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Neochloris oleoabundans grown on anaerobically digested dairy manure for concomitant nutrient removal and biodiesel feedstock production

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ABSTRACT

Microalgae have been investigated as a promising biodiesel feedstock; however, large-scale production is not currently cost-competitive with petroleum diesel, and its environmental impacts have received little attention. Using wastewater to supply nutrients for algal growth obviates synthetic fertilizer use, provides on-site nutrient removal, and reduces greenhouse gas emissions. In this work, anaerobically digested dairy manure was used to grow the oleaginous green alga *Neochloris oleoabundans*. In batch culture experiments with both synthetic media and anaerobic digester effluent, *N. oleoabundans* assimilated 90–95% of the initial nitrate and ammonium after 6 d and yielded 10–30% fatty acid methyl esters on a dry weight basis. Cellular lipid content and the N concentration in the growth media were inversely correlated. In addition, the proportion of polyunsaturated fatty acids (i.e. C16:3, C18:2, and C18:3) decreased with N concentration over time while the proportion of C18:1 fatty acid increased. Although N deficiency is likely the primary driver behind lipid accumulation, the influence of culture pH confounded results and requires further study. Other living microorganisms in the digester effluent were not observed to affect algal growth and lipid productivity, though the breakdown of organic nitrogen may have hindered lipid accumulation traditionally achieved through the manipulation of synthetic media. This work highlights the potential for waste-grown mono-algal cultures to produce high quality biodiesel while accomplishing simultaneous wastewater treatment.

1. Introduction

The demand for liquid transportation fuels continues to rise amidst concern over climate change, air pollution, ecosystem destruction, and national security challenges related to the production and combustion of petroleum. Biodiesel, a mixture of fatty acid alkyl esters commonly derived from the transesterification of vegetable oils, is an attractive replacement for petroleum diesel because it is compatible with existing diesel engines, reduces tailpipe emissions of most criteria air pollutants, biodegrades, and can be produced from domestically available, biologically derived feedstocks [1,2]. Recently, interest has grown in using oleaginous microalgae as a non-edible biodiesel feedstock, largely on the promise of high oil yields (5000 to 100,000 L ha^{-1} y^{-1}), the opportunity to capture waste CO_2, and the ability to cultivate algae on abandoned or

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unproductive land using brackish, salt or wastewaters instead of freshwater [3–6].

Despite its potential advantages, algae cultivation generally requires nutrients (e.g., N and P) in large quantities, which can significantly affect the cost and environmental impact of its production. A recent life-cycle assessment (LCA) estimated that fertilizer use could account for approximately 50% of the energy and greenhouse gas (GHG) emissions related to algal feedstock production [7]. Instead of using synthetic fertilizers, some of which are finite resources (e.g., mined P) and all of which are increasingly expensive, nutrients for algal production can be supplied from municipal, industrial or agricultural wastewaters [8]. Concentrated animal feeding operations (CAFOs) that employ anaerobic digestion as a primary waste-processing step are a particularly promising source of nutrients for algal cultivation [9]. Anaerobic digestion is commonly used to reduce manure's biological oxygen demand, decrease putridity and odors, and produce electricity by combusting the resulting biogas (ca. 60% CH_4 and 40% CO_2). Despite such advantages, anaerobic digestion does not generally diminish N and P levels of the waste stream and, in fact, often increases the bio-availability of these nutrients (e.g., N in the form of NH_4^+) [10]. This is problematic for CAFOs, which generally produce waste streams that are too highly concentrated or too large to be dissipated by means of field application without incurring the environmental and economic costs of hauling liquid manure to distant fields for spreading, NH_3 volatilization, and nutrient run-off leading to eutrophication of nearby waterways [11–13]. To mitigate these environmental impacts, the liquid effluent and biogas of anaerobic digesters can be used as a nutrient and carbon source, respectively, for enhanced microalgae cultivation. Once harvested, ADE-grown algae can be processed to extract lipids for biodiesel conversion, while the remaining solids can be used as a fertilizer, animal feed, fermented to produce ethanol, or digested to produce additional electricity and return nutrients to the effluent [3]. Given the co-location of a highly concentrated nutrient and CO_2 source, appropriate climate, and flat terrain, CAFOs can be ideal settings for algal biorefineries.

Like most wastewaters, anaerobic digester effluent (ADE) presents unique challenges for algal cultivation that are not typically encountered with chemically defined media, including high and potentially unbalanced concentrations of organic and inorganic nutrients, high turbidity, and the presence of potentially competitive microorganisms. In contrast to the large body of work carried out with green algae in synthetic media, which has largely focused on the role that chemical and physical stressors play in intracellular triglyceride (TG) accumulation [5,14], relatively little is known about how ADE can be effectively used to grow algae as a biodiesel feedstock. Specifically, data are lacking regarding the influence of wastewater dilution, buffering capacity, nutrient composition and loading rates, and bacterial–algal interactions on algal biomass and lipid yields. Furthermore, it remains to be seen whether mono- or polycultures of algae are better suited to biodiesel feedstock production when using waste nutrients sources. Early work by the Aquatic Species Program (1978–1996) advocated for the use of wastewater-grown polycultures of endogenous, unicellular algae [15], but the lipid content of such cultures was rarely reported and recent evidence suggests it is typically relatively low (5–15% lipids on a dry weight basis) [16–18]. Mixed periphyton cultures grown on raw and digested animal manure were even less suitable for biodiesel production, containing only 0.6–1.5% lipids [19]. The relatively low lipid content of wastewater-grown polycultures suggests that the maintenance of a mono-algal population known to be capable of accumulating TG, if possible, may result in higher overall biodiesel yields.

Previous attempts to grow mono-algal populations on wastewater used mostly municipal sources (either diluted and filtered [20] or with chemical additives [21]) or focused on algae that are not well suited to biodiesel production (e.g., *Botryococcus braunii* [22–24]). Recently, however, a wild-type isolate of *Chlorella* sp. was grown on diluted ADE [25], though filtering was used to remove indigenous bacteria and data on lipid productivity in either synthetic media or ADE were not given. In this work, we examine the feasibility of maintaining a specific, oleaginous alga on dairy-derived ADE and determine the impact of culture conditions on lipid productivity and quality. The unicellular, green alga *Neochloris oleoabundans* was chosen for this study due to its reportedly high TG content under conditions of N stress [26–30]. However, with the exception of a single study involving NH_4HCO_3 as an N source [31], previous work has reported on *N. oleoabundans* grown in synthetic media with nitrate (NO_3^-). To determine if this alga is a suitable candidate for integrated biodiesel feedstock production and wastewater treatment, we examined biomass growth and lipid productivity in both synthetic media containing ammonium (NH_4^+) and diluted, NH_4^+-rich ADE. N assimilation was studied as a proxy for wastewater treatment efficacy and changes in lipid composition due to N source, N concentration, and culture pH were reported as indicators of biodiesel fuel quality. Finally, a brief case study detailing the potential for algal biodiesel production at a typical U.S. CAFO is presented.

2. Materials and methods

2.1. Microorganism, media composition, and culture maintenance

Growth and lipid productivity of *N. oleoabundans* (University of Texas Culture Collection of Algae #1185) were examined in synthetic media, anaerobic digester effluent (ADE), and sterile ADE. Cultures were maintained in a modified Bold's Basal Medium (MBBM) (concentration, mg L^{-1}): $CaCl_2 \cdot 2H_2O$ (25), $MgSO_4 \cdot 7H_2O$ (75), K_2HPO_4 (75), KH_2PO_4 (175), NaCl (25), $Na_2EDTA \cdot 2H_2O$ (4.5), H_3BO_3 (2.86), $FeCl_3 \cdot 6H_2O$ (0.58), $MnCl_2 \cdot 4H_2O$ (0.25), $CuSO_4 \cdot 5H_2O$ (0.079), $ZnCl_2$ (0.030), $CoCl_2 \cdot 6H_2O$ (0.012), $Na_2MoO_4 \cdot 2H_2O$ (0.024), vitamin B_{12} (0.131), biotin (0.024), and thiamin (0.534). In batch culture experiments, N was provided as $NaNO_3$ or NH_4Cl at concentrations ranging from 0 to 100 mg L^{-1}. N concentrations in MBBM-NH_4^+ batches (50, 25, and 10 mg L^{-1}) were designed to approximate ADE that had been diluted 1:50, 1:100, and 1:200, respectively (Table 1). Synthetic media were prepared with purified deionized water, autoclaved, and adjusted to pH 7.2 with 1 N NaOH. Additional microorganisms in *N. oleoabundans* cultures

Table 1 – Comparison of nutrients in synthetic media (MBBM-NH_4^+) and anaerobic digester effluent (ADE).

Component	Concentration (mg L^{-1})	
	MBBM-NH_4^+	Undiluted ADE[a]
Total N	100	3007
NH_4^+-N	0–100	2097
NO_3^--N	0.00	–
Organic N	0.00	910
P[b]	53.10	300
K[c]	84.00	3262
Ca	6.80	1044
Mg	7.40	659
Cu	0.02	9.59
Zn	0.01	20.4
Fe	0.12	64.7
Mn	0.14	16.8
B	0.50	6.00

a Manure analysis completed by UVM Agricultural & Environmental Laboratory.
b P reported as mg L^{-1} P_2O_5.
c K reported as mg L^{-1} K_2O.

were monitored microscopically and by heterotrophic plate counting. This background contamination was somewhat limited by filtering (P5 qualitative filter, Fisher Scientific) and washing inocula thoroughly in an N-free medium prior to each experiment. Experiments using synthetic media were inoculated with algae that were previously adapted to the appropriate N source at the desired concentration and had never been exposed to ADE. Flasks (250 mL) for batch culture experiments were acid-washed, rinsed, and autoclaved. All treatments were replicated at least twice.

A humidified 2–3% CO_2-air mixture was delivered to each flask (0.4 L min^{-1}) via a linear air pump (LT15, Whitewater) and a regulated CO_2 cylinder. Light was supplied in a 14:10 h light:dark photoperiod by two cool-white fluorescent bulbs (Residential EcoLux 40 W, GE) and two plant and aquarium fluorescent bulbs (Plant and Aquarium 40 W, Phillips), for a total light output at the culture flask surface of approximately 200 μmol m^{-2} s^{-1}. A PAR detector (QMSW-SS, Apogee Instruments) was used to measure irradiance. Experimental time is reported as 1 d being equivalent to the 14 h photoperiod (e.g., 21 total light-hours = 1.5 d). Considering time on a light-hour basis allows data collected at different time points in different experiments to be more readily compared. Flasks were maintained at room temperature (23–25 °C).

2.2. Anaerobic digester effluent (ADE)

ADE was collected from a CAFO at Blue Spruce Farm in Bridport, VT (44.0 N, 73.3 W), which milks approximately 1000 cows and scrapes raw manure into a plug-flow, mesophilic anaerobic digester maintained at 37.8 °C. The typical hydraulic retention time is 20 d with a total reactor volume of 2300 m^3. ADE is processed on-farm with a screw-press to remove solids, which are used as animal bedding and later returned to the digester. The remaining liquid is stored on-site in holding lagoons and later applied to fields. The solids-separated ADE (pH 7.5) was collected directly from the screw-press in 20-L plastic containers and stored at 4 °C prior to use. *N. oleoabundans* previously adapted to MBBM-NH_4^+ was used to inoculate flasks containing ADE diluted 1:50, 1:100, and 1:200 in purified, distilled water. No pH adjustment or sterilization of the ADE was performed prior to inoculation, except for one treatment group, in which ADE 1:200 was autoclaved (A-ADE) to assess whether the native, pre-existing microbial community affected algal growth and lipid yields. Undiluted ADE contained both NH_4^+-N (2100 mg L^{-1}) and organic N (910 mg L^{-1}) (Table 1). The organic N fraction was not characterized nor monitored over time, though it is likely that some organic N became bioavailable to the algae as a result of hydrolysis. At all dilutions, ADE contained less P and K than MBBM, while concentrations of Ca, Mg, and Fe were higher. Growth curves were determined for cultures at each dilution by subtracting the mass of the culture at the time of inoculation, such that growth rates and productivity estimates reflect only biomass increases due to new algal growth. Biomass contributions from other microorganisms were negligible, as confirmed by controls that were not inoculated with algae.

In addition to small-volume batch culture experiments, *N. oleoabundans* was grown on ADE in 50-L photobioreactors (110 cm × 60.5 cm × ~10 cm deep) constructed out of 0.15 mm thick polyethylene tubing (U-Line). Reactors were positioned horizontally and lit from above (200 μmol m^{-2} s^{-1}). High efficiency aeration tubing (Aquatic Ecosystems) was used for gas sparging. Reactors containing ADE diluted 1:200 were inoculated (10% v/v) with algae adapted to MBBM-NH_4^+ and fed with 10 L of diluted ADE (1:40) once every 7 d. Cultures were harvested (10 L) just prior to each feeding. Culture stability was assessed over a three-month period through qualitative microscopic observations. At the end of the three-month period, ADE collected from an outdoor holding lagoon, as opposed to directly from the screw-press separator, was loaded with the intent to examine the influence that additional contamination might have on the viability and lipid production of *N. oleoabundans*.

2.3. Analytical measurements

Biomass density (g L^{-1} dry weight) was measured gravimetrically and via optical density measurements at 600 nm. Culture samples were centrifuged in pre-weighed glass screw-top test tubes at 671 gravity for 15 min. Ion-selective electrodes (ISEs, Vernier) were used according to standard methods to measure pH, NO_3^-, and NH_4^+ concentrations in the supernatant [32]. Wet algae pellets were dried at 60 °C for 24 h, allowed to cool in a desiccator, and re-weighed. Dry pellets were stored in the dark under N_2 at 4 °C prior to lipid analysis. Bomb calorimetry was used to determine the heating value of dried algal biomass and the following conventional products: pure biodiesel, canola oil, and diesel fuel.

The lipid composition of dried biomass samples was determined using a modified, one-step in-situ transesterification (IST) procedure [33,34] or a warm isopropyl alcohol (IPA, 95%) extraction coupled with acid-catalyzed transesterification. Total lipids in cells harvested from synthetic media containing NO_3^- were analyzed with the modified IST procedure. Briefly, dried algal samples

(~10–50 mg) were reacted with 5 mL 10:1:0.3 CH_3OH:$CHCl_3$:H_2SO_4 (v/v/v) at 90 °C for 2 h with vigorous stirring. Water (2 mL) was added to stop the reaction and FAMES were extracted with 4:1 C_4H_{14}:$CHCl_3$ for gas chromatography-mass spectrometry (GC/MS) analysis. Theoretically, this IST procedure converted fatty acids (FAs) from all lipid classes to FAMEs. In comparison, non-polar solvents, such as *n*-hexane, are typically used to extract only TGs from oilseeds, leaving most of the more polar lipids in the solid. To provide a more realistic estimate of recoverable lipids that may be readily converted into biodiesel, samples collected from cultures grown with MBBM-NH_4^+, ADE, and without N were processed similarly to how biodiesel is typically made from dry soybeans, although warm IPA was employed as a less toxic alternative to *n*-hexane. IPA has been found to reduce lipid degradation caused by lipolytic enzymes in plant tissues and provides easy separation of the oil-solvent mixture upon cooling [35,36]. Oven-dried biomass samples (10–50 mg) were extracted with 10 mL IPA with vigorous stirring for 8 h at 55 °C. Following extraction, samples were centrifuged, and the oil-IPA supernatant was quantitatively transferred to a separate transesterification vial using 5 mL hexane. The combined organic phase was evaporated to dryness under N_2, weighed to determine the crude lipid mass, and transesterified according to Christie [35]. Briefly, the crude lipid extract was dissolved in 1 mL toluene containing nonadecanoic acid (Sigma) as a surrogate and reacted with 2 mL methanol containing 1% (v/v) H_2SO_4 for 3 h at 70 °C. FAMES were obtained in a hexane-rich phase, which was analyzed by GC/MS. Surrogate recoveries of methyl nonadecanoate (C19:0; 105% typical recovery) indicated that transesterification proceeded to completion. Given the influence of extraction procedure on the lipids recovered, a direct quantitative comparison between lipid data for cells grown in MBBN-NO_3^- and other media is not possible. Nevertheless, because the lipid productivity of wastewater-grown algae is of greatest interest and because IST has been shown to recover more lipids (as FAMEs) than two-step extraction-transestertification procedures [34,37–39], the data provided herein are a more conservative portrayal of this technology's potential.

FAME analysis was performed using GC/MS (Agilent 6890 N GC, Agilent 5975 Mass Selective Detector) with a polar SUPELCOWAX™ 10 fused silica capillary column (30 m × 0.32 mm × 0.25 μm). Automated splitless injection (1 μl; 250 °C inlet temperature) was made with an initial oven temperature of 150 °C. After a 2 min hold, the temperature was ramped at 4 °C min^{-1} to 220 °C, held for 8 min, and then increased at 10 °C min^{-1} to 260 °C. Helium was used as the carrier gas at a constant flow rate of 1.0 mL min^{-1}. Calibration standards (FAME Mix RM-3, Supelco) were prepared to contain most FAMEs of interest, as well as the internal standard (IS) and C19:0 surrogate. Methyl pentadecanoate (C15:0, Sigma) served as the IS; analysis of control samples demonstrated that endogenous C15:0 FA was present at <0.5% of total FAs, in agreement with previous findings [27].

2.4. Biodiesel fuel quality assessment

Iodine number is a measure of the degree of unsaturation present in a mixture of FAs, expressed as grams of iodine that react with 100 g of sample. Iodine number was calculated as the product of the mass percent of each FAME and the following conversion factors [40]: C16:1 (0.950); C18:1 (0.860), C18:2 (1.732); C18:3 (2.616); C20:1 (0.785), C22:1 (0.723). Viscosity was estimated according to the method of Allen et al. [41]. The total enthalpy of combustion was calculated from known values for each pure FAME compound [40] weighted by its relative abundance in the FAME mixture. Pure biodiesel (B100) and transesterified canola oil were analyzed as controls to validate the estimation of these parameters.

3. Results and discussion

3.1. Algal growth in synthetic media

Batch culture experiments were performed to characterize how N source (NO_3^- or NH_4^+) and N concentration (0, 10, 25, 50, 100 mg L^{-1}) affected the growth and lipid productivity of *N. oleoabundans* in synthetic media. Over the course of 7 d, *N. oleoabundans* grown in MBBN-NO_3^- containing N at 100 mg L^{-1} demonstrated the highest productivity (Table 2) and achieved the greatest cell density (Fig. 1a). Similarly robust growth was observed for cells grown in MBBN-NO_3^- with N at 50 mg L^{-1}, while lower NO_3^- concentrations supported exponential growth for only 4 d, after which growth ceased. In MBBM-NH_4^+, growth was impaired at high initial N concentrations and demonstrated productivities that were ~50% lower than NO_3^--adapted cells at the same N levels (Fig. 1b, Table 2). High NH_4^+-N concentrations (100 mg L^{-1}) were apparently toxic to *N. oleoabundans*, with cell density never exceeding 0.1 g L^{-1} and complete bleaching of the culture occurring around Day 4 (data not shown). With NH_4^+-N at 50 mg L^{-1}, cell biomass increased at an average of 69 mg L^{-1} d^{-1} up to Day 4 but then declined, likely in response

Table 2 – Biomass and lipid productivity[a] in batch culture.

Treatment[b]	Productivity (mg L^{-1} d^{-1})	
	Biomass	Lipid
MBBM		
NO_3^- (100)	158 ± 25.5	8.9 ± 0.1
NO_3^- (50)	134 ± 8.3	11.1 ± 2.3
NO_3^- (10)	55 ± 5.5	16.5 ± 2.5
NH_4^+ (50)	69.9 ± 1.5	–
NH_4^+ (25)	42.0 ± 7.5	4.3 ± 1.6
NH_4^+ (10)	24.4 ± 1.7	3.6 ± 0.6
N (0)	13.6 ± 0.8	3.1 ± 1.1
ADE		
ADE (1:50)	88.3 ± 7.9	2.57 ± 0.9
ADE (1:100)	64.0 ± 8.6	1.4 ± 0.5
ADE (1:200)	39.9 ± 5.6	3.8 ± 1.6
A-ADE (1:200)	43.8 ± 18	4.7 ± 2.0

a Productivities are calculated based on the time at which the maximum biomass density or lipid content occurred.
b N concentrations (mg L^{-1}) and ADE dilution are indicated. Data are averages ± standard error. A-ADE was autoclaved.

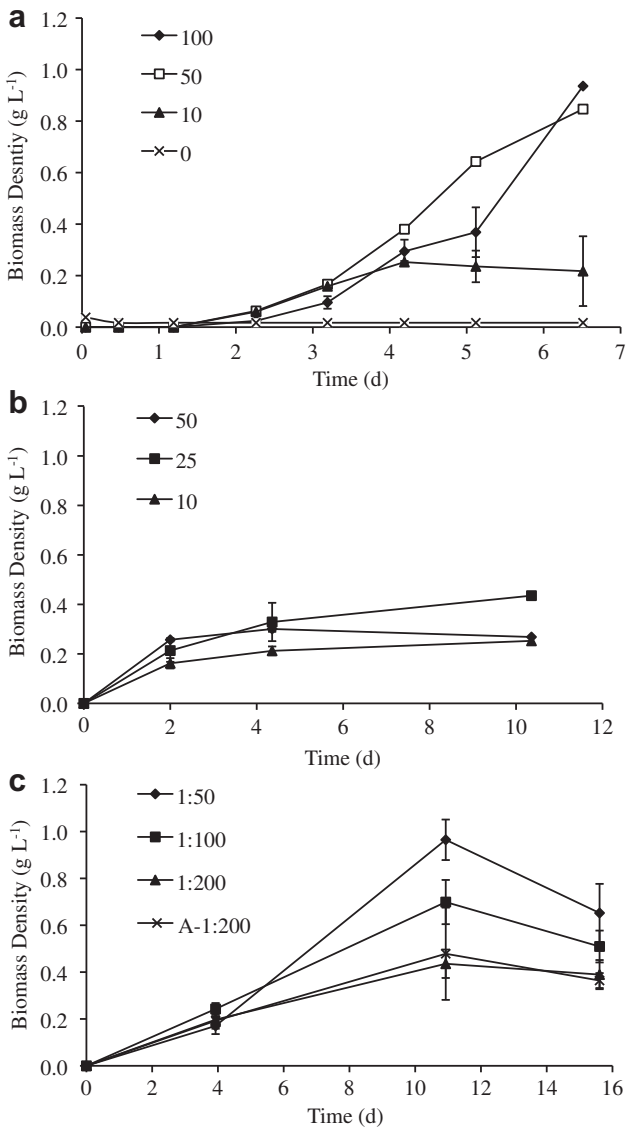

Fig. 1 – Biomass density (g L^{-1}) of *N. oleoabundans* over photo-time when grown in a) MBBN-NO_3^-; b) MBBM-NH_4^+; c) ADE. Density reported represents new growth post-inoculation. Cells grown in ADE received no light or aeration after Day 11. Data are averages ± standard error.

to acidification of the culture medium. Mid-level concentrations of NH_4^+-N (25 mg L^{-1}) supported the highest sustained cell growth that was observed for NH_4^+ treatments, achieving a maximum of 0.4 g L^{-1}. The lowest NH_4^+-N concentration (10 mg L^{-1}) supported cell growth over the 10 d period and reached a similar final biomass density as the culture grown with NO_3^- at the same N level.

These results are consistent with recent literature indicating that *N. oleoabundans* achieves the highest cell density when N is present in concentrations ranging from 70 to 140 mg L^{-1} as NO_3^- [31]; however, performance in NH_4^+-containing media can likely be improved using fed-batch or continuous-flow reactors to reduce inhibition related to high initial NH_4^+ concentrations. Although low pH in synthetic NH_4^+-containing cultures would suggest most N was present in the ionized form and, therefore, unlikely to be a direct contributor to cellular toxicity [42], longer periods of adaption to NH_4^+-containing media and selective breeding might also be used to improve this organism's tolerance to ammonia.

3.2. *Characterization of anaerobic digester effluent (ADE) and its ability to support algal growth*

Given that *N. oleoabundans* grew on a synthetic medium containing NH_4^+ as the sole N source, we hypothesized that ADE, which is rich in NH_4^+, would also sustain growth of this alga. Several dilutions of ADE were used in batch culture experiments to determine how light attenuation due to suspended solids, nutrient concentrations, and endogenous microorganisms might affect *N. oleoabundans* growth (Fig. 1c). The highest biomass productivity was observed in the most concentrated ADE preparation and decreased as ADE became more dilute (Table 2). In comparison to cells grown on all levels of MBBM-NH_4^+, cultures in 1:50 ADE grew more quickly and maintained higher biomass productivity for longer periods of time. ADE diluted 1:50 and 1:200 supported growth to maximum biomass densities similar to MBBM-NO_3^- with N at >50 mg L^{-1} and 10 mg L^{-1}, respectively, though productivity in ADE cultures was lower given the longer time (10 d vs. 7 d) required to achieve these densities. After culture aeration and illumination stopped (Day 11), growth ceased and biomass density declined in all ADE dilutions. In all batch cultures of *N. oleoabundans* grown on ADE, other microorganisms (e.g., free swimming ciliates and bacteria) were regularly observed, but no additional green algae were detected.

The nutrient composition of ADE from dairy operations can vary widely due to different animal diets and pre-digestion manure handling (e.g., flushing vs. scraping) [43]. In agreement with our findings (data not shown), Woertz et al. [16] reported difficulty growing algae in undiluted ADE from a flushed dairy operation (366 mg L^{-1} total N and 160 mg L^{-1} NH_4^+-N) and used 1:4 and 1:10 dilutions in outdoor batch cultures. In the present work, undiluted ADE contained about 10-fold higher N concentrations (Table 1) and was diluted more aggressively. To optimize dilution strategies, future work should consider the net impacts of using local freshwater, saline groundwater, or seawater, with the possibility of improving lipid yields through osmoregulatory pathways [44,45], as well as returning water liberated during biomass harvesting for dilution. Optimal light attenuation should also be considered, because while low dilution may beneficially limit photo-oxidative damage [15], it simultaneously may limit the photosynthetic carbon flux necessary for lipid accumulation [31].

N. oleoabundans did not demonstrate significantly different growth (Fig. 1c) or lipid composition when cultured in unprocessed ADE compared to autoclaved ADE (Table 3). Although heterotrophic nutrient remineralization and carbon oxidation may provide nutrients and CO_2, respectively, to algal cells [46], and bacteria have been found to produce vitamin B_{12} [47], which is essential to algal growth, there was little evidence that microorganisms native to ADE contributed to better *N. oleoabundans* growth in ADE. Rather, organic nutrient sources and other complex cofactors present in both

Table 3 – Biomass lipid content and degree of fatty acid unsaturation.

Treatment[a]	Lipid content[b]		Iodine number[c]	
	Day 4	Day 10–11	Day 4	Day 10–11
MBBM				
NH_4^+ (50)	0.2	0.1	0	0
NH_4^+ (25)	5.4 ± 2.1	1.3 ± 0.7	83 ± 8.6	84 ± 15.5
NH_4^+ (10)	10.0 ± 1.9	14.8 ± 2.1	92.0 ± 11.5	91.55 ± 2.5
ADE				
ADE (1:50)	1.6 ± 0.4	2.2 ± 0.7	112 ± 21.1	125 ± 10.2
ADE (1:100)	n/a	2.3 ± 1.0	n/a	122 ± 5.7
ADE (1:200)	1.09 ± 0.04	9.5 ± 2.8	119 ± 6.5	94 ± 4.4
A-ADE (1:200)	1.8 ± 1.3	9.9 ± 2.7	154 ± 31.8	92.5 ± 10.9

a N concentrations (mg L^{-1}) and ADE dilution are indicated. Data are averages ± standard error.
b Lipid content reported as the percent FAMEs of dry cell weight extracted.
c Iodine number in g I_2 per 100 g of FAME.

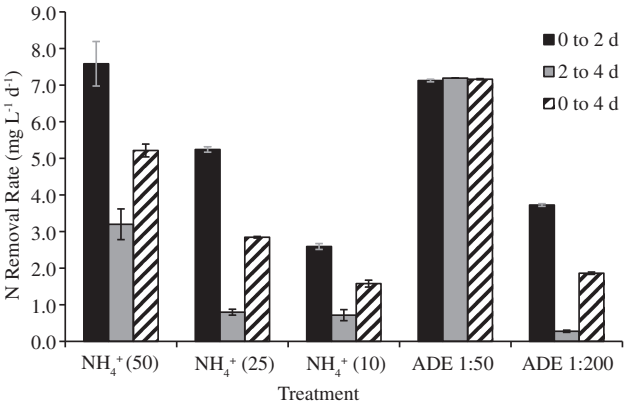

Fig. 2 – N removal rates from NH_4^+-containing media. N concentrations (mg L^{-1}) and ADE dilution are indicated. Data are averages ± standard error.

ADE and A-ADE most likely played a role in supporting robust algal growth. For example, iron was notably 10-fold higher in the 1:50 ADE dilution compared to MBBM and has been found to influence biomass density and lipid accumulation in *Chlorella vulgaris* [48]. Future studies should consider how these and other properties of wastewater affect biomass and lipid productivity in a wider array of species with lipids ideal for biodiesel production. It is possible that mixotrophic lipid-rich algae (e.g., *Chlorella* [25,49]), which can take advantage of dissolved carbon sources present in ADE, may outperform *N. oleoabundans* and other algae that are capable only of photosynthesis [26].

3.3. Nitrogen assimilation

To better understand N assimilation and assess nutrient-loading rates that would both maximize algal productivity and N removal from wastewaters, NO_3^- and NH_4^+ levels were monitored over time. As expected, N concentration decreased over time as cells assimilated N, and all treatment groups achieved ~90–95% removal of the initial N present after 6 d. Nitrate was almost completely assimilated from synthetic media at all concentrations tested after 4–5 d (data not shown), consistent with previous reports on this alga [31]. Ammonia volatilization, which occurs at a pH > 8.5, was assumed to be negligible because the pH was consistently below 7.2 in MBBM-NH_4^+ and ADE cultures. Likewise, the NO_3^- concentration in all ADE flasks was <1 mg L^{-1} N, suggesting that nitrification was likely not a large factor in nutrient removal.

Among cultures grown in MBBM-NH_4^+, N removal rates were significantly greater at the highest concentrations of NH_4^+ (Fig. 2). In all MBBM-NH_4^+ cultures, N removal rates declined significantly after Day 2, most likely due to diminishing biomass productivity. Cells grown on ADE 1:50 demonstrated sustained N removal rates equal to those observed in the NH_4^+ high treatment group during the first 2 days, and had an overall removal rate over the course of 4 d significantly higher than any other treatment group. This may be correlated with sustained growth in this medium, possibly stimulated by organic N becoming more bioavailable over time. The ADE 1:200 treatment demonstrated N removal rates comparable to cells grown in MBBM-NH_4^+ at low and mid-level concentrations. In general, near complete N removal by *N. oleoabundans*, which was similar to that observed for *Chlorella* sp. [25] and mixed algal communities [16] grown on dairy-derived ADE, suggests this is a robust organism for wastewater remediation.

3.4. Cell lipid content and productivity

Cell lipid composition was investigated over time based on the mass of FAMEs positively identified by GC/MS. Importantly, gravimetric analyses revealed 2–5 fold higher lipid contents than quantification of FAMEs by GC/MS, indicating that non-lipid components (e.g., chlorophylls, carotenoids, sterols) were most likely also extracted. These compounds are more likely to be contaminants than useful fuel components. As a result, our FAMEs-based analysis is a more conservative estimate of attainable biodiesel yields than gravimetric data alone. In general, lipid accumulation correlated with N deficiency for most treatments. After 7 d, cells grown in MBBM-NO_3^- with N at 100, 50, and 10 mg L^{-1} contained 5.7 ± 0.7%, 8.2 ± 0.8%, and 29.7 ± 1.1% lipids as FAMEs on a dry weight basis, respectively. Cells grown in N-free media contained 23.9 ± 3.7% lipids after 7 d. This is similar to recent reports of *N. oleoabundans* containing 29–45% lipids when grown in synthetic media containing N at 0–70 mg L^{-1} as NO_3^- [28,31]. In the present work, lipid productivity was highest in the treatment with the lowest initial NO_3^- concentration (Table 2), but overall lipid productivity was relatively low compared to recent reports in the range of 100–130 mg L^{-1} d^{-1} [31]. *N. oleoabundans* grown in MBBM-NH_4^+ and ADE also showed N concentration-dependent patterns of lipid accumulation (Table 3). Ten days after inoculation, batch cultures grown in high concentrations of NH_4^+ contained only 0.1% lipid, while those grown at low NH_4^+ concentrations contained ~15% lipid. The lipid content of algal cells in all dilutions of ADE were similar at 4 d following inoculation (1–2% lipids), but

demonstrated lipid accumulation at 10 d (2–10% lipids; Table 3). After Day 10, lipid accumulation was further monitored for 5 d in the absence of light and supplemental CO_2. During this time, biomass density declined significantly (Fig. 1c) while cell lipid content decreased slightly or remained unchanged.

Lipid recovery from cells grown at the highest concentration of NH_4^+ in synthetic media was surprisingly low, with all detected FAMEs in these samples except C16:0 below quantifiable limits. Crude lipid extracts from these samples indicated 1–3% lipids, which is comparable to the previously reported TG content of *N. oleoabundans* (~3%) cultured in nutrient-sufficient synthetic media with NO_3^- [30]. While it is possible that a cellular stress response to N limitation was not induced given some residual N in solution (<5 mg L^{-1}), lipid accumulation may also have been inhibited by acidic conditions at high NH_4^+ levels. The pH was below 7 in all MBBM-NH_4^+ cultures, with severe acidification (pH < 3.5) occurring in the highest NH_4^+ treatment after 4 d. In contrast, MBBM-NO_3^- cultures remained above pH 9 two days following inoculation, with some treatments reaching pH 12 after 7 d. Acidification may have resulted from the weakly acidic nature of NH_4^+, aeration with CO_2-enriched air, and the efflux of protons by H^+-ATPases coupled to the NH_4^+ uniporter in the cell membrane [50,51].

Alkaline conditions have been found to inhibit cell division and result in lipid accumulation independent of nutrient stress in the green alga *C. vulgaris* [52]. The accrual of autosporangial complexes, in which nuclei have divided but the autospores have not separated, was observed only in unbuffered media with pH > 10. It was hypothesized that alkaline pH stress may increase the flexibility of the mother cell wall, thereby preventing autospore release. It is also possible that enzymes responsible for degrading the mother cell wall, such as sporangial autolysin, are inhibited at high pH [53]. In our work, large sporangial complexes, known as aplanospores, were routinely observed in aging cultures grown on NO_3^- with pH > 8.5 but not in cultures grown with NH_4^+. Since autospore release has been found to coincide with TG utilization in the algal cell cycle, factors limiting cell division may cause TG accumulation [52]. It is possible that alkaline stress, potentially achieved through photosynthetic uptake of dissolved carbon, may be useful to induce neutral lipid accumulation before N depletion. Accordingly, the acidification of NH_4^+-media and the increase in pH observed in NO_3^--media may have influenced lipid accumulation independent of N concentration. Examination of lipid accumulation under different N sources using a more highly buffered medium (e.g., HEPES) or pH control through CO_2 addition would help evaluate the confounding effects of pH and N source.

In contrast to synthetic media, ADE treatments demonstrated relatively stable pH (~7.2) over time. After 10 d, cells in all treatment groups demonstrated lipid accumulation, but cells in the more dilute ADE medium (1:200) demonstrated significantly higher lipid contents, probably due to N stress and greater light availability. While these findings demonstrate the possibility of using N deficiency as a means of inducing lipid accumulation, low overall lipid yields also highlight some of the difficulties encountered when working with complex wastewater rather than synthetic media. For example, the naturally well-buffered ADE medium may have limited lipid accumulation that could have been promoted via alkaline stress, while the increasing bio-availability of organic N over time may have hindered the induction of a N-based stress response. Overall, ADE supported biomass growth better than comparable levels of NH_4^+ in synthetic media, but cells had lower lipid contents. Similar results have been reported for heterotrophic cell growth in *C. vulgaris*, where organic N sources, such as yeast extract, more effectively promoted growth compared to inorganic sources like KNO_3 but caused a dramatic reduction in cell lipid content when present at high concentrations [54]. Decreasing biomass density and cell lipid content after Day 10 in ADE treatments suggests that intracellular lipid stores may have been used to meet cellular energy demands and that lipid accumulation requires at least light for photosynthetic carbon fixation [5]. Further, these data are consistent with the theory that TG synthesis occurs principally in the light and TGs are then utilized in the dark for polar lipid synthesis [55], suggesting that allowing algal cells to age in darkness without aeration, even in low N media, is not an effective strategy for increasing the concentration of intracellular TGs.

3.5. *Fatty acid profiling for biodiesel fuel standards*

The FA composition of *N. oleoabundans* was analyzed to estimate several parameters of biodiesel fuel quality, including the degree of unsaturation (iodine value), heat of combustion, and viscosity. FAMEs generated from algal lipids in all N-deficient cultures contained similar FA profiles (Table 4). In general, the major FAs detected were: C16:0 (23–30%), C18:1 (30–43%), C18:2 (18–23%), and C18:3 (5–12%). This FA profile is similar to previously reported values for *N. oleoabundans* in N-deficient media and resembles the FA profile of waste vegetable oil and biodiesel derived from tallow (Table 4). However, our data are in contrast with recent reports describing lipid accumulation without changes in the cellular FA profile [28]. In particular, our results reveal a dramatic increase in the proportion of 9-octadecenoic acid (C18:1) over time while the proportion of 9-,12-,15-octadecatrienoic acid (C18:3) and 7,10,13-hexadecatrienoic acid (C16:3) declined significantly. Namely, the proportion of C18:1 and C18:3 in cells harvested from ADE 1:200 changed from 20.9 ± 1.5% and 30.7 ± 8.1% at Day 4 to 41.1 ± 2.7% and 6.4 ± 0.9% at Day 10, respectively. Likewise, the proportion of polyunsaturated fatty acids (PUFAs) present in cells at each harvest was lower in cultures grown with less initial N compared to those with more N of the same source. Fewer PUFAs is advantageous for reducing fuel polymerization that can otherwise cause damaging engine deposits, and C18:1 FA is considered ideal for biodiesel because it provides better cold flow properties without losses to oxidative degradation [40].

The stark change in the FA composition of recovered lipids should inform growth strategies and harvesting schedules to optimize fuel quality. Specifically, obtaining biodiesel with C18:3 levels below the EN14214 limit (<12.0%) would be beneficial. Though most pronounced in the ADE treatments, the reduction in PUFA with time was observed in cells grown on all N sources and generally corresponded to declining iodine values (Table 3). In addition, the relative C16:0 FA content found in algal lipids was higher than that in canola

Table 4 – Fatty acid profiles for various biodiesels (% FAME).

Source	Fatty Acid									
	14:0	16:0	16:1	16:2	16:3	17:0	18:0	18:1	18:2	18:3
N. oleoabundans										
ADE 1:200[a]	–	26.4	1.5	0.5	2.2	–	1.5	41.1	18.9	6.4
NH_4^+[b]	–	29.5	1	1.3	1.9	–	2.7	35.7	21.1	5.9
NO_3^-[c]	–	23.3	0.6	1.6	2.4	0.2	4.5	43.0	17.8	5.8
NO_3^-[d]	1.6	15	3.5	2.5	–	3.3	11	36	7.4	–
NO_3^-[e]	0.4	19.4	1.9	1.7	0.9	–	0.9	20.3	12.9	17.4
Other feedstocks										
B100[f]	2	24.1	2.8	–	–	0.7	15.1	44.2	10.4	0.9
Canola Oil[g]	0.1	10.4	–	–	–	0.1	4.4	30.0	45.9	7.7
SBO[h]	–	11–12	–	–	–	–	3–5	23–25	52–56	6–8
RSO[h]	–	3–5	–	–	–	–	1–2	55–65	20–26	8–10
Used frying oil[h]	1–3	13–25	0–4	–	–	–	6–8	43–52	7–22	0.5–3

a ADE 1:200 at 10 d after inoculation.
b N at 10 mg L^{-1} 10 d after inoculation.
c N at 10 mg L^{-1} 7 d after inoculation.
d Data for cells grown in low NO_3^- [27].
e Data for cells grown in low NO_3^- [28].
f Biodiesel supplied by Rothsay Biodiesel (Quebec, Canada; tallow source).
g Canola oil was purchased locally and transesterified.
h Soybean oil (SBO), low-erucic acid rapeseed oil (RSO), and used frying oil data [40].

and soybean oil, imparting algal biodiesel with better oxidative stability, a higher cetane number, but worse cold flow properties [40]. These results are consistent with previous findings that FA content increases and becomes more saturated with declining N levels [27,56]. The heat of combustion for nearly all analyzed lipid samples was estimated at approximately 39 MJ kg^{-1}, which is consistent with bomb calorimetry results. Finally, the viscosity of algal FAMEs increased with decreasing N concentrations, though typical values were between 2.8 and 3.5 Pa s, which is similar to that predicted for tallow-based B100 (3.7 Pa s) and common for soy methyl esters (2.7–3.6 Pa s).

3.6. *Culture stability*

Cultures of *N. oleoabundans* were maintained for over 3 months indoors in low-cost, enclosed bioreactors (50 L) on diluted ADE. Over this time period, other species of green algae were not detected in these photobioreactors or in any batch culture experiments. In contrast, however, when ADE was collected from the outdoor holding lagoon, as opposed to directly from the screw-press effluent pipe, as was done routinely for all other experiments, *N. oleoabundans* was overtaken by another green alga. The lipid content of this unidentified new alga was approximately 2–3% when examined over a period of 2 weeks. This finding suggests that some degree of bioreactor enclosure, high-density inoculum or culture, and/or the use of selective pressures (e.g., extreme pH or salinity) may be required to maintain a *N. oleoabundans*-dominated population in open, outdoor growth ponds. However, it also corroborates previous findings that naturally occurring, algal polycultures typically contain lower concentrations of lipids [16]. Obviously, sufficiently high lipid productivity relative to native polycultures would be necessary to justify the potential added expense (if any) of maintaining mono-algal populations. A full LCA and cost estimate of using plastic bioreactors relative to traditional open raceways is beyond the scope of this work but has been discussed in the recent literature [18,57,58].

3.7. *Case study of algal biodiesel production at a CAFO*

The potential for on-site wastewater treatment coupled with the production of liquid transportation fuels at CAFOs may one day provide opportunities for rural economic development and increased energy self-sufficiency. For example, based on the average solar insolation in Burlington, Vermont (44.4 N, 73.15 W) in the 8 warmest months of the year (4.70 kWh m^{-2} d^{-1}; [59]) and the estimated amount of N excreted by 1000 cows that are milked daily at Blue Spruce Farm (73 Mg y^{-1} of N [12,60]), approximately 330 m^3 y^{-1} biodiesel could be produced on about 25 ha of land (corresponding to about 17 g m^{-2} d^{-1} biomass productivity assuming 7% N and 30% lipids on a dry weight basis). This amount of algal biodiesel production could replace nearly 100% of the petroleum diesel currently used each year by Blue Spruce Farm. The promise of this technology is likely even greater at lower latitudes with more favorable climatic conditions. Overall, an estimated 1.2 Mt of N are recoverable each year from animal waste at U.S. CAFOs [13], which has the potential to support production of about 5.6 million cubic meters of biodiesel per year (or about 4% of US low-sulfur diesel fuel consumption in 2008 [61]). In this situation, GHG emissions are reduced via three major avenues: 1) the direct replacement of fossil diesel fuel with biodiesel derived from extracted algal lipids; 2) reduction in the energy required to treat, transport, and process wastewater compared to traditional methods; and 3) the replacement of fossil-fuel derived fertilizers with recycled nutrients in both algae cultivation and traditional agriculture.

4. Conclusion

In the present study, a specific species of oleaginous microalgae, previously adapted to synthetic media containing NO_3^-, was observed to grow on NH_4^+-containing synthetic media and anaerobically digested dairy manure. *N. oleoabundans* removed N from both synthetic media and ADE while accumulating lipids well-suited for conversion to biodiesel. In particular, algal lipids contained fewer PUFAs and more C18:1 FA as cells aged and were exposed to declining levels of extracellular N. When grown in simple, non-optimized batch cultures, *N. oleoabundans* contained a maximum of ~10% lipids when using ADE as a nutrient source. The large difference between the maximum achievable biomass lipid content in synthetic media and wastewater suggests that the conditions required to induce lipid accumulation, such as N stress and high pH, require more careful implementation and continued study in the complex matrix of ADE. While the feasibility of maintaining large-scale mono-algal populations on ADE for combined biodiesel production and wastewater treatment have not yet been examined, the present work demonstrates its potential feasibility in batch culture and in larger, low-cost photobioreactors.

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REFERENCES

[1] Janaun J, Ellis N. Perspectives on biodiesel as a sustainable fuel. Renew Sustain Energ Rev 2010;14:1312–20.
[2] Wu F, Wang J, Chen W, Shuai S. A study on emission performance of a diesel engine fueled with five typical methyl ester biodiesels. Atmos Environ 2009;43:1481–5.
[3] Chisti Y. Biodiesel from microalgae. Biotechnol Adv 2007;25:294–306.
[4] Dismukes G, Carrieri D, Bennette N, Ananyev G, Posewitz M. Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. Curr Opin Biotechnol 2008;19:235–40.
[5] Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, et al. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J 2008;54:621–39.
[6] Schenk P, Thomas-Hall S, Stephens E, Marx U, Mussgnug J, Posten C, et al. Second generation biofuels: high-efficiency microalgae for biodiesel production. Bioenerg Res 2008;1:20–43.
[7] Clarens A, Resurreccion E, White M, Colosi L. Environmental life cycle comparison of algae to other bioenergy feedstocks. Environ Sci Technol 2010;44:1813–9.
[8] Pittman J, Dean A, Osundeko O. The potential of sustainable algal biofuel production using wastewater resources. Bioresour Technol, in press doi:10.1016/j.biortech.2010.06.035.
[9] Cantrell K, Ducey T, Ro K, Hunt P. Livestock waste-to-bioenergy generation opportunities. Bioresour Technol 2008;99:7941–53.
[10] Uludag-Demirer S, Demirer G, Frear C, Chen S. Anaerobic digestion of dairy manure with enhanced ammonia removal. J Environ Manag 2008;86:193–200.
[11] Van Horn HH, Newton GL, Kunkle WE. Ruminant nutrition from an environmental perspective: factors affecting whole-farm nutrient balance. J Anim Sci 1996;74:3082–102.
[12] Pizarro C, Mulbry W, Blersch D, Kangas P. An economic assessment of algal turf scrubber technology for treatment of dairy manure effluent. Ecol Eng 2006;26:321–7.
[13] Kellogg R, Lander C, Moffitt D, Gollehon N. Manure nutrients relative to the capacity of cropland and pastureland to assimilate nutrients: spatial and temporal trends for the United States. US Department of Agriculture; 2000. nps00–0579.
[14] Rodolfi L, Chini Zittelli G, Bassi N, Padovani G, Biondi N, Bonini G, et al. Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol Bioeng 2009;102:100–12.
[15] Sheehan J, Dunahay T, Benemann J, Roessler P. A look back at the U.S. Department of Energy's Aquatic Species Program—biodiesel from algae. National Renewable Energy Laboratory; 1998.
[16] Woertz I, Feffer A, Lundquist T, Nelson Y. Algae grown on dairy and municipal wastewater for simultaneous nutrient removal and lipid production for biofuel feedstock. J Environ Eng 2009;135:1115–22.
[17] Chinnasamy S, Bhatnagar A, Hunt R, Das KC. Microalgae cultivation in a wastewater dominated by carpet mill effluents for biofuel applications. Bioresour Technol 2010;101:3097–105.
[18] Chinnasamy S, Bhatnagar A, Claxton R, Das KC. Biomass and bioenergy production potential of microalgae consortium in open and closed bioreactors using untreated carpet industry effluent as growth medium. Bioresour Technol 2010;101:6751–60.
[19] Mulbry W, Kondrad S, Buyer J. Treatment of dairy and swine manure effluents using freshwater algae: fatty acid content and composition of algal biomass at different manure loading rates. J Appl Phycol 2008;20:1079–85.
[20] Bhatnagar A, Bhatnagar M, Chinnasamy S, Das KC. *Chlorella minutissima*—a promising fuel alga for cultivation in municipal wastewaters. Appl Biochem Biotechnol 2010;161:523–36.
[21] Kong QX, Li L, Martinez B, Chen P, Ruan R. Culture of microalgae *Chlamydomonas reinhardtii* in wastewater for biomass feedstock production. Appl Biochem Biotechnol 2010;160:9–18.
[22] Sawayama S, Inoue S, Yokoyama S. Continuous culture of hydrocarbon-rich microalga *Botryococcus braunii* in secondarily treated sewage. Appl Microbiol Biotechnol 1994;41:729–31.
[23] An J, Sim S, Lee JS, Kim BW. Hydrocarbon production from secondarily treated piggery wastewater by the green alga *Botryococcus braunii*. J Appl Phycology 2003;15:185–91.
[24] Shen Y, Yuan W, Pei Z, Mao E. Culture of microalga *Botryococcus* in livestock wastewater. T ASABE 2008;51:1395–400.
[25] Wang L, Li Y, Chen P, Min M, Chen Y, Zhu J, et al. Anaerobic digested dairy manure as a nutrient supplement for cultivation of oil-rich green microalgae *Chlorella* sp. Bioresour Technol 2010;101:2623–8.
[26] Chantanachat S, Bold HC. Phycological studies. II. Some algae from arid soils. Publication No 6218. University of Texas; 1962.

[27] Tornabene TG, Holzer G, Lien S, Burris N. Lipid composition of the nitrogen starved green alga *Neochloris oleoabundans*. Enzym Microb Technol 1983;5:435–40.
[28] Gouveia L, Oliveira A. Microalgae as a raw material for biofuels production. J Ind Microbiol Biotechnol 2009;36:269–74.
[29] Gouveia L, Marques AE, Da Silva TL, Reis A. *Neochloris oleoabundans* UTEX #1185: a suitable renewable lipid source for biofuel production. J Ind Microbiol Biotechnol 2009;36:821–6.
[30] Pruvost J, Van Vooren G, Cogne G, Legrand J. Investigation of biomass and lipids production with *Neochloris oleoabundans* in photobioreactor. Bioresour Technol 2009;100:5988–95.
[31] Li Y, Horsman M, Wang B, Wu N, Lan CQ. Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. Appl Microbiol Biotechnol 2008;81: 629–36.
[32] American Public Health Association, American Water Works Association, and the Water Environment Federation. Standard methods for the examination of water and wastewater. Washington, DC: American Public Health Association; 2005.
[33] Indarti E. Direct FAME synthesis for rapid total lipid analysis from fish oil and cod liver oil. J Food Compos Anal 2005;18: 161–70.
[34] Lewis T, Nichols PD, Mcmeekin TA. Evaluation of extraction methods for recovery of fatty acids from lipid-producing microheterotrophs. J Microbiol Meth 2000;43:107–16.
[35] Christie William W. Lipid analysis: isolation, separation, identification, and structural analysis of lipids. Bridgwater, England: Oily Press; 2003.
[36] Lusas EW, Hernandez E. Isopropyl alcohol. In: Wan PJ, Wakelyn PJ, editors. Technology and solvents for extracting oilseeds and nonpetroleum oil. Champaign, Illinois: AOCS Press; 1997. p. 199–266.
[37] Lepage G, Roy CC. Improved recovery of fatty acid through direct transesterification without prior extraction or purification. J Lipid Res 1984;25:1391–6.
[38] Lepage G, Roy CC. Direct transesterification of all classes of lipids in a one-step reaction. J Lipid Res 1986;27:114–20.
[39] Tran H, Hong S, Lee CG. Evaluation of extraction methods for recovery of fatty acids from *Botryococcus braunii* LB 572 and *Synechocystis* sp. PCC 6803. Biotechnol Bioprocess Eng 2009; 14:187–92.
[40] Mittelbach M, Remschidt C. Biodiesel: the comprehensive handbook. Austria: Martin Mittelbach; 2004.
[41] Allen CAW, Watts KC, Ackman RG, Pegg MJ. Predicting the viscosity of biodiesel fuels from their fatty acid ester composition. Fuel 1999;78:1319–26.
[42] Abeliovich A, Azov Y. Toxicity of ammonia to algae in sewage oxidation ponds. Appl Environ Microbiol 1976;31:801–6.
[43] Wilkie A, Mulbry W. Recovery of dairy manure nutrients by benthic freshwater algae. Bioresour Technol 2002;84:81–91.
[44] Arredondo-Vega BO, Band-Schmidt CJ, Vazquez-Duhalt R. Biochemical composition of *Neochloris oleoabundans* adapted to marine medium. Cytobios 1995;83:201–5.
[45] Band CJ, Arredondo-Vega BO, Vazquez-Duhalt R, Greppin H. Effect of a salt-osmotic upshock on the edaphic microalga *Neochloris oleoabundans*. Plant Cell Environ 1992;15:129–33.
[46] Cole JJ. Interactions between bacteria and algae in aquatic ecosystems. Ann Rev Syst 1982;13:291–314.
[47] Droop M. Vitamins, phytoplankton and bacteria: symbiosis or scavenging? J Plakton Res 2007;29:107–13.
[48] Liu ZY, Wang GC, Zhou BC. Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. Bioresour Technol 2008;99: 4717–22.
[49] Liang Y, Sarkany N, Cui Y. Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. Biotechnol Lett 2009;31: 1043–9.
[50] Glass ADM, Britto DT, Kaiser BN, Kinghorn JR, Kronzucker HJ, Kumar A, et al. The regulation of nitrate and ammonium transport systems in plants. J Exp Bot 2002;53:855–64.
[51] Ninnemann O, Jauniaux J, Frommer W. Identification of a high affinity NH_4^+ transporter from plants. EMBO J 1994;13: 3464–71.
[52] Guckert JB, Cooksey KE. Triglyceride accumulation and fatty acid profile changes in *Chlorella* (Chlorophyta) during high pH-induced cell cycle inhibition. J Phycol 1990;26:72–9.
[53] Jaenicke L, Kuhne W, Spessert R, Wahle U, Waffenschmidt S. Cell-wall lytic enzymes (autolysins) of *Chlamydomonas reinhardtii* are (hydroxy)proline-specific proteases. Eur J Biochem 1987;170:485–91.
[54] Xiong W, Li X, Xiang J, Wu Q. High-density fermentation of microalga *Chlorella protothecoides* in bioreactor for microbio-diesel production. Appl Microbiol Biotechnol 2008;78:29–36.
[55] Thompson G. Lipids and membrane function in green algae. BBA-Lipid Lipid Met 1996;1302:17–45.
[56] Piorreck M, Baasch K, Pohl P. Biomass production, total protein, chlorophylls, lipids and fatty acids of freshwater green and blue-green algae under different nitrogen regimes. Phytochemistry 1984;23:207–16.
[57] Jorquera O, Kiperstok A, Sales E, Embiruçu M, Ghirardi M. Comparative energy life-cycle analyses of microalgal biomass production in open ponds and photobioreactors. Bioresour Technol 2010;101:1406–13.
[58] Stephenson A, Kazamia E, Dennis J, Howe C, Scott S, Smith A. Life-cycle assessment of potential algal biodiesel production in the United Kingdom: a comparison of raceways and air-lift tubular bioreactors. Energy Fuels 2010;24:4062–77.
[59] 30-year average of monthly solar radiation, 1961-1990 [Internet]. Renewable Resource Data Center. Available from: <http://rredc.nrel.gov/solar/old_data/nsrdb/redbook/sum2/state.html>.
[60] Van Horn HH, Wilkie AC, Powers WJ, Nordstedt RA. Components of dairy manure management systems. J Dairy Sci 1994;77:2008–30.
[61] Petroleum product supplied [Internet].Energy Information Administration. Available from: <http://tonto.eia.doe.gov/dnav/pet/pet_cons_psup_dc_nus_mbbl_a.htm>.