Use of the microalga *Monoraphidium sp.* grown in wastewater as a feedstock for biodiesel: cultivation and fuel characteristics.

Gabriel P. Holbrook ^{a,*}, Zachary Davidson ^a, Robert A. Tatara ^b, Norbert L. Ziemer ^c, Kurt A. Rosentrater ^d, W. Scott Grayburn ^a

Affiliations:

^aDepartment of Biological Sciences, Northern Illinois University, DeKalb, IL 60115, USA

^bDepartment of Technology, Northern Illinois University, DeKalb IL 60115, USA

^cDepartment of Operations Management & Information Systems, Northern Illinois University, DeKalb, IL 60115, USA

^dDepartment of Agricultural and Biosystems Engineering, Iowa State University, Ames, IA 50011, USA.

*Corresponding Author: Tel.: +01 815 753 3199

Email addresses: gholbrook@niu.edu (G. Holbrook), davidsonz11@hotmail.com (Z. Davidson), rtatara@niu.edu (R. Tatara), nziemer@niu.edu (N. Ziemer), karosent@iastate.edu (K. Rosentrater), sgrayburn@niu.edu (S. Grayburn),

Nomenclature: FAMES Fatty Acid Methyl esters, TAG Triacylglycerol

ABSTRACT

The use of microalgae as feedstocks for biodiesel is potentially limited by climatic conditions with low light and temperature levels. *Monoraphidium sp.* Dek19 was identified by 18S rRNA gene sequencing. This is a species indigenous to the upper Midwestern USA which grows to high densities in wastewater with bioremediation resulting in a reduction of nitrate and phosphate levels. Mesocosm pool cultures (171L) were used to characterize growth of this species in larger volumes than typical of previous lab-based studies,. The alga was shown to be cold-tolerant and to grow to workable density within 15 days at relatively low light intensities in sterilized treated effluent. It was harvested by FeCl₃ flocculation and filtration, and lipid content was measured at 26% of dry weight. Pool cultures yielded enough biomass to extract lipids and transesterify them to biodiesel for testing in a stationary engine. GC analysis showed FAMES produced from *Monoraphidium sp.* to have a similar fatty acid profile to soybean oil. Engine testing of this algal biodiesel in blends with petrodiesel showed

a significant reduction in NO_x emissions. The results of this study indicate in general that searching for species of algae adapted to local environments is a good strategy for developing biodiesel feedstocks, and specifically that *Monoraphidium sp. Dek19* represents a species isolate that could be used to produce this fuel economically using wastewater in Northern locations with cool climates.

Keywords: Biodiesel, Diesel engine, Microalgae, *Monoraphidium*, Triacylglycerols, Wastewater

1. Introduction

Many studies have suggested that algae are a viable option for renewable biofuel which avoids both the problems of diverting food crops to fuel production and also requires less land usage [1-5]. Algae, like many organisms, produce lipids, the majority of which ($\approx 80\%$) are either phospholipids or triacylglycerols (TAGs) [6]. The conversion to biodiesel requires TAGs, or derivatives of TAGs, to be the starting material in the transesterification reaction where the fatty acid chains are removed from the glycerol backbone and a methanol group is added to the terminal carbon creating Fatty Acid Methyl Esters (FAMES) which are usable as biodiesel [7]. Transesterification and quality of biodiesel are higher with saturated or monounsaturated fatty acids than polyunsaturated fatty acids [8]. The most common fatty acids produced by algae are oleic (18:1 ω 9), linoleic (18:2 ω 6), and palmitic (16:0) [9].

Algae are a diverse and metabolically flexible group and can be grown in a wide range of environments including wastewater treatment facilities [5, 10]. Microalgae can potentially be harvested throughout the year as long as conditions allow workable rates of growth and reproduction. Therefore, biodiesel production can be constant, provided algal growth is continuous. Their rapid growth and unicellular nature allow them to adapt quickly, and thus, they can be manipulated to produce more lipids by appropriate changes to their environment [5]. These lipids can then be extracted to serve as the feedstock for biodiesel production [2].

A major impediment to the utility of many algae species for producing biodiesel, however, is the need for warm temperatures (20-30°C) in open culture systems [11, 12, 13]. High light intensities ($>1400\mu\text{mol photons/m}^2/\text{sec}$) with long photoperiods (12-14 hours) are also required for optimal outdoor growth of microalgae [1, 14, 15]. Some of the main species of algae used in large scale commercial production include members of the genus *Chlorella*, *Haemattococcus*, *Dunaliella*, and *Spirulina* whose culture is generally restricted to geographic locations with warm climates and annual average temperatures above 15°C [15, 16]. Consequently, in the USA, large open pond cultures have been established in New Mexico, Southern California and Hawaii [1, 16]. Many oleaginous species like *Chlorella sp.* and *Nannochloropsis oculata* would be unable to grow at acceptable rates during the cold seasons of the significantly cooler climates of the Northern USA [11, 12]. One solution to this problem is to identify indigenous algae that are adapted to light conditions and

temperatures in these Northern zones, and culture them in existing municipal waste water facilities where treated effluent remains substantially above cold ambient temperatures during the winter months. An effluent temperature range of approx. 4.0°C to 21°C was recorded from October 2012 to March 2013, averaging $13.0 \pm 5.3^\circ\text{C}$ (SD) in DeKalb, IL (41° 55' 45" N / 88° 45' 1" W). A mean temperature of 13°C is well below optimal for most species in the genus *Chlorella* [11,12], but may be suitable for more psychrophilic algae [17], or indigenous species of *Monoraphidium* capable of acclimation to low growth temperatures. The ability to utilize waste water year-round in geographic areas which extend beyond the regions normally used for mass culture of algae would greatly increase the cost effectiveness of growing algae as a biodiesel feedstock [14,15, 18, 19].

Monoraphidium sp. Dek19 is a freshwater green algae found in the upper Midwestern USA. This species-isolate grows at low irradiance levels and can obtain high cell densities when cultured at 10°C, a cool temperature at which it can outcompete other species like *Chlorella* [Holbrook GP, unpublished]. Taxonomically, *Monoraphidium* is a genus in the *Selenastraceae* whose morphology features long cells with either straight or crescent shapes [20]. These species reproduce mainly through autosporulation. Though many genera are monophyletic, some, including *Ankistrodesmus* and *Monoraphidium*, have been found to be paraphyletic [21]. Species identification can be difficult because most members of this family have general morphological characteristics, but the diversity within the genera of this family is extensive, leading to an underestimation of the number of species in the *Selenastraceae* [20, 21]. Identification by 18S rRNA sequencing can be used to determine genera and helpful in species identification. Yet, since 18S rDNA is highly conserved it may not be useful to differentiate among certain closely related species. An alternative multi-gene approach has been recommended to better distinguish between species in the *Selenastraceae* [20].

To determine whether *Monoraphidium* sp. would be a superior candidate species for use in biofuel production in cool growing zones it must be amenable to processing to biodiesel, involving harvesting, lipid production and extraction, and conversion of lipids to FAMES. The following characteristics are desirable: (1) algae must grow to high cell density in wastewater (a) to eliminate the expense of media, and (b) to remove polluting levels of nitrates and phosphates; (2) Harvesting can be practically performed by flocculation and filtration; (3) The lipids must be extractable from the algae and efficiently transesterified into biodiesel; (4) The algae must also be adaptable to growth over a time period related to the typical flow of wastewater through water treatment facilities; (5) The biodiesel produced must be of suitable quality for use in unmodified diesel engines.

The eventual implementation of this process would preferably use existing municipal infrastructures with modifications to provide raceways or lagoons where the algae can grow over several days in large batch cultures or flowing wastewater. *Monoraphidium* sp. should fill a niche for which other algae currently being developed for biodiesel production are less suitable [22]. If this species can grow at workable rates in cool temperatures with low light conditions, biodiesel production in Northern climates may be possible year-round.

Consequently, the goals of this research are as follows: (1) to evaluate whether *Monoraphidium sp.* is an option for use as a year-round feedstock for biodiesel in climates with cold winters, such as those experienced in Northern Illinois, USA; (2) to investigate whether *Monoraphidium sp.* can be used to reduce the levels of nitrates and phosphates in wastewater to sub-eutrophic levels; (3) to establish the effects of initial cell population density, and other parameters on the growth of this alga in mesocosm rather than laboratory cultures; (4) to examine the addition of supplemental carbon sources on the growth of *Monoraphidium sp.*, and (5) to harvest the algae and produce sufficient biodiesel to characterize and perform emission tests in a compression ignition engine.

2. Materials and Methods

2.1. Identification of algae species

Microalgae (Fig. 1) were obtained from a greenhouse isolate growing in sterilized wastewater collected from a local treatment plant (DeKalb, IL Sanitary District). Identification using 18S rRNA gene sequencing was performed as follows: single algal colonies were obtained after streaking liquid cultures to plates of autoclaved effluent water that was solidified with agar (Alfa Aesar H26724). Algae from single colonies were then propagated in flasks on a shaker (60 rpm) at 30°C, 16h:8h light:dark in autoclaved effluent water. Cells were concentrated by centrifugation, and pelleted cells were frozen. For DNA isolation, pellets were suspended in 0.4 mL of extraction buffer (5 M Urea, 50 mM Tris pH 7.8, 0.1% SDS, 50 mM NaCl) with a sterile pestle, then frozen at -75°C. After thawing in a water bath at 55°C, 25 µL 20 mg/ml proteinase K was added and samples were incubated for 1 h. Samples were extracted at room temperature with two volumes of chloroform by inversion on a labquake (Barnstead Thermolyne). Following centrifugation, DNA in the aqueous phase was precipitated with two volumes of 95% ethanol and centrifuged. Pellets were washed twice with 70% ethanol and centrifuged. Pellets were then dried completely in a SpeedVac (Savant) and suspended in water. Algae DNA was diluted prior to amplification with the polymerase chain reaction (PCR). Amplification of 18S rRNA genes used primers 5'-PCRe and 3'-PCRe [23]. Cycling conditions were 35 cycles of 98°C for 10 sec, 50°C for 15 sec, 68°C for 2 min. then hold at 10°C. PrimeStar GXL DNA polymerase (Takara Bio) and other reaction components were used as recommended by the supplier. A prominent 2.3 kb PCR product was purified from an agarose gel following electrophoresis in TAE buffer using a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) then ligated to pJET1.2 blunt (Thermo Scientific). The ligation mixture was used to transform *E. coli* DH5alpha cells that were made competent according to Hanahan [24]. Plasmid DNA for sequencing was prepared using a Zyppy plasmid miniprep kit (Zymo Research Corporation).

2.2. Growth conditions for culturing algae

The algal population was grown photoautotrophically in a standard greenhouse under partially regulated growth conditions via control of air temperature with heating and air conditioning. Effluent collected from the second clarifier tank from the DeKalb Sanitary District water treatment facility was chlorinated with standard bleach (sodium hypochlorite) at a concentration of 0.024mL/L for twenty-four hours to sterilize and eliminate any phytoplankton or zooplankton. Approximately 171L of this effluent was pumped into plastic pools (capacity 380L), and excess sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) was added to dechlorinate the medium. Sodium bicarbonate (75g) was dissolved in the effluent as a supplemental carbon source. Initial samples of 15 mL were taken of the effluent before inoculation of algae to determine the concentration of nitrates and phosphates, and also to be used as a blank in absorption measurements at 680nm light. The inoculum consisted of a 19L starter culture comprising $\geq 75\%$ *Monoraphidium* Dek19 cells (6.5×10^5 cells/mL), that also contained $\leq 25\%$ *Chlorella*, *Diatoms*, *Scenedesmus*, and unidentified algae growing in effluent. This was diluted into 171L volumes of sterilized effluent as described above. A standard (11,000 L/h) pond pump was used to both circulate and aerate the algal suspension. The pools were an open system located on greenhouse benches under natural light with air temperature maintained between 15-22 °C, and the algae were grown to stationary phase. In keeping with the relatively cool conditions at the water treatment facility ($13.0 \pm 5.3^\circ\text{C}$ (SD)), the pool culture temperature averaged $16.4 \pm 2.28^\circ\text{C}$ (SD) during the experimental period.

To record both light intensity and temperature HOBO pendant data loggers (MicroDAQ) were submerged to 10cm depth, and in air on the edge of the pools. Samples (25mL) were collected periodically from the middle of the pool at 15 cm depth after completely suspending any settled cells with a fiberglass brush. The sample was then used to determine nitrate and phosphate concentrations and cell density by extinction at 680nm light with a spectrophotometer. Measurements of pH were taken, and pool depth was checked in order to correct the culture volume for any evaporation. Cell counts were also performed on the samples at three day intervals using a hemocytometer.

2.3. Addition of supplemental inorganic carbon

Use of an extra inorganic carbon source was tested either by employing an air fractioning pump in addition to the circulation pump, or by addition of sodium bicarbonate and hydrochloric acid. One pool was set up with a 1000 L/h air fractionating pump to continuously add more air, and thus more CO_2 , to the effluent. A second pool was set up by adding 75g (4.69 mM) NaHCO_3 and 75mL 12M HCl to produce carbonic acid, which will dissociate into carbon dioxide and water. This addition of bicarbonate and HCl was repeated every other day

and the pH monitored to maintain an average of pH 8.5. A third pool with no additions served as the control. The algae were allowed to grow until cell density remained constant or decreased for three consecutive days. These growth regimes were replicated three more times in additional pools.

2.4. *Nutrient depletion*

The accumulation or depletion of nitrate and phosphate was measured colorimetrically using NitraVer 5 reagents and PhosVer 3 reagents respectively (Hach Company, Loveland, CA, USA). The readings were taken twice for each sample, and the average was recorded for 4 separate pools (n=4). Nitrate concentrations in pool samples were also checked using Czechrome NAS reagent, (Polysciences Inc., Warrington, PA, USA) and E570 measurements were performed with a spectrophotometer.

2.5 *Algae harvesting, lipid extraction, and conversion to biodiesel*

Flocculation of stationary phase algae was performed after pumping the pool contents into seventy-five gallon (284L) tanks. Then 125mL 40% (w/v) $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ was added per forty gallons (151L) of algae suspension. The solution was stirred and allowed to stand for twenty-four hours. The algae suspension and flocculated precipitate was then filtered at 0.5L/min through a 0.5m x 0.5m stainless steel sieve lined with a nylon mesh (40 μm), and allowed to air-dry for twenty-four hours in the greenhouse. The flocculated algae dried to a solid “cake” and were pulverized in a sample mill (Tecator Cyclotec 1093) and weighed. To gravimetrically determine the lipid content of the resulting algal powder, a Soxhlet extraction was performed using hexane solvent.

On a larger scale, algal lipids extracted from flocculated algae powder by refluxing with 1:10 (w/v) hexane solution were dried down in a rotary evaporator in small batches, pooled together to yield 600 mL of crude algal oil. This was combined with a mixture of methanol and NaOH (concentration of 1.0 M) at a ratio of 10:56 (w:w), placed in several 125-mL flasks, which were heated in a shaking water bath (Model 2876, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) at 60°C for 90 min to conduct the transesterification reaction. Following the reaction, samples were centrifuged (Model CL2, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) for 10 min at 4000 rpm, and the supernatant was run through filter paper (Grade 1, 11 μm , Whatman, GE Healthcare, Piscataway, New Jersey, USA) under vacuum. Liquid samples were then pooled into separatory funnels, small quantities of water were added to separate glycerol and unreacted components, which were removed by draining via density separation. The resulting FAMES were analyzed by Gas Chromatography to determine the fatty acid profile of the algal biodiesel. Following methods described by Yao et al. [25] FAMES were removed using hexane, and was then injected into a HP 5890 Series II gas

chromatograph (Hewlett-Packard, PA, USA) equipped with a SP 2340 capillary column (60 m x 0.25 mm x 0.2 μ m, Supelco, Bellefonte, PA, USA). The carrier gas was helium with a column flow rate of 1 mL/min. The injector and detector were held at 250°C. The oven temperature was programmed to ramp from 100° to 240°C using a temperature increase of 4°C/min. Methyl heptadecanoate (Sigma-Aldrich, St. Louis, MO, USA) was used as the internal standard. Peak identification was accomplished by comparing the retention times with those in the mix standard (GC 566, Nu-Chek, Elysian, MN, USA).

2.6 Engine testing

Tests were completed using standard #2 petrodiesel and two algal biodiesel blends. The first series of tests used pure petrodiesel to establish a baseline. This petrodiesel was obtained from a fuel distributor (SPEX CertiPrep, Inc., Metuchen, New Jersey, USA) and certified to be 100% free of any bio-based content. Then, B5 and B20 algal fuels were prepared by splash blending. All runs were conducted at full load and 2500 rpm with a Daihatsu 13.4-kW, inline, three-cylinder, and direct-injection diesel engine. To load the engine, a dynamometer was coupled to the rotating shaft of the flywheel, and water flowed through to act as the engine load. The engine was instrumented for performance and exhaust emissions measurement for CO₂, O₂, CO, unburned hydrocarbons (HC), and NO_x. The system included a probe that was inserted into the exhaust pipe and connected to a NOVA gas analyzer. For each test, the engine was started, warmed up at idle under full load for ten minutes, and then shut down to weigh the fuel tank. The tank was quickly reinstalled, the engine was restarted, set to 2500 rpm, and three sets of data were taken, each at a ten-minute interval. The reported results represent data averaged over these three sets. Fuel consumption was measured by weighing the fuel tank after the 30-minute period.

3. Results and discussion

3.1 Identification of the algae Species as *Monoraphidium* sp. Dek 19

A local greenhouse isolate of an unidentified microalgal species was cultured from a bucket of autoclaved effluent obtained from the DeKalb IL Sanitary District final settling tanks. This species was found growing in cool, low light conditions consistent with the early spring and late fall seasons of the Northern Mid-Western USA. In the lab, it could be cultured in wastewater under low light levels (10 hour photoperiods with a light intensity of 30 μ mol photons/m²/sec) and cool temperature (10°C) conditions.

The initial identification of the algae was achieved by analysis of morphological characteristics under a phase contrast microscope (Figure 1). The cells were mostly straight (and some crescent shaped) with tapered ends, green-yellow to chartreuse in color, and 85-140 μ m in length. The alga was first determined to be species of the *Ankistrodesmus* genus (*Selenastraceae*). Many species in this family have similar morphology with the same

length, shape, and color when viewed under a light microscope. Therefore, members of the *Selenastraceae* need to be distinguished from one another by 18S rDNA sequencing or some other multi-gene approach [20, 21]. Blastn similarity searches [26] showed a 100% nucleotide identity of the plasmid insert with *Monoraphidium* sp. Itas 9/21 14-6w with a 76% sequence coverage. A 98% nucleotide identity was seen with *Ankistrodesmus fusiformis* with a 93% sequence coverage. A type I intron was found in the *A. fusiformis* 18S rRNA gene [21]. The greater sequence coverage between our query sequence and *A. fusiformis* is likely due to the presence of an intron in our query sequence.

The most relevant study of algae that are closely related to our isolate, named *Monoraphidium* sp. Dek19, was carried out by Fawley et al. [20]. They examined algae that were isolated from two areas in Minnesota and North Dakota (U.S.A.). These states typically have cooler climates than Northern Illinois where Dek19 was isolated. Based on 18 S rRNA sequencing, *Monoraphidium* sp. Itas 9/21 14-6w (from a mid depth sample in Lake Itasca, Minnesota), had the highest sequence similarity with *Monoraphidium* sp. Dek19. Dek19 18 rRNA includes an intron, which is rare in related species of algae. When this intron sequence was used in a similarity search, *Monoraphidium contortum* strain AS-11 (from a surface sample in Arrowwood Lake, North Dakota) had the highest sequence similarity with *Monoraphidium* sp. Dek19. Part of the intron sequence is unique to Dek19, and can be used with the polymerase chain reaction to verify the identity of this strain. The local isolate of *Monoraphidium* sp. Dek19 was able to dominate the cultures, causing other phytoplankton species populations within the mesocosm community to go functionally extinct, or nearly so (Fig. 2).

Fig.1. Light micrographs of the alga *Monoraphidium* sp. Dek19 using phase contrast (160X). Cells are 85-140 μm in length.

3.2 Growth conditions of pool cultures

Open plastic pools (380 L capacity; depth 27cm) were used as a mesocosm for large scale growth experiments culturing *Monoraphidium sp.* Dek19 in 190L of effluent to produce cellular lipids for biodiesel production. The pools were placed on benches in the NIU Greenhouse, and contained circulation pumps to keep algae in suspension. To monitor conditions under which the *Monoraphidium* algae grew well in bulk cultures, HOBO probe data loggers recorded the average water temperature in the culture pools. During several 15-day algal culture experiments (between October and March) the water temperature was relatively constant at $16.4^{\circ}\text{C} \pm 2.83^{\circ}\text{C}$ (SD). For comparison, the mean effluent temperatures recorded at the nearby DeKalb, Illinois, water treatment facility were $13.0^{\circ}\text{C} \pm 5.3^{\circ}\text{C}$ (SD) over the same seasonal period. The average ambient air temperature of the greenhouse during that time was found to be slightly higher at $17.1^{\circ}\text{C} \pm 4.90^{\circ}\text{C}$. The day length (photoperiod) average was 11.6 h. The light intensity in these pools at a depth of 10 cm during this time period ranged from $0.25 - 145 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ with an average intensity of $22 \pm 17.4 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Outside the pools, light levels ranged from $0.25 - 859 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ with an average intensity of $83 \pm 74.0 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Under these conditions *Monoraphidium sp.* Dek19 showed a typical sigmoidal growth curve. The cell populations increased slowly, then exponentially for 15 days, and leveled off to a stable cell density for 4 - 6 days (Fig. 3).

Population dynamics over time were determined using hemocytometer counts for each pool. The average species composition for the pool mesocosm was calculated (Fig. 2) using four pools beginning with the same concentrations of species. The phytoplankton were categorized into five groups: *Monoraphidium*, *Chlorella*, *Senedesmus*, diatoms, and unknown (consisting of unidentified phytoplankton). These measurements were also repeated on other groups of samples with the same starting concentrations of algae. The dynamics showed that when the culture was inoculated with a high percentage ($\geq 75\%$) of *Monoraphidium sp.*, the final phytoplankton community was dominated by this alga at $\geq 98\%$ *Monoraphidium sp.* This was found under the relatively low light and temperature conditions ($10-15^{\circ}\text{C}$) typical of cool season water treatment plant effluent. Other algal species such as *Chlorella sp.* and *Scenedesmus sp.* became dominant in batch cultures if the growth conditions were much warmer ($>25^{\circ}\text{C}$) and so less suited to *Monoraphidium sp.* [27]. An exponential increase in population density of *Monoraphidium sp.* cells was accompanied by a slight increase in the cell numbers of competing phytoplankton species, followed by a marked decrease to 0 cells/ml, indicating functional extinction of their population (Fig. 2). *Monoraphidium sp.* grew to an average population density of around $5.0 \times 10^5 \pm 7.3 \times 10^4$ cells/mL in pool cultures ($n=4$), and certainly represented the greatest unicellular biomass as this species has long cells with a volume at least 20-fold bigger than *Chlorella sp.* Much higher cell densities of related microalgae species are attainable in closed systems with optimal nutrient and light levels [14, 28]. The competitive growth of *Monoraphidium sp.* may favor its implementation in open pools at waste water treatment

plants, since the effluent would not need to be devoid of other algal species before inoculating with this alga to produce biodiesel feedstock.

Fig. 2. Average pool population densities of *Monoraphidium sp.* and competing algal species over a 23 day growth period after inoculation into effluent. Plot for Total cells (●) and *Monoraphidium sp.* (○) uses 0-600 x 10³ per mL cell density scale or algae cell dry weight scale (left), and remaining plots for Chlorococci (■), Diatoms (▲), Scenedesmus (◆), and unidentified algae (▼) use cell 0-100 x 10³ cell density per mL scale (right). Data are means ± SD (n=4 pools)

In preliminary experiments, nitrate and phosphate were not depleted to 0 mg/L during the growth of *Monoraphidium sp.* within the pools. Complete depletion of the nutrients was observed once an extra inorganic

carbon source was added. The algal population in pools with additional inorganic carbon was increased significantly to greater final densities from an average of 1.84×10^5 cells/mL to 2.75×10^5 cells/mL via aeration, or 2.73×10^5 cells/mL by adding extra bicarbonate (Table 1). Algal cells use CO_2 absorbed from the surrounding solution for photosynthesis; however, *Monoraphidium sp.*, like other algal species, may also utilize bicarbonate [29, 30]. The data indicated that available CO_2 could limit productivity of *Monoraphidium* as media pH increased (sometimes $> \text{pH } 10$). Supplemental carbon supplied as bicarbonate or an air fractionating pump elevated final cell density, and extended the growth period in mesocosm cultures. The resulting decrease in photorespiration rates would be expected to increase growth rates of the cells [31, 32]. However, we also recognize that carbon addition using sodium bicarbonate and hydrochloric acid produces carbonic acid, CO_2 and water, but also produces sodium and chloride ions, increasing the salinity of the solution. This could retard the growth, but boost the lipid production of *Monoraphidium sp.* as observed in other species of algae [32, 33]. An alternative source of additional carbon has been explored using CO_2 in simulated flue gas supplied to *Monoraphidium minutum* [34], so a number of environmental remediation applications are feasible with this organism.

Table 1

Effects of supplemental inorganic carbon added to *Monoraphidium sp.* growing in 190L effluent pools under greenhouse conditions.

Growth Condition	Days until Stationary Growth	E680 nm Light	Final Cell Density/ml ($\times 10^3$)	Flocculated Algal Mass Estimate (g)	
				Total	<i>Monoraphidium sp.</i>
Control	13.0 ± 1.4	^a 0.69 ± 0.03	^a 184 ± 8.8	175	171.5
Supplemental Aeration	14.5 ± 1.0	^b 0.99 ± 0.03	^b 275 ± 9.0	262	256.8
+ NaHCO_3	14.8 ± 0.96	^b 0.98 ± 0.02	^b 273 ± 4.9	260	254.8

Data shown are means $\pm$ S.D. Means preceded by the same letter are not significantly different, and means preceded by different letter are significantly different (ANOVA at $P \leq 0.05$). Algal mass flocculated using optimal 5mM FeCl_3 concentrations in 190L pools was estimated based on the dry mass of 100ml flocculated algal suspensions at equivalent cell densities, and shows the amount of dry mass represented by *Monoraphidium* cells, with the balance being from other algal species described in Fig.2. NaHCO_3 (75g) was added every other day (see Materials and Methods).

3.3 Nutrient depletion

In four replicate 191 L pool experiments using addition of inorganic carbon, the time to deplete the nitrate concentration ($\leq 5\text{mg/L}$, which is 50% or less of the minimum standard for drinking water set by the US EPA)

was found to be approximately 13-15 days (Fig. 3). At 15 days, the nitrate concentration of all pools was below 5mg/L, averaging 1.84 ± 1.59 mg/L, and at 16 days, nitrate was as low as 0.89 ± 0.97 mg/L. The time to deplete the phosphate concentration (≤ 1 mg/L) was similar at 14 days (Fig. 3) when it was below 1mg/L with an average of 0.64 ± 0.47 mg/L, and 0.40 ± 0.37 mg/L after 16 days. The depletion of both nutrients correlated closely with the growth of the alga as shown by an increase in effluent culture cell density. Therefore, *Monoraphidium sp.* can remove essentially all the nitrate and phosphate ions from effluent. These ions represent the two major inorganic pollutants remaining in wastewater after the secondary treatment (activated sludge process). This relationship was seen in all pools when supplemental inorganic carbon was added, indicating the potential dual function of *Monoraphidium* algae for phytoremediation of tertiary treated wastewater, and concomitant biomass production for lipid extraction.

Fig. 3. Nitrate and phosphate depletion during growth of *Monoraphidium sp.* in effluent pool cultures over a 20 day period. Nitrate (Δ) and phosphate (□) data are means $\pm$ SD (n=4 pools). The effluent had initial concentrations of 33 mg nitrate/L and 6 mg/L phosphate. Cell density plot (●) is shown in terms of extinction of light at 680nm, and also as the corresponding dry weight of algae cells in grams/L with data from one representative pool out of the four in the experiment.

3.4 Flocculation, lipid extraction, and transesterification to biodiesel

Ferric chloride hexahydrate (40% w/v) was added to the effluent culture to induce the algae to flocculate, as untreated single cells would have been too small and impractical to filter. The ferric chloride was converted to ferric hydroxide and caused the cells to adhere together [35] prior to harvesting by filtration through nylon mesh (40 μ m). The estimated potential harvest yield, once cell density had reached stationary phase in effluent was 260 g dry algal biomass per 171 L pool (1.52g/L).

Small 10g samples of the filtrate were run through a Soxhlet extraction for 12 hours using hexane solvent to measure lipid content gravimetrically. The amount of lipids extracted was $26.4\% \pm 6.1\%$ dry weight, corrected for the ferric hydroxide content of the flocculated algae. This is comparable to other native algal species such as *Chlorella sp.* which has an optimal lipid yield of 28-32% [2, 14, 22]. Therefore, *Monoraphidium sp.* contain lipid levels which are high enough to allow its use as a substitute for other algal species currently produced as feedstocks.

After a larger-scale FeCl_3 -induced flocculation and filtration of the 171 L *Monoraphidium sp.* cultures, lipids were extracted using hexane, and the fatty acids were then converted into biodiesel by alkali-catalyzed transesterification. Gas chromatography was used to quantify the fatty acid composition of the biodiesel (Table 2). Less than 1% of the finished product was composed of free fatty acids. In terms of overall processing efficiency, considering both the algal oil as well as the chemical reactants used, approximately 70% of the input algae oil was converted into finished biodiesel; this accounted for processing losses and unreacted chemicals.

The GC-based analysis of biodiesel FAMES produced from *Monoraphidium sp.* (Table 2) showed the following compositions: 18.6% saturated fatty acids as palmitic C16:0 and stearic C18:0; 27.8% mono-unsaturated fatty acids as oleic C18:1c and elaidic acids C18:1t; 48.5% linoleic; and 5.3% linolenic acids. In comparison to the fatty acid profile from *Chlorella sp.* 800, *Monoraphidium* extracts produced at average culture temperatures (around 16°C) had appreciably less polyunsaturated linolenic acid (C18:3). In fact, the *Monoraphidium* fatty acid profile is quite similar to that seen in biodiesel from Soybean and Corn (Maize) oil, which comprise 55.3% and 56.2% C18:2\ linoleic acid respectively [36]. Based on this composition, the characteristics of biodiesel produced using *Monoraphidium* as a feedstock would be expected to resemble biodiesel produced from soybean oil, which at present is the predominant feedstock in the USA. Another species termed *Monoraphidium* FXY-10 has recently been identified as a potential feedstock for biodiesel when grown under heterotrophic conditions [37].

Table 2.

Quantification by gas chromatography of algal biodiesel fatty acid composition extracted from *Monoraphidium sp.* grown photoautotrophically in effluent culture. Relative mass of Fatty Acid composition (% w/w).

Feedstock	C14:0	C16:0	C16:1	C18:0	C18:1c	C18:1t	C18:2	C18:3	C20:0	C22:0	Total (w/w) 100%
<i>Monoraphidium sp.</i>	-	16.4	-	2.2	26.2	1.6	48.5	5.3	-	-	
^a <i>Chlorella sp.</i> 800	-	24.7	0.1	2.4	14.7		34.3	23.8			
^b Soybean	-	9.4		4.1	22.0	-	55.3	8.9	-	0.3	
^b Corn	-	12.1	0.2	1.8	27.2	-	56.2	1.3	0.4	0.8	

^a Data from Hempel et al. [9] for algae grown at 15°C. ^b Data from Sanford et al. [32].

3.5. Engine tests with *Monoraphidium sp.* microalgal biodiesel blends

From the emission measurements, there was a stepwise reduction in NO_x present in exhaust from 209 ppm to 169 ppm and 161 ppm for the petrodiesel, algal B5, and algal B20, respectively (Table 3). The CO₂ emissions showed a marginal decline using the B5 blend, while the B20 blend did not yield CO₂ emissions that were significantly different from petrodiesel nor from the B5 blend. In comparison, CO was negligible or very low for all the fuels. Elevated unburned hydrocarbons indicated inefficiencies in the combustion process. The B5 did not yield a significant increase in hydrocarbons compared to the petrodiesel standard, but B20 displayed significantly more: 20.2 ppm vs. 9.6 ppm. In terms of fuel consumption compared with #2 petrodiesel, the B5 blend was slightly more efficient, consuming 7% less mass of fuel while the B20 mixture burned 11% more at the engine speed and dynamometer load used.

Table 3

Comparison of algal biodiesel blends with pure petrodiesel supplied to a stationary compression ignition engine running at 2500 rpm under full dynamometer load. Emissions and fuel consumption characteristics.

	Fuel Consumption (g/min)	Hydrocarbon (ppm)	CO (%)	CO ₂ (%)	NO _x (ppm)
Petrodiesel	25.0	^a 9.6 ± 1.60	n.d.	^a 7.0 ± 0.06	^a 209 ± 10.5
B5	23.2	^a 7.7 ± 0.02	n.d.	^b 6.8 ± 0.02	^b 169 ± 0.10
B20	27.7	^b 20.2 ± 4.7	0.17 ± 0.02	^{a,b} 6.9 ± 0.45	^b 161 ± 12.3

For each column, means ± SD (n=3) preceded by the same letter are not significantly different. Means preceded by different letters are significantly different at P < 0.01 as analyzed by ANOVA. (n.d. = none detected).

Exhaust gas emission traces for a typical B20 fuel run are presented in Fig. 4. Data for NO_x, HC, CO₂, and CO, measured in the diesel engine exhaust gas were generally consistent for the 10-minute sampling, with nitrogen oxides trending slightly lower towards the end of this sampling period. Unburned hydrocarbons, as well as carbon dioxide rates, were quite steady over the entire run. Carbon monoxide (not shown) was very

stable at 0.2% for the entire run. Each trace represented approximately 40 data points, or one measurement logged every 15 seconds. Other runs, including those with B5 and pure petrodiesel, exhibited similar consistency with respect to all the measured parameters.

Fig. 4. Experimental exhaust gas emission traces for a typical B20 fuel run in a stationary compression ignition engine. (○) NO_x (ppm); (●) HC (ppm); (▲) CO₂ (%). Conditions were as described in Table 3.

Presently, for all biodiesel fuels, the focus is on NO_x, the combination of nitric oxide (NO) and nitrogen dioxide (NO₂), which, if excessive, can contribute to smog formation. Additionally, any CO and hydrocarbons present in the exhaust represent incomplete combustion and/or excess fuel in the cylinders. Significant decreases in NO_x when using *Monoraphidium*-derived biodiesel blends are findings consistent with other algae-fuel testing. Tuccar and Aydin [38] instrumented a direct-injection, 89-kW engine, and measured an average reduction of 9.3% for NO_x with B100 over a wide range of engine speeds while experiencing a slight reduction in power. Another study [39] processed *Chaetoceros gracilis* into B100, and fueled a two-cylinder and 7.9-kW engine running at no load and 3500 rpm; NO_x decreased from the #2 petrodiesel baseline of 25.7 ppm to 21.9 ppm. Similar declines in NO_x have been measured for biodiesel made from macroalgae [10, 40]. However,

other studies show a different trend. For instance, Al-Lwazy and Yusaf [41] derived a B20 mixture from *Chlorella protothecoides* and used this fuel in a three-cylinder and 25.8-kW agricultural tractor engine. Their data indicated little difference in NO emission with values of 970 ppm and 994 ppm at 1500 rpm for petrodiesel and B20, respectively. Another study saw NO_x values increase 2.6% and 4.4% for algal B20 operated in six-cylinder, heavy-duty 250-kW and 246-kW engines, respectively, along with a corresponding 2% reduction in fuel efficiency [42]. It is possible that the variable NO_x emissions measured for biodiesel from several feedstocks may also be related to engine test procedures, and can be ameliorated by adjusting fuel injection timing [43].

4. Conclusions

Monoraphidium sp. Dek19 represents a good candidate species for biodiesel production at wastewater treatment plants in locations with less favorable climatic conditions than are typically selected for outdoor open cultures (e.g. the Southwestern USA). The alga is capable of taking up excess amounts of nitrates and phosphates from the wastewater, and uses these pollutants as a cost-free nutrient source, eliminating the need for specialized growth media. At the same time, this removal of nitrate and phosphate can serve as a water treatment step which would otherwise require expensive chemical methods. *Monoraphidium sp.* is resilient to environmental changes, and grows well in cool water at relatively low light intensities. It can be grown in large photoautotrophic cultures which are not axenic, and out-competes other algal species in effluent without the need for high initial population densities. The addition of bicarbonate during the initial and exponential growth phase stimulates growth and leads to higher total cell densities. Mesocosm experiments suggest that growth in much larger volumes of effluent outdoors will be possible without premature collapse of cultures often encountered when scale-ups from laboratory conditions are attempted. Flocculation with FeCl₃ can be used as a conventional means of harvesting the algae prior to filtration and air-drying. Extracted lipids are approximately 26% of cell dry weight under the conditions used for growth of algae. Transesterification yields FAMES that show a fatty acid profile similar to biodiesel made from soybean oil. When B5 and B20 blends of *Monoraphidium* algae biodiesel are run in a fully-loaded 13.4-kW test engine, measurements indicate similar performance compared to pure petrodiesel in terms of fuel efficiency and exhaust emissions of CO₂ and CO. However, significantly, with biodiesel from *Monoraphidium sp.*, there were reductions in nitrogen oxides of 19% and 23% using the B5 and B20, respectively.

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