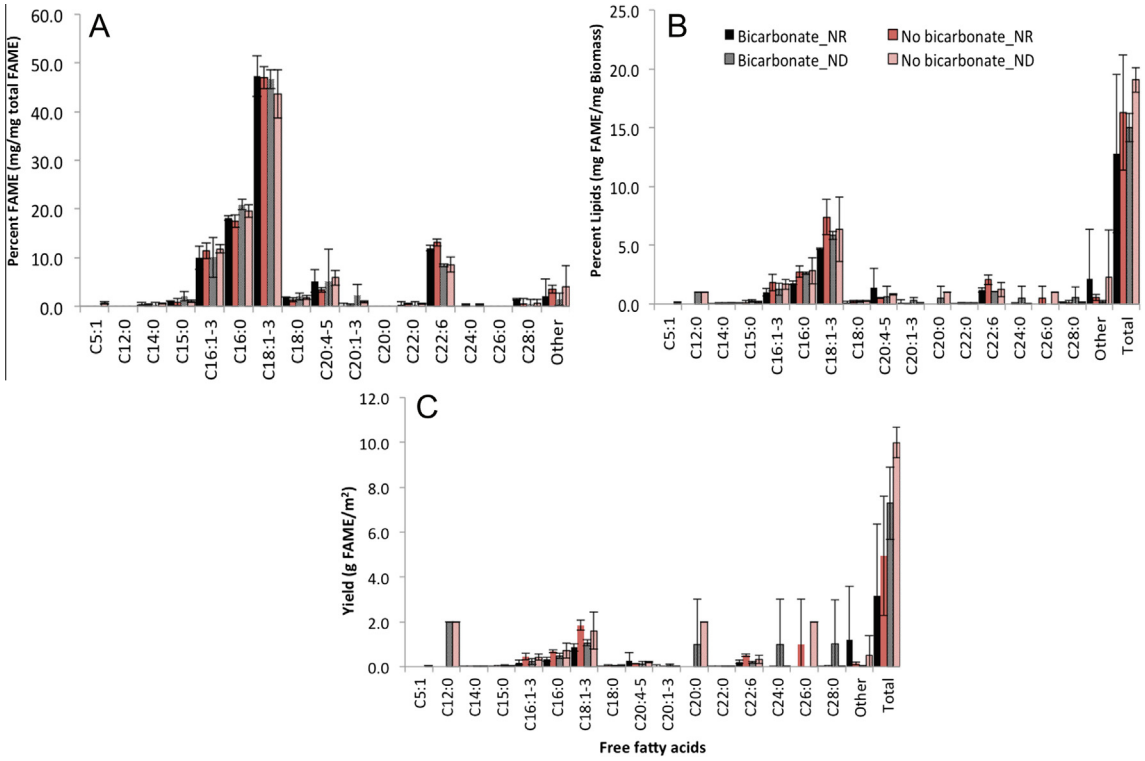

Fig. 5. Total FAMEs and free fatty acid composition of the FAMEs. (A) Percent FAME per total FAME (w/w), (B) percent FAME per biomass (w/w), (C) areal concentration ($g\ m^{-2}$). Error bars represent range ($n = 2$). ND and NR represent nitrogen deprived and replete algal biofilms, respectively.

using images from CLSM showed the same result, the microalgal biofilms only showed a slight increase in lipids after N-starvation (Fig. S3). Previous studies have attributed the inability of N-depletion in the growth medium to induce lipid production in algal biofilms to possible nutrient re-cycling within the biofilms and resilience of algal biofilms to environmental stress (Bernstein et al., 2014; Schnurr et al., 2013).

4. Conclusions

For this study, there was no significant difference in algal biofilm growth, nutrient removal, and lipid accumulation between algal biofilms amended with bicarbonate and those that did not receive bicarbonate. However, an increase in photosynthesis rates was observed in algal biofilms amended with bicarbonate. The influence of bicarbonate on photosynthetic and respiration rates was especially noticeable in biofilms that experienced nitrogen stress, as compared to biofilms in nutrient replete conditions.

Medium N-depletion may not be a suitable stimulant for lipid production in algal biofilms; rather focusing on optimizing growth, nutrient removal rates, and/or biomass productivities may be more beneficial.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2014.12.082.

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