Demonstration of the Feasibility of Milking Lipids from Algae for Biodiesel Production

By

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Submitted to the graduate degree program in Bioengineering and the Graduate Faculty of the University of Kansas School of Engineering in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

A major challenge to the development of industrial-scale biodiesel production from cultured algae is the identification of energy efficient and cost effective methods of harvesting/dewatering algal cells. Producing 1 gallon of biodiesel from algae, even if grown in highly concentrated suspensions, requires processing thousands of gallons of water.

An alternative to growth and dewatering methods is milking of algal cells whereby lipids are continually extracted without requiring the sacrificing of cells. Growth and extraction are combined by contact between cells and a biocompatible solvent. Algal cells can be maintained in a stationary phase for extended periods and with nitrogen-limiting conditions which favor lipid accumulation in the cells. With continual extraction of lipids, cells in the stationary phase would direct photosynthetic activity more towards lipid production instead of new cellular growth. Essential nutrients such as nitrogen and phosphorous would also be retained in algal suspensions because milking selectively extracts lipids (compounds of C, H, and O) which are produced from the CO_2, H_2O, and light supplied to the bioreactor, thus diminishing the need for expensive fertilizer use.

Batch studies showed that n-dodecane and biodiesel were biocompatible solvents with the green algae *Ankistrodesmus falcatus* and *Chlorella kessleri* for 4 days of contact time. Long term (28 day) milking experiments were performed in 2 L photobioreactors with continuous n-dodecane solvent recirculation. The results showed that *Chlorella* maintained a viable population with a half-life of 49 days, but *Ankistrodesmus* populations became non-viable after 6–26 days of solvent contact. Lipid analysis by HPLC demonstrated successful milking. The total lipid concentrations reached up to 4,720 mg/L in the solvent phase, equaling a lipid milking productivity of 16.1 mg/L-d, and representing up to 86% extraction of total lipids in the system. Milking favored extraction of monoglycerides and free fatty acids from algal cells. Nutrients were largely retained in algal suspensions; 55–85% of total nitrogen and 66–98% of total phosphorous were retained at the end of experiments.

Overall, successful milking of *Chlorella* with n-dodecane was demonstrated. Possibilities for scale up of the process, critical analysis of energy balances, and lipid purification methods should be investigated.

DEDICATION

With sincere gratitude to my beloved wife Sandy, and to Luke, a little glimmer of light early in our marriage. All things can work for the greater glory of God.

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The persons that I am indebted to for assistance in completing this research and report are many. First, let me thank Mr. Alan Walker, Mr. Ash Shadrick, and Mr. Jay Barnard for their assistance in constructing reactor system components. Thanks to Ms. Chloe Wooldridge for assistance in sampling and analysis; Ms. Heather Shinogle for providing flow cytometer analyses and SEM imaging; and Dr. Karen Peltier for performing analyses of dissolved carbon. I want to acknowledge the U.S. Department of Energy for providing funding for this research through Award EE-0000408. I appreciate the guidance provided by my committee members, Dr. Susan Williams and Dr. Aaron Scurto. And of course, special thanks to my advisor, Dr. Belinda Sturm, who first proposed the idea of milking algae for lipids and provided constant assistance, in manifold ways to bring this work to completion.

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1. INTRODUCTION

1.1. Overview and Motivation of Current Study

In the search for alternatives to fossil fuels, the use of algae for production of biodiesel has been proposed as a promising avenue. However, the economical production of biodiesel from algal feedstocks has yet to be performed on a commercial or industrial scale, due in part to a number of technical challenges. In particular, proposed systems for algal biodiesel production have typically assumed a growth-and-harvest model which presents inherent inefficiencies related to harvesting, dewatering, and extraction operations. When cells are harvested from the culture, non-target compounds (other than lipids) such as proteins are retrieved. While these compounds may have use for other purposes, for example as fish feed in aquaculture (Batan et al., 2010), the harvesting of cells requires continuous regrowth of algal cells and non-target compounds to provide the desired lipids. Extensive dewatering is required to concentrate algal biomass. For example, (da Silva et al., 2009) were able to grow algae at a highly concentrated 2.8 g/L with an 11% lipid content; this would require that at least ~3,000 gallons of water be processed to produce 1 gallon of biodiesel. Large scale culture of algae is likely to be substantially less concentrated, perhaps 0.1–0.5 g/L (Lardon et al., 2009; Sturm & Lamer, 2011). Even with careful process selection this leads to large energy requirements for dewatering, ~40% of the total required for biodiesel production (Sturm & Lamer, 2011). It has frequently been assumed that lipid extraction from algal solids would be accomplished by established industrial methods for extracting lipids from oilseeds (Batan et al., 2010; Sturm & Lamer, 2011). This will likely be satisfactory in terms of effectiveness, but is not likely to be optimal in terms of energy efficiency or cost effectiveness because algal solids must be fed to the extractor with a solids

content that is comparable to that observed in oilseeds (90%) when they are extracted (Lardon et al., 2009).

1.2. Milking of Algae

An alternative to a growth-and-harvest model of algal biodiesel production is *in-situ* extraction, or milking, whereby cells are exposed to conditions that liberate the target compound from the cell without requiring the concentration and killing of cells. Milking methods can utilize biocompatible solvent extraction (Frenz et al., 1989), osmotic shock (Sauer & Galinski, 1998), or some other technique which causes the migration of the target compound into the harvesting medium. Milking can be accomplished as either a periodic process where cells are cyclically cultured then milked, or in a continuous process where culture and milking occur simultaneously. An example of a continuous process is a two-phase bioreactor where growth occurs in the aqueous phase and compounds of interest are extracted to the solvent phase. One example of milking of microorganisms was reported by (Frenz et al., 1989) who described the growth of the green alga *Botryococcus braunii* with periodic, subsequent extraction by hexane. The target compounds were long chain (C30–C37) hydrocarbons that the alga accumulates up to 50% of dry cell mass. The cells retained adequate metabolic activity and continued to produce hydrocarbons after repeated exposures to hexane. Hejazi et al. devoted several studies to the milking of β-carotene from the marine alga *Dunaliella salina* in two-phase bioreactors. Solvents with log $K_{OW} > 6$ (including n-dodecane) were shown to be biocompatible for *D. salina* (Hejazi et al., 2002). Higher mixing rates resulted in larger beta-carotene concentrations in the dodecane (Hejazi et al., 2003). They also assessed the long-term viability of the cells in a semi-continuous reactor and found that the cell population retained viability for at least 47 days (Hejazi et al., 2004a). Milking studies have most frequently used n-dodecane as a solvent (da Silva et al., 2006;

Hejazi et al., 2003; Hejazi et al., 2002; Hejazi et al., 2004a; Hejazi et al., 2004b; Hejazi & Wijffels, 2003). However, decane (Leon et al., 2003), hexane (Frenz et al., 1989), and solvent mixtures containing decane as a biocompatible, primary component and dichloromethane, methyl *tert*-butyl ether, or methyl ethyl ketone as a minor, secondary solvent to increase the extraction efficiency of the target compound (Mojaat et al., 2008) have also been employed. Researchers investigating the use of the milking technique have typically sought high-valued compounds (Hejazi & Wijffels, 2004) with, for example, nutraceutical uses. However, it has been suggested that milking might be cost effective in producing relatively low-value lipids from algae cultures as a biodiesel feedstock (Lamers et al., 2008; Ramachandra et al., 2009).

Only one study has presented results from experiments which investigated the possibility of milking lipids from algae for biodiesel production. Zhang et al. (2011) exposed batch cultures of the marine alga *Nannochloropsis sp.* to several different organic solvents to assess the viability of the cells and the extraction of lipids to the solvent. It was found that alkanes between C9–C16 were biocompatible with the alga, and that lipids were milked from a viable cell population with solvent lipid concentrations up to 33 mg/L after 8 days of exposure.

1.3. Advantages of Milking for Algal Biodiesel Production

The use of milking to produce lipids from algae has several potential advantages when compared to a growth-and-harvest model. First, the overall process can be significantly simplified because growth, harvest, and extraction steps can be combined into a single process. Second, a milking reactor system operates in batch with respect to the algal suspension, as opposed to a growth-and-harvest model which essentially requires continuous or semi-continuous growth of the algal suspension. Cells can be maintained in a stationary phase where

cell growth is balanced. This results in several advantages for a milking reactor system. Third, the need for large volumes of water inputs and subsequent dewatering requirements is eliminated because the only continuous water requirement is make-up for evaporation. Fourth, non-carbon nutrients such as nitrogen and phosphorous do not need to be continuously supplied to the reactor because nutrients bound in cells in proteins, phospholipids, nucleic acids, etc., or dissolved in the aqueous phase are ideally not extracted by the lipophilic solvent but remain in the algal suspension. Fifth, algae can be maintained in a nitrogen-limiting condition (low N:P molar ratio), which can induce high lipid accumulation, for example, 60-70% (Liang et al., 2009) of dry cell mass. This, however, also results in slowed growth rates (Rhee, 1978). For a milking system with a stationary, viable batch of algae maintained for a long time, the drawback of slow growth rates becomes inconsequential and the advantage of high lipid content dominates. Sixth, the preferential extraction of lipids means that more of the energy available to cells from illumination can be directed to lipid production, instead of cell replication with its attendant production of non-target compounds. Figure 1 compares simplified process inputs, outputs and operations for a milking reactor system and a growth-and-harvest reactor system. The reduced number of process streams (4 versus 6) and operations is apparent for the milking reactor system. It is worth noting that the large water and nutrient requirements of the growth-and-harvest model may be reduced if algal culture is coupled with wastewater treatment (Sturm & Lamer, 2011), or when recycle of water is incorporated into the design of the process, but the other drawbacks remain a challenge in the efficient design of such systems.

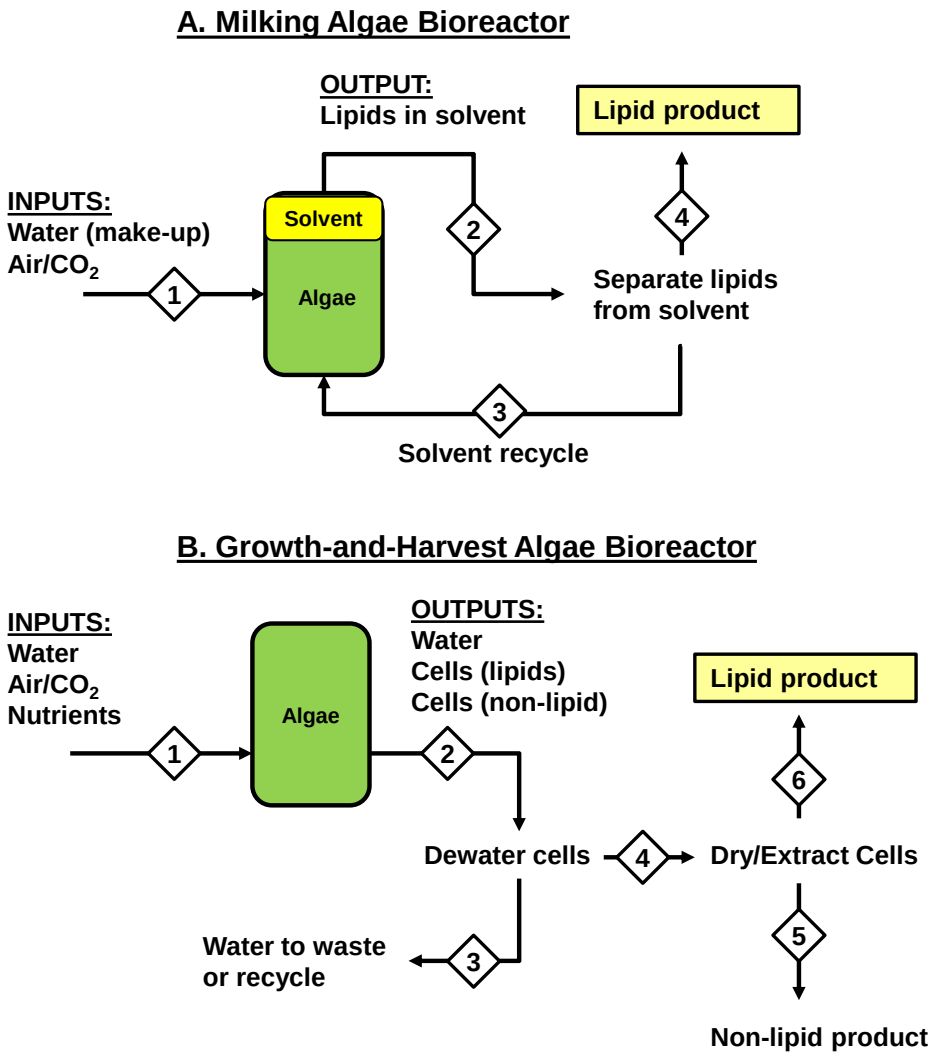

Figure 1. Comparison of simplified process inputs, outputs, and operations for a milking algae bioreactor (A) and a growth-and-harvest algae bioreactor (B). The milking bioreactor requires 2 fewer process streams and fewer operations.

1.4. Purpose and Hypothesis of this Thesis

The purpose of this thesis is to report the results of experiments which were performed to identify biocompatible solvents for 2 freshwater, nonmotile green algal genera (*Ankistrodesmus* and *Chlorella*) for short term exposure, and to assess the feasibility of milking algal cells for longer periods of time to produce lipids. It is hypothesized that algal populations will maintain

viability for extended periods (up to 28 days) when exposed to solvents which were determined to be biocompatible in short term exposure. It is also hypothesized that lipids will be milked from the cells and extracted to the solvent phase, and that the nutrients nitrogen and phosphorous will be retained in the algal suspension during milking, reducing the nutrient demand for algal lipid production.

2. METHODS

2.1. Algal Cultures

The *Ankistrodesmus* strain used to identify biocompatible solvents was *Ankistrodesmus falcatus* (provided by Dr. Val Smith, University of Kansas). For experiments assessing long term milking of algae, *Ankistrodesmus braunii* (UTEX 187) was utilized. *Chlorella kessleri* (UTEX 2228) was used for both biocompatible solvent identification and in long term milking assessments. Algae were grown on WC Media (Guillard, 1975), with an N:P ratio of 50 (nitrogen-sufficient) for biocompatible solvent identification, or at an N:P ratio of 5 (nitrogen-limiting) for long term milking of algae. For Experiment M-8, N:P-5 media with twice the concentration of nitrogen and phosphorous was used. See Table 15, Appendix for details on nutrient concentrations in media. All cultures were exposed to a photoperiod of 14 hr light/10 hr dark per day. The pH was monitored periodically (n=63) in samples from the long term milking experiments and the average pH was 6.43 (±0.22 standard deviation).

2.2. Materials and Reactor Configuration

Two hydrophobic solvents were used in the experiments; n-dodecane (Fisher Scientific #O2666-500, Pittsburgh, PA, USA; or Alfa Aesar #A14834-AP, Ward Hill, MA, USA), and

biodiesel produced from waste cooking oil by The Biodiesel Initiative, University of Kansas. Biodiesel was only used for biocompatibility experiments because it caused rapid degradation of peristaltic pump tubing during trial runs. The choice of n-dodecane follows up on the prior use by the Hejazi group and others, and was assumed to be a good starting point to identify biocompatible solvents. The use of biodiesel as a solvent presented the chance to assess the potential for using the desired end product as a processing agent; at full-scale, this would represent a recycle of a portion of the biodiesel produced.

Biocompatibility experiments were performed in batch reactors. Chemostats with a hydraulic residence time of 5 days and aeration provided at a rate of 0.4 L/min with 1% (v/v) CO_2 were used to provide algal suspensions for biocompatibility batch experiments. Batch reactors consisted of 250 mL glass bottles filled with 90 mL of algal suspension and 10 mL of solvent for experimental treatments. A stir bar was placed in each batch reactor and a stirring rate of 90 rpm was used to provide mixing of aqueous algal suspension and solvent phases- stirring caused dispersal of fine solvent droplets through the algal suspension which coalesced after mixing was stopped. Control samples contained only algal suspension. The mouth of each reactor was covered with multiple-layer cheesecloth to allow for gas exchange and prevent contamination. Reactors were illuminated at ~110 μmol/m^2-s.

Long term milking of algae was performed in 2 L fermentor-type bioreactors with solvent and aqueous phase recirculation provided by peristaltic pumps (Masterflex L/S, Cole Parmer, Vernon Hills, IL, USA). Figure 2 presents a schematic of the bioreactor system.

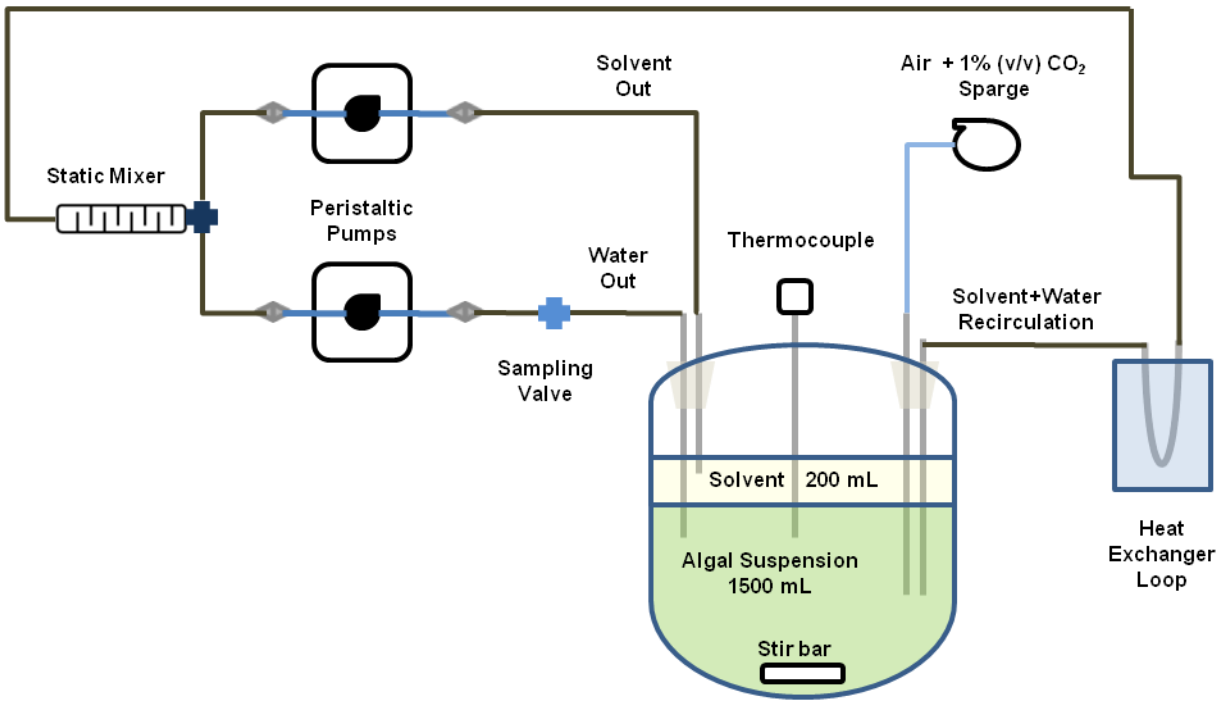

Figure 2. Schematic of reactor system used in long term milking experiments.

Solvent and aqueous phases were pumped at equal rates and contacted in a 21 element 0.25" diameter inline static mixer (Koflo #1/4-21, Cary, IL, USA). Mixed suspension in a closed loop received temperature control from heat exchange with a water vessel maintained at 25°C by pumped recirculation from an Isotemp 3016P refrigerated circulator (Fisher, Pittsburgh, PA, USA) before returning to the bioreactors for recirculation. The resultant temperature in the bioreactors averaged 26.4°C (±1.4°C standard deviation).

For inoculation of all experiments, cultures of algae were maintained in stationary growth in 300 mL cultures of N:P-5 media. Algal suspensions for experiments were prepared by inoculating 1500 mL of N:P-5 media with 10 mL of reserve culture, aerating at 0.2 L/min with 1% (v/v) CO_2, and incubating under illumination (~60 µmol/m^2-s) for 14 days (incubation for

experiment M-5 used aeration at 0.4 L/min). At the beginning of each recirculation experiment, incubation cultures were refilled with deionized water to 1500 mL to make-up water losses to evaporation, 14 mL of suspension was sampled, the remainder was transferred to the 2 L bioreactors, and 200 mL of solvent (12% v/v) was added to the bioreactors. Illumination at ~80 μmol/m^2-s, and aeration at 0.4 L/min with 1% (v/v) CO_2 was provided during the experiments (Experiment M-5 used 0.5 L/min aeration). A stir bar was used in each reactor to insure that algae cells remained suspended, but the relatively mild mixing (120 rpm for continuous recirculation experiments; 240 rpm for intermittent recirculation experiments) did not cause obvious mixing of the solvent and aqueous layers in the bioreactor.

2.3. Experimental Series

Table 1 presents a summary of key features of the experiments performed to assess the biocompatibility and long term milking of algae. Four biocompatibility experiments (Experiments B-1 through B-4), one for each solvent and algae combination, were performed with triplicate experimental treatments and triplicate control treatments. Algae were contacted with solvent for 4 days, and 10 mL of algal suspension was sampled at 1, 2, and 3 days, yielding an average percentage solvent volume of 12% (v/v). At 4 days, the remaining algal suspension (60 mL) was sampled, as well as the solvent. Time zero samples were algal suspension that had not been contacted with solvent. Six long term milking experiments (M-5 through M-10) with a duration up to 28 days were performed in order to assess the extended biocompatibility of the solvents with the algae, the retention of nutrients in the algal suspension and the performance of milking lipids in a nitrogen-limiting state. Experiments M-5 through M-8 used *Chlorella*, and Experiments M-9 and M-10 used *Ankistrodesmus*. The effect of pumping rate was assessed in Experiments M-5 (25–250 mL/min) and M-9 (50–250 mL/min). Experiment M-8 tested the

effect of pumping rate (25–250 mL/min) with algae grown in N:P-5 media with twice the concentration of nitrogen and phosphorous. All other experiments used a 100 mL/min pumping rate for all treatments. Experiment M-6 used nutrient addition (nitrogen and phosphorous, at an N:P-5 ratio) in make-up water; 0.75, 3.75, and 7.5 mg-N/L with corresponding concentrations of phosphorous, 0.33, 1.66, and 3.32 mg-P/L, respectively. A control treatment, with deionized water used for make-up, was also utilized in Experiment M-6 to allow for comparison to a treatment in Experiment M-5. Experiments M-7 and M-10 tested the effect of intermittent pumping; pumping of the reactor volume was performed for 1 hr at the end of the light or dark photoperiods (Experiment M-10 also had a treatment with pumping at the end of both light and dark photoperiods). Experiments M-7 and M-10 used control treatments that had no solvent, for M-7 continuous pumping was used and for M-10 pumping was provided at the end of both light and dark photoperiods. Periodically (every 1–7 days), make-up, sterile deionized water was added to each reactor to refill the reactor to the initial volume. After make-up water was added, 10–14 mL of algal suspension was sampled. The solvent phase was sampled and weighed at the end of the milking experiments. In Experiment M-8 periodic sampling of 2 mL of solvent (with replacement) was also performed on 9 several days during the experiment.

Table 1. Experiments and treatments performed to assess biocompatibility of solvents and long term milking of algae

Exp. ID	Days in Exp.	Species	Variable	Treatments	Units/description	Notes
Biocompatibility experiments						
B-1	4	*A. falcatus*	--	dodecane	--	--
B-2	4	*A. falcatus*	--	biodiesel	--	--
B-3	4	*C. kessleri*	--	dodecane	--	--
B-4	4	*C. kessleri*	--	biodiesel	--	--
Long term milking experiments						
M-5	28	*C. kessleri*	pumping rate	25–250	mL/min	--
M-6	28	*C. kessleri*	nutrient addition	0.75–7.5	mg-N/L	Media also contained P to maintain N:P-5 ratio; pumping rate 100 mL/min
M-7	28	*C. kessleri*	intermittent pumping	light, dark	time of pumping	1 hr pumping at end of respective photoperiod; pumping rate 100 mL/min; control reactor did not have solvent
M-8	28	*C. kessleri*	pumping rate	25–250	mL/min	Media 2x concentration of N, P
M-9	26	*A. braunii*	pumping rate	50–250	mL/min	--
M-10	6	*A. braunii*	intermittent pumping	light, dark, light+dark	time of pumping	1 hr pumping at end of respective photoperiod; pumping rate 100 mL/min; control reactor did not have solvent

2.4. Analytical Methods

2.4.1 Optical Density and Growth Rate

Optical densities (OD) of algal suspensions were measured at 684 nm with one of 3 spectrophotometers; a Genesys 10 vis, a Genesys 10S UV-Vis (Thermo Fisher Scientific, Waltham, MA, USA), or a UV-1650PC (Shimadzu, Kyoto, Japan). The growth rate of cells in periodic samples was determined to assess the viability of cells. This was performed by inoculating 23 mL of fresh media with 2 mL of sample, and culturing under illumination in 50 mL poly centrifuge vials, except in Experiment M-8 where 15 mL poly centrifuge vials with 11.5

mL of media were inoculated with 1 mL of sample. The media used for growth rate analyses was the same as the media used in each experiment; biocompatibility experiments used N:P-50, and long term milking experiments used N:P-5 media except for Experiment M-8 which used N:P-5 media with twice the concentration of N and P. Long term milking experiments used triplicate measurements of growth rate, except for Experiment M-8 that used a single determination for each sample per sampling day. Vials were mixed daily every 1–4 days to resuspend settled algae. OD initial values were measured at inoculation; final values were measured at 3 days for solvent biocompatibility experiments and at 6 days for long term milking experiments. The first-order growth rate constant, *k*, was calculated using Equation 1 and has units of d^{-1}. Positive values of *k* indicate that cells are viable and values near zero or negative indicate that cells are non-viable.

$$k = \frac{\ln\left[\frac{OD_f}{OD_i}\right]}{t_f - t_i}$$

Equation 1

Where *i* is the initial value of OD at $time_i$
f is the final value of OD at $time_f$

2.4.2 Total and Dead Cell Counts

Cell counts were performed on a hemacytometer with either an Axioplan 2 epifluorescent microscope (Zeiss, Jeve, Germany) or a DM2500 microscope (Leica, Bensheim, Germany). Cell counts were performed in duplicate for biocompatibility experiments or in quadruplicate for long term milking experiments. Total cell counts were performed with brightfield imaging. Propidium iodide (Invitrogen #P3566, Carlsbad, CA, USA) was used to stain cells with compromised plasma membranes; these cells were assumed to be dead. The propidium iodide (PI) staining procedure involved dosing 2 mL of algal suspension with 4 µL of PI (PI final concentration: 2

μg/mL). After 10 min of exposure, the suspensions were centrifuged at 4000 rpm (3,220 *g*) for 10 min, decanted, washed with WC media and then centrifuged and decanted again. All samples were frozen at -20°C until counting was performed; cells were resuspended in media at the time of counting. PI was excited at 540–550 nm and the emission was measured at 590 nm on the Zeiss microscope. PI staining of *Chlorella* cells yielded inconclusive results because autofluorescence of the alga was substantial in the measurement wavelength and it obscured the staining of the nuclei of cells which may have had compromised membranes. Therefore, PI-staining was not used to determine dead cell counts for Chlorella. The live cell count was assumed to be the difference between the total and dead cell counts.

2.4.3 Flow Cytometry

Flow cytometry was performed at KU's Microscopy and Analytical Imaging Laboratory (MAI) for samples from Experiment M-8. Hoechst, 10 mg/mL (Invitrogen #H3570), was dosed at 10 μL per mL suspension (Hoechst final concentration: 99 μg/mL) to stain all cells in the sample. Sytox Green, 30 μM (Invitrogen #S34860) was dosed at 1 μL per mL algal suspension (Sytox Green final concentration: 0.03 μM) to stain dead cells. Cells were contacted with stains for about 1 hr and then were analyzed immediately. Each day flow cytometry was performed, 4 control samples were prepared by compositing 1 mL of sample from each reactor, then splitting the composite sample into 4- 1 mL aliquots. The first control had no stain, the second was stained only with Hoechst, and the third was stained only with Sytox Green. The fourth control sample was prepared by adding 1 mL of 90% (v/v) ethanol, vortexing for 15 sec, then allowing for 10 min of contact time, in order to kill the cells. The sample was then centrifuged at 3,220 *g* for 10 min, decanted and refilled with N:P-5 media with twice the concentration of N and P.

Finally, the sample was stained with Sytox Green only.Stained cells were analyzed with a MoFlo XDP Flow Cytometer/Cell Sorter (Beckman Coulter, Brea, CA, USA). Hoechst was excited at 355 nm and the emission was measured at 457/50 nm. Sytox Green was excited at 488 nm and the emission was measured at 529/28 nm. At least 68,800 total cells were counted for each sample. Processing of flow cytometry data was performed with Kaluza v1.1 software (Beckman Coulter). The difference between the total and dead cell counts was assumed to equal the number of live cells.

2.4.4 Scanning Electron Microscopy

Scanning electron microscopy (SEM) was performed on samples of algal cells for Experiment M-8 at day 0, 1, and 28 to characterize the morphology of *Chlorella* before and after exposure to solvent. Preparation and imaging of SEM samples was performed by Heather Shinogle at MAI. Preparation of samples for SEM involved obtaining a pellet of algal cells by centrifugation (3,220 *g*, 10 min) followed by fixing the cells in 2.5% glutaraldehyde for 5 min. Post fixing with 1% osmium tetraoxide was performed for 30 min. Dehydration was performed with increasing concentrations of alcohol; 30%, 50 %, 70%, 80%, 90%, 95%, and 100%. Drying with 100% alcohol was performed twice and each step had a duration of 5 min. Samples were then dried in a critical point dryer (Tousimis, Rockville, MD, USA). Finally the samples were placed on SEM sample stubs, and sputter coated with gold/palladium before imaging with a LEO 1550 field emission scanning electron microscope (Zeiss).

2.4.5 β-carotene Measurement

β-carotene is ubiquitous in nonmotile green algae (Wehr & Sheath, 2003). It is a lipophilic compound that can accumulate in lipid bodies in algal cells such as *D. salina* (Hejazi

& Wijffels, 2004). In biocompatibility experiments, it was used as an analog for lipid compounds and was analyzed in the algal suspension and the solvent phase. The algal suspension was prepared by centrifuging 4 mL of suspension at 3,500 rpm (2,465 *g*) for 5 min, discarding the supernatant, then adding 8 mL of anhydrous ethanol (Fisher A405P-4) and vortexing for 2 min to extract the β-carotene. Extracts were centrifuged again and clarified supernatant was saved for analysis. Solvent phases were collected and filtered with 0.22 μm capsule filters. A UV-1650PC (Shimadzu) UV-vis spectrophotometer was used to measure OD of ethanol extracts and solvent phases; measurement wavelength was 451 nm for ethanol extracts, 456 nm for dodecane solvent phase and 468 nm for biodiesel solvent phase. A calibration curve was developed by dissolving β-carotene (Sigma-Aldrich #22040, St. Louis, MO, USA) in tetrahydrofuan, and making dilutions in anhydrous ethanol, dodecane or biodiesel, as appropriate for the analysis.

2.4.6 Lipid Analyses

Lipids were analyzed in algal suspensions and in solvent phases. Total lipids in the aqueous algal suspension were extracted using a modified gravimetric method (Bligh & Dyer, 1959). Lipid samples recovered from gravimetric analysis were dissolved in 1 mL of chloroform and stored at -20°C until compositional analysis by HPLC was performed. Lipids extracted in solvent phases (and recovered from algal suspensions) were analyzed using an Agilent (Santa Clara, CA, USA) 1200 Series HPLC with a variable-wavelength detector and Supelco (St. Louis, MO, USA) C-18 column (5 μm particle size; 25cm length; 4.6 mm diameter) according to a previous method developed (Di Nicola et al., 2008). Table 2 presents a summary of key method parameters. Briefly, two mobile phases 4:1 (v/v) acetonitrile:methanol, and 8:5 (v/v) n-hexane:isopropanol were used in gradient at a flow rate of 1.3 mL/min, with a column temperature of 30°C, an analysis time of 30 min, a detector wavelength at 210 nm, and an

injection volume of 40 µL. Solvent samples were not diluted before injection. Recovered lipids from the algal suspension were prepared for HPLC analysis by evaporating the chloroform used for storage then redissolving the samples in 1 mL of n-dodecane, centrifuging at 10,000 *g* for 60 sec, and saving the supernatant for analysis. The centrifugation step was used to pelletize small amounts of insoluble matter, likely cellular debris, which was noticeable upon close inspection of both chloroform and dodecane solutions.

Table 2. Summary of key HPLC method parameters* for analyzing lipids in solvent phase

Parameter	Value	Unit	Notes
Flow rate	1.3	mL/min	
Column temperature	30	°C	
Injection volume	40	µL	Samples not diluted
Mobile phase A	4:1	(v/v)	Acetonitrile:Methanol
Mobile phase B	8:5	(v/v)	n-Hexane:Isopropanol

Pump program	Time (min)	Mobile phase A	Mobile phase B
Start	0	100%	0%
Begin gradient	2.2	100%	0%
End gradient	25.5	34%	66%
End	30	34%	66%

*Source: (Di Nicola et al., 2008)

The method was developed to measure unreacted lipid compounds and fatty acid methyl esters in a biodiesel mixture; it is able to quantify free fatty acids, mono-, di-, and triglycerides, and fatty acid methyl esters. Standards of neutral lipids included free fatty acids (FFA), monoglycerides (MG), diglycerides (DG), triglycerides (TG), and methyl esters of common fatty acid groups.

Table 3 presents the lipid standards by retention time analyzed for development of (external) standard curves. Overall, lipid standard concentrations ranged between 2–6,020 ppm (w/w). Standards were obtained from Nu-Check Prep, Inc. (Elysian, MN, USA) and included

MG, DG, TG, and methyl ester mixtures of palmitic (TLC 16-0A), stearic (TLC 18-0A), oleic (TLC 18-1A), linoleic (TLC 18-2A), and linolenic (TLC 18-3A) fatty acids. Additional standards included palmitic acid (N-16A), stearic acid (N-18A), palmitoleic acid (U-40A), oleic acid (U-46A), linoleic acid (U-59A), linolenic acid (U-62A) and tripalmitolein (T-215). Mixtures containing MG compounds included isomers with fatty acid groups bound to the 1- and 2-positions of the glycerol group, and DG compounds included isomers with fatty acid groups bound to the 1-, 2- or 1-, 3-positions of the glycerol group. Standards were diluted with dodecane or mixtures of dodecane and 8:5 (v/v) n-hexane:isopropanol.

Table 3. Lipid standards by retention time and range of concentrations used for HPLC analysis

[RT, retention time; LG, lipid group; CASRN, Chemical Abstract Service Registry Number; n/a, not available; n/d, compound not detected in standards. LGs include: 1, MG & FFA; 2, FFA; 3, DG; 4, DG & TG; 5, TG]

Lipid Standard	RT (min)	LG	Fatty acid group carbon number: Unsaturations	CASRN*	MW	Standard concentrations	
						Min (ppm)	Max (ppm)
Monolinolenin	3.37	1	18:3	n/a	352.3	2	243
Monolinolein	4.00	1	18:2	2277-28-3	354.3	25	992
Linolenic acid	4.10	1	18:3	463-40-1	278.2	78	961
Palmitoleic acid	4.90	1	16:1	2091-29-4	254.4	256	1,920
Linoleic acid	5.00	1	18:2	2197-37-7	280.3	26	1,020
Methyl linolenate	5.13	1	18:3	7361-80-0	292.2	2	243
Monoolein	5.35	1	18:1	25496-72-4	356.3	100	1,520
Monopalmitin	5.50	1	16:0	542-44-9	330.5	122	1,080
Methyl linoleate	6.60	1	18:2	112-63-0	294.3	25	992
Oleic acid	6.70	2	18:1	112-80-1	282.3	597	6,020
Palmitic acid	7.00	2	16:0	57-10-3	256.4	1480	6,020
Methyl oleate	8.80	2	18:1	112-62-9	296.3	100	1,520
Methyl palmitate	9.20	2	16:0	112-39-0	270.3	122	1,080
Stearic acid	9.40	2	18:0	57-11-4	284.5	575	5,970
Dilinolenin	10.30	3	18:3	n/a	612.5	2	243
Methyl stearate	11.38	3	18:0	112-61-8	298.3	195	425
Dilinolein	12.30	3	18:2	30606-27-0	616.5	25	992
Diolein	15.40	3	18:1	25637-84-7	620.5	254	1,520
Dipalmitin	16.20	3	16:0	26657-95-4	568.9	122	1,080
Trilinolenin	17.48	4	18:3	14465-68-0	872.7	2	243
Distearin	18.50	4	18:0	51063-97-9	625	195	425
Trilinolein	20.20	5	18:2	537-40-6	878.8	25	992
Tripalmitolein	20.75	5	16:1	n/a	801.3	100	984
Triolein	23.50	5	18:1	122-32-7	884.8	100	1,520
Tripalmitin	23.90	5	16:0	555-44-2	807.3	122	1,080
Tristearin	25.50	5	18:0	555-43-1	891.5	195	425
Monostearin	n/d	--	18:0	31566-31-1	358.3	195	425

*Source:(NIH, 2011). CASRNs refer to the 1- isomers for monoglycerides, and to the 1,2-isomers of diglycerides, except for dipalmitin and diolein which are for the 1,3- isomers.

To insure that the injection volume and other factors related to detection of lipids were consistent between runs and sample injections, 11 injections of a standard mixture (linoleic mixture, 1,050 ppm total concentration) were performed periodically during HPLC analyses. A summary of quality assurance characteristics for the 11 injections of the standard mixture is

shown in Table 4. The percent relative standard deviation (% RSD) of chromatogram peak areas was 1.0–9.3%, and the standard deviation of the compound retention time was 0.008–0.084 min. This indicates that HPLC operational characteristics were relatively consistent, and there was acceptable reproducibility (<10% RSD) for measured peak areas.

Table 4. Summary of quality assurance characteristics of peak areas and retention times for periodic injections of an HPLC standard mixture

[%RSD, percent relative standard deviation; RT, retention time; N, number of standard analyses]

Compound	Peak area %RSD	Average RT (min)	RT Standard deviation (min)	N
Monolinolein	2.7%	4.09	0.008	11
Methyl linoleate	1.0%	6.64	0.018	11
Dilinolein	9.3%	12.35	0.084	11
Trilinolein	2.2%	20.24	0.073	11

Fatty acid groups occur in organisms in various combinations in DG and TG, and isomers of the combinations are also possible. Because fatty acid groups other than those obtained as standards can occur in organisms, and because of the large variety of combinations and isomers possible for MG, DG and TG compounds, identification of individual lipid compounds is difficult in samples originating from algae. Where reasonable certainty was possible (retention times of sample peaks within ±0.1 min of standard peaks), individual lipid compounds were identified. In other cases where retention times did not allow for reasonable certainty, peaks identified in chromatograms were assigned to a lipid group identification. Lipid compounds which were identified are also included in the aggregate lipid group concentrations. Table 5

presents the grouping of lipid classes by retention time used for reporting lipid class and concentrations in this study.

Table 5. Grouping of lipid classes by retention time in HPLC analyses

Lipid Group	Lipid Classes	Analytical retention time	
		Begin (min)	End (min)
1	MG & FFA	3.30	6.65
2	FFA	6.65	9.85
3	DG	9.85	16.84
4	DG & TG	16.84	20.20
5	TG	20.20	30.00

Because uncertainty exists in the identification of lipid compounds, the quantification of lipids used the following protocol. If a compound was identified with reasonable certainty, then the correlation developed from standards of the compound was used to calculate the concentration of the compound. If a compound was identified only by lipid group, then the correlation developed for the standard that had the nearest retention time was used to calculate the concentration of the compound. The standards containing saturated fatty acid groups yielded a much weaker signal in comparison to compounds with unsaturated fatty acid groups. This is illustrated by Figure 3 which shows chromatograms of standard mixtures of lipid compounds of the stearic acid group (A) and the linoleic acid group (B). The concentration of each component in the standards were similar (390.5 ppm for linoleic compounds, 425 ppm for stearic compounds), but the peak areas associated with linoleic compounds (2 unsaturations per fatty acid group) were substantially larger than peaks associated with the peak areas associated with stearic compounds (saturated fatty acid groups). This results in correlations that may inflate the calculated concentration of lipid compounds if a peak cannot be identified with reasonable

certainty. As a result, correlations for compounds containing stearic acid and palmitic acid groups (unsaturated lipid compounds) were each used to quantify only one unknown in each analysis, in order to avoid inflating reported concentrations of lipids.

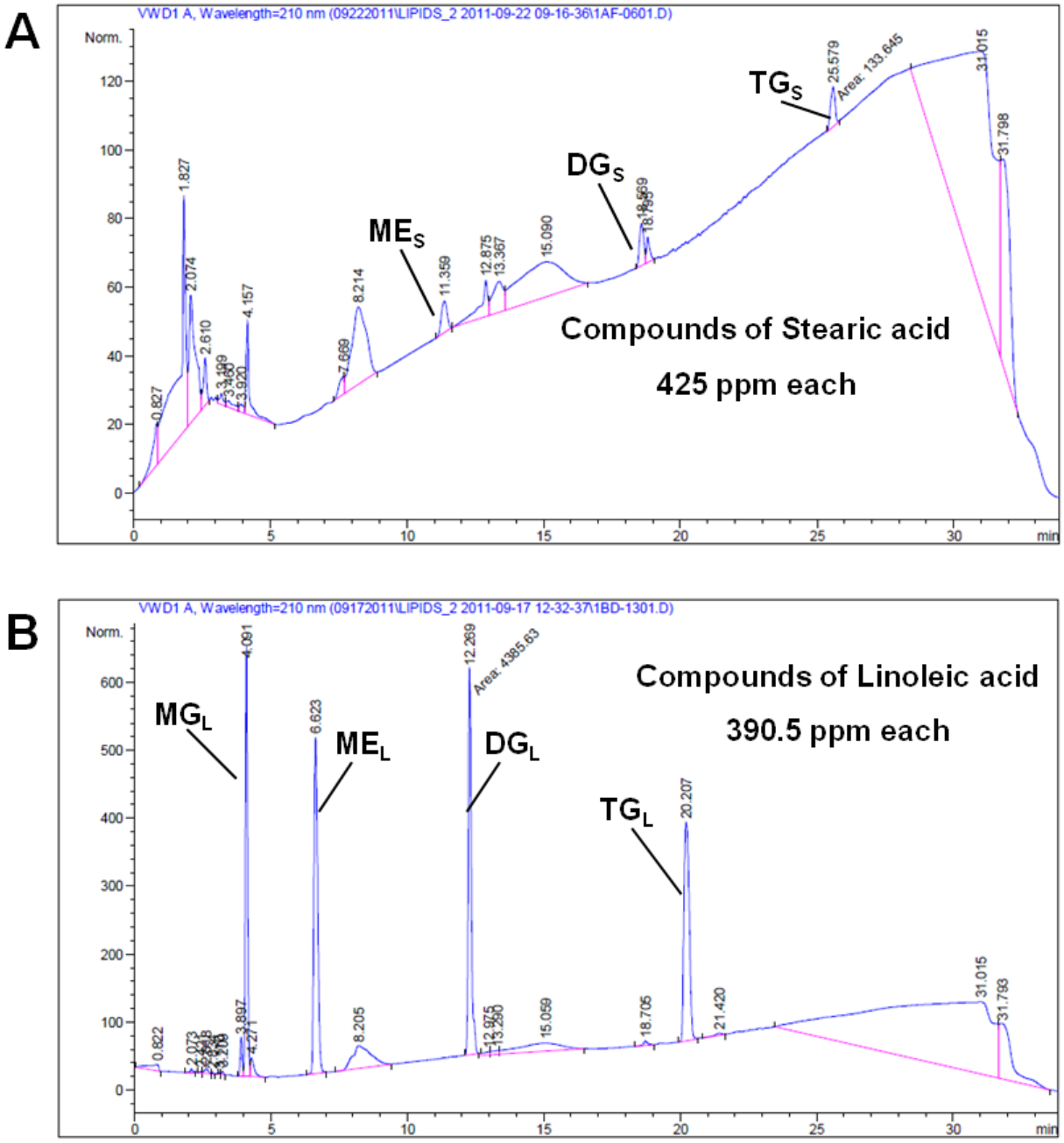

Figure 3. Chromatograms of standard mixtures of lipid compounds of the stearic acid group (A) and the linoleic acid group (B). The concentration of each component in the standards were similar (390.5 ppm for linoleic compounds, 425 ppm for stearic compounds), but the peak areas associated with linoleic compounds (2 unsaturations per fatty acid group) were substantially larger than peaks associated with the peak areas associated with stearic compounds (saturated fatty acid groups). Key to compounds: MG, monoglyceride; ME, methyl ester; DG, diglyceride; TG, triglyceride.

2.4.7 Nutrients and Total Suspended Solids

Procedures for measuring the concentrations of total and dissolved nitrogen and phosphorous, and total suspended solids (TSS) were adapted from Standard Methods (Eaton et al., 2005). Persulfate digestion was used to prepare nitrogen (4500-N C) and phosphorous (4500-P B 5) samples. Analysis of nitrogen (4500-NO_3^- B) and phosphorous (4500-P E) were analyzed with a Genesys 10S UV-Vis (Thermo Fisher Scientific) or a UV-1650PC (Shimadzu) spectrophotometer. Water samples for dissolved constituents were obtained by vacuum filtration with GF/F glass fiber filters (Whatman #1825-047, Buckinghamshire, UK). TSS determinations (2540 D) were also performed with GF/F filters. Analyses of total dissolved inorganic carbon (TDIC) and total dissolved organic carbon (TDOC) were performed with a Torch TOC analyzer (Teledyne Tekmar, Mason, OH, USA). Standards for TDIC were automatically diluted by the analyzer either from a 100 ppm standard stock (Ricca, #1845-4, Arlington, TX, USA), or from a 93.6 ppm preparation of sodium carbonate and sodium bicarbonate; the range of concentrations was between 1.9 and 33.3 ppm,.Standards for TDOC were automatically diluted from a stock solution of potassium biphthalate (Aqua Solutions #9571-1L, Deer Park, TX, USA) and had concentrations between 2.0 and 50 ppm.

2.5. Calculated Values

Concentrations of lipids analyzed by HPLC were converted from parts per million (w/w) to mg/L by assuming a specific gravity of 0.751 for n-dodecane, and calculation involving biodiesel assumed a specific gravity 0.879 (using methyl oleate as a representative methyl ester component of biodiesel) (Green, 2008). In order to provide an assessment of the total mass of lipids in each milking reactor in both the solvent and algal suspension phases, it was assumed that the total mass of dodecane remaining in the reactor was equal to the mass which was

recovered during sampling, plus 30 g to account for the solvent which could not be sampled from the reactor volume or remained in the process lines. The lipid milking productivity (mg/L-d) was used to express the average rate of the mass of lipids extracted to the solvent phase, normalized to the volume of algal suspension in the reactor, and was calculated using Equation 2.

$$Lipid\ milking\ productivity = \frac{Mass\ of\ lipids\ in\ solvent\ phase\ (g) \times \frac{1{,}000\ mg}{g}}{days\ in\ experiment \times 1.5\ L\ algal\ suspension}$$

Equation 2

The solvent lipid accumulation rate (LAR) was obtained by using linear regression (intercept = 0) of the time series of solvent total lipid concentration values for each treatment in Experiment M-8. The slope of the linear relationship represented the LAR, a quantity distinct from, but related to the lipid milking productivity. The LAR (mg/L-d) is not normalized to the volume of algal suspension in the reactor, but expresses the rate at which total lipids were extracted to the solvent phase, and allows for statistical comparison of the effect of the different pumping rates in Experiment M-8. Regression of linear or exponential relationships was performed in Excel. The percent lipid content of algal cells (TSS) was calculated on a dry weight basis. In order to quantify the distribution of the lipid group compositions between the cells and solvent phases, the ratio between the solvent phase aggregate MG & FFA composition and the cellular aggregate MG & FFA composition (S/C ratio) was computed using Equation 3.

$$S/C = \frac{Solvent\ phase\ \%\ MG\ \&\ FFA}{Cellular\ \%\ MG\ \&\ FFA}$$

Equation 3

A mass balance calculation was used to assess the degree to which the nutrients nitrogen and phosphorous were retained in algal suspensions after milking. An expected final mass of total nitrogen (TN) and total phosphorous (TP) was computed by making subtractions from the total mass of nutrients at the beginning of the experiment to account for nutrient mass lost due to sampling and wasting of algal suspension during the experiment, and an addition to account for the mass of nutrients added to the media through the 10 mL of inoculum. The measured final concentration of TN or TP was used to determine a final mass of nitrogen or phosphorous in the reactor volume which was then divided by the expected final mass of the nutrient and expressed as a percentage, as shown in Equation 4.

$$\%\ Nutrient\ retained = \frac{Measured\ final\ nutrient\ concentration\ \left(\frac{mg}{L}\right) \times 1.5\ L}{Expected\ final\ nutrient\ mass\ (mg)} \times 100\%$$

Equation 4

2.6. Statistics

The 95% confidence interval (CI) of arithmetic means or slopes was computed assuming a normal distribution of data and expressed as ± the mean or slope value. ANOVA and *t*-test analyses were performed to compare the means of replicate analyses. The solvent lipid accumulation rates (LAR) obtained in Experiment M-8 were compared using *t*-tests according to Equation 5.

$$t_{i-ii} = \frac{LAR_i - LAR_{ii}}{[SE_i^2 + SE_{ii}^2]^{1/2}}$$

Equation 5

Where *SE* is the standard error of the LAR obtained through linear regression

3. RESULTS AND DISCUSSION

3.1. Biocompatibility Experiments

Biocompatibility experiments were performed to identify solvents that are biocompatible with *Ankistrodesmus* and *Chlorella*. Four experiments were performed: *Ankistrodesmus*+dodecane (B-1), *Ankistrodesmus*+biodiesel (B-2), *Chlorella*+dodecane (B-3) and *Chlorella*+biodiesel (B-4). The biocompatibility of the solvents was assessed using 3 indicators of cell viability: total and live cell counts and growth rate determinations. β-carotene in the solvent and in the cells was measured as a proxy to assess the effectiveness of lipid milking. Analysis of lipids in the solvent phase was also performed.

3.1.1 Cell Viability

The total cell density after solvent exposure for the biocompatibility experiments (B-1 through B-4) is shown in Figure 4. The decreased growth in cell density for *Ankistrodesmus* exposed to solvent demonstrated inhibition relative to control samples (no solvent contact). Figure 4A and B show that total cell densities at day 4 were reduced to 51% and 45% of control sample densities for dodecane and biodiesel treatments, respectively. The inhibition was not observed in *Chlorella* treatments where solvent-contacted samples closely tracked control

samples for both dodecane and biodiesel. For example, Figure 4C and D show that the total cell densities at day 4 were 83% and 137% of control sample densities for dodecane and biodiesel treatments, respectively. The statistical significance of differences between solvent and control treatments for *Chlorella* at day 4 were assessed using *t*-tests. These yielded *p*-values of 0.09 for both the *Chlorella*+dodecane and *Chlorella*+biodiesel experiments, demonstrating that solvent exposure did not significantly impact total cell density for the alga.

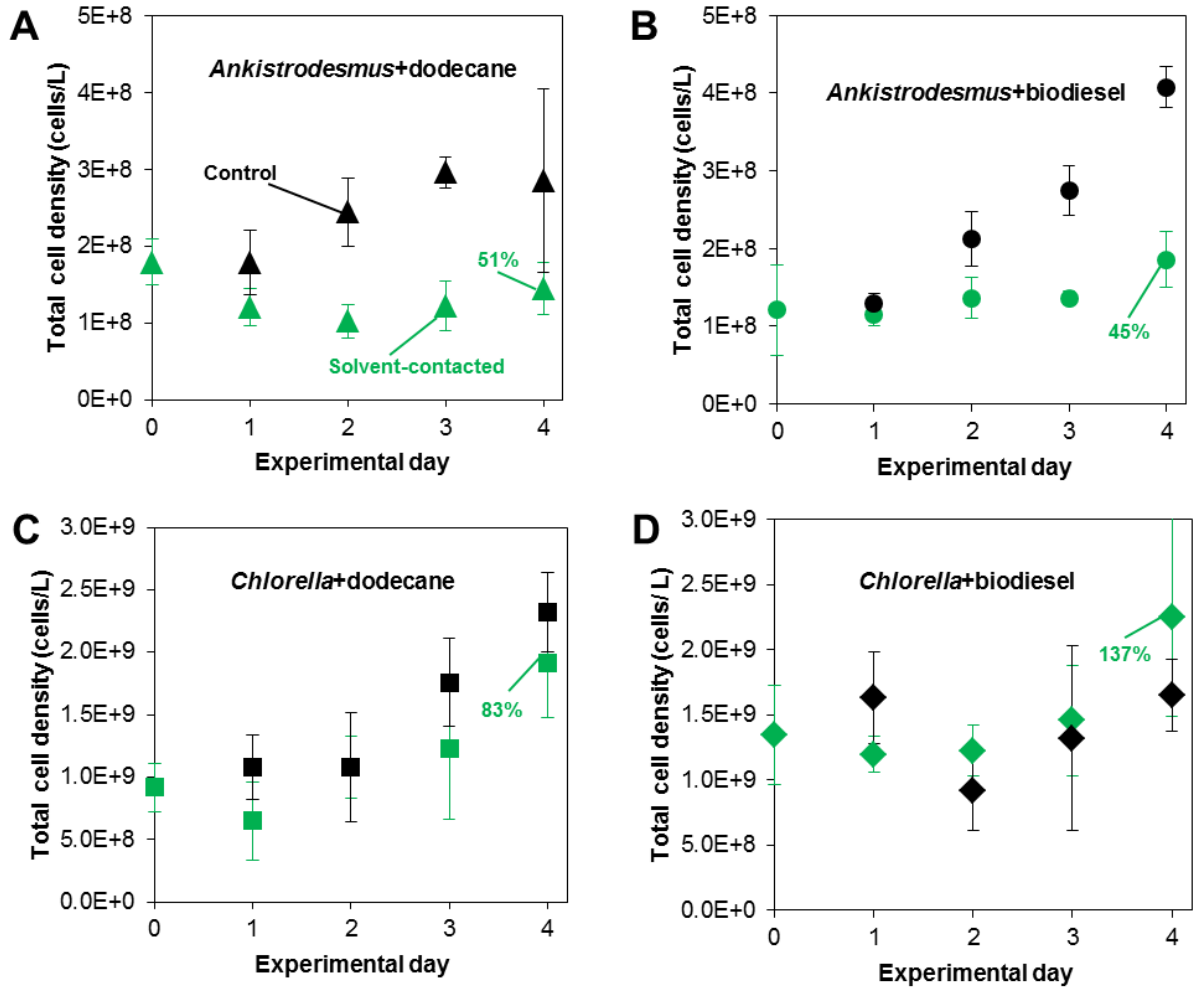

Figure 4. Total cell densities for algal cells in biocompatibility experiments after solvent exposure, the percentage denotes the total cell density of the solvent-contacted treatment normalized to the control treatment. The growth of *Ankistrodesmus* (A and B) was inhibited by solvent when compared to control samples. *Chlorella* (C and D) did not show inhibition after solvent exposure. Error bars represent the 95% CI.

The live cell densities for the *Ankistrodesmus* experiments are shown in Figure 5. A trend similar to that observed for total cell densities (Figure 4A and B) was observed for live cell densities in *Ankistrodesmus*; the live cell densities were reduced for solvent-contacted samples when compared to control samples for both dodecane and biodiesel treatments by day 4 of the experiments.

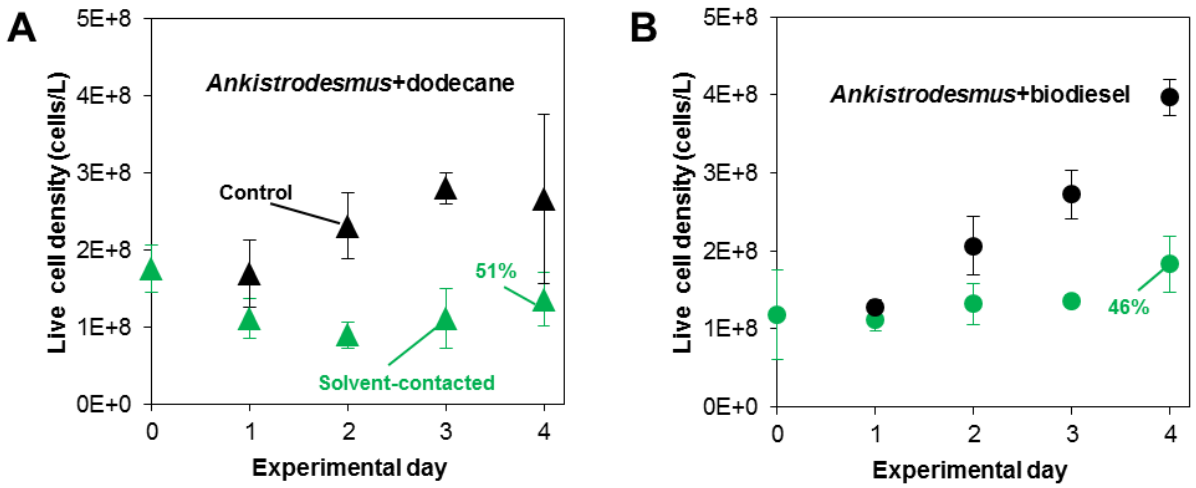

Figure 5. Live cell densities for *Ankistrodesmus* were reduced relative to controls for both (A) dodecane and (B) biodiesel treatments. The percentage denotes the live cell density of the solvent-contacted treatment normalized to the control treatment. Error bars represent the 95% CI.

First order growth rates, *k*, were used to assess the viability of Chlorella cell populations. Figure 6 shows that average values of *k* remained positive for both dodecane and biodiesel treatments, demonstrating that a viable population of cells was maintained during the experiments. Values of *k* were 0.18 d^{-1} at the beginning of both experiments and declined to 0.11 and 0.03 d^{-1} for dodecane and biodiesel samples, respectively. Results of *t*-tests for values of *k* on day 4 showed that the difference between growth rates for solvent-contacted and control samples were not statistically significant for either dodecane (p=0.15) or biodiesel (p=0.27) treatments.

The small growth rates observed for the biodiesel treatments are likely due in part to the formation of an oily emulsion which was carried over with the inocula to the fresh media and dissipated by the time of the second measurement. Thus, the second optical density measurement was lower in these cases. The values of *k* shown in Figure 6 are the average of 3 replicates. While all the (average) values of *k* remained positive, one of the replicates for the *Chlorella*+biodiesel experiment (B-4) had a negative value of *k* on day 3; this was also likely due to the initial presence of an emulsion which dissipated by the end of the analysis. Overall, values of *k* for solvent-contacted cells closely tracked the control samples for both dodecane and biodiesel. The results of total cell counts showed there were differences between *Ankistrodesmus* and *Chlorella* in terms of the ability of the cells to grow in the presence of the solvents. However, live cell counts and growth rate measurements showed that both algal strains had viable cell populations after 4 days of contact time with n-dodecane and biodiesel.

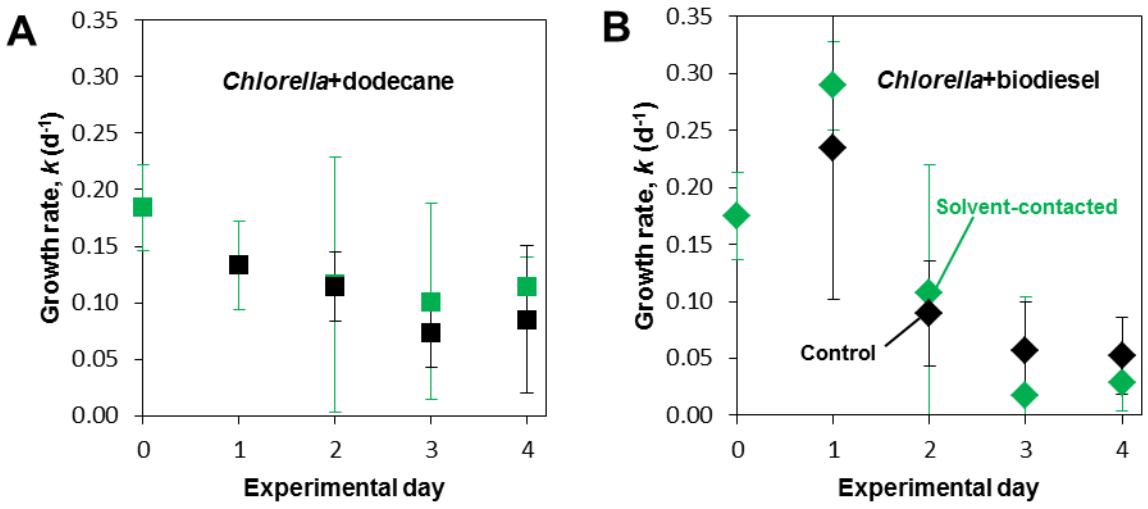

Figure 6. The average first order growth rate constants (*k*) for *Chlorella* remained positive during biocompatibility experiments. Both (A) dodecane and (B) biodiesel treatments closely tracked their control samples over 4 days. Error bars represent the 95% CI.

3.1.2 β-Carotene

β-carotene was measured as a proxy to assess the effectiveness of lipid milking. Table 6 presents results of β-carotene analyses for the biocompatibility experiments. The concentration of β-carotene in the solvent phase was 0.31 mg/L for *Ankistrodesmus* and ranged between 0.37–0.74 mg/L for *Chlorella*. The concentration in the cells ranged between 0.47–0.64 mg/L for *Ankistrodesmus* and 1.25–1.36 mg/L for *Chlorella*. These concentrations represent an extraction of β-carotene between 4.6–9.8% after 4 days of solvent contact. The total mass of β-carotene in the reactors was lower for solvent-contacted treatments (0.03–0.09 mg) than for control treatments (0.09–0.18 mg). This indicates that milking of the cells resulted in lower overall productivity of β-carotene.

Table 6. Results of β-carotene analyses for biocompatibility experiments.

Experiment	Treatment	Solvent β-carotene (mg/L)	Cellular β-carotene (mg/L)	Percent extraction	Total β-carotene (mg)
Ankistrodesmus +dodecane	Solvent	0.31	0.64	7.4%	0.04
	Control	--	1.43	--	0.09
Ankistrodesmus +biodiesel	Solvent	0.31	0.47	9.8%	0.03
	Control	--	2.53	--	0.15
Chlorella +dodecane	Solvent	0.37	1.25	4.6%	0.08
	Control	--	1.70	--	0.10
Chlorella +biodiesel	Solvent	0.74	1.36	8.3%	0.09
	Control	--	2.97	--	0.18

3.1.3 Analysis of Lipids

The results of cellular total lipids and TSS for the biocompatibility experiments using biodiesel were considered unreliable because a noticeable amount of oily emulsion was retained in the samples used to analyze total lipids and TSS. This is problematic for accurate analysis of total lipids by the (Bligh & Dyer, 1959) method because extraction of lipids from cells proceeds from an algal suspension concentrated by centrifugation; an oily emulsion may carry over to the extraction and artificially inflate the gravimetric measurement of algal lipids. This can be seen in Figure 7A where a highly cloudy appearance resulted after *Ankistrodesmus* suspensions were contacted with biodiesel. Even after centrifugation (Figure 7B) the emulsion was difficult to separate completely from the aqueous phase. The same problem affected the gravimetric analysis of TSS because drying at 105°C did not evaporate the emulsion which adhered to the sample filters. This is also demonstrated by the values of both TSS and cellular total lipids which were much larger for biodiesel treatments than dodecane treatments. For example, the measured average cellular lipids concentration for the biodiesel treatment in Experiment B-2 (303 mg/L) was 4 times larger than the dodecane treatment in Experiment B-1 (73 mg/L) (Table 16, Appendix).

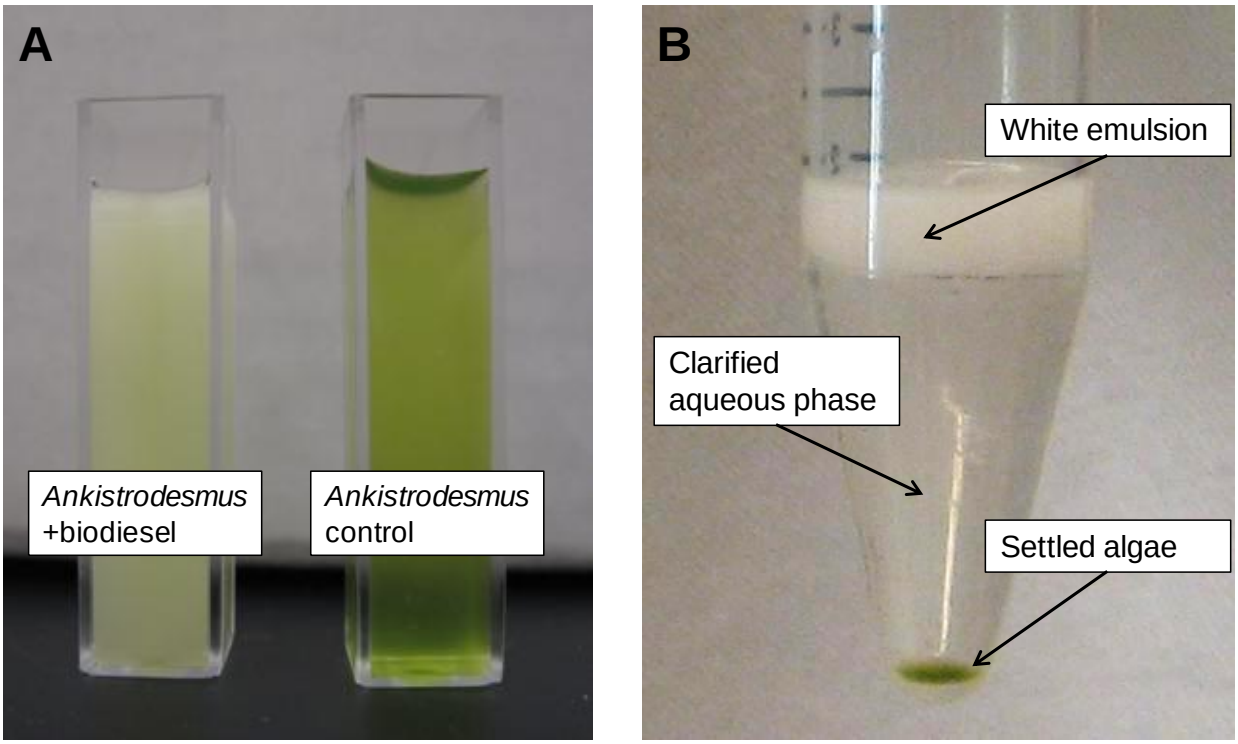

Figure 7. Samples from Experiment B-2. Treatments that used biodiesel formed an emulsion (A) that was difficult to separate even after centrifugation (B).

Cellular lipids and TSS measurements for dodecane treatments were not affected by an emulsion and are therefore considered reliable. Results of solvent phase lipid analyses are presented in Table 7. Analysis of lipids in biodiesel solvent was complicated by a large amount of background noise, likely attributable to unreacted lipids in the fresh biodiesel used for biocompatibility experiments. Thus, concentrations of lipids in biodiesel solvent are not reported here. Dodecane treatments, however, were readily analyzed; for these samples the total solvent phase lipid concentrations ranged between 54–79 mg/L. For Experiment B-1, the lipid classes detected were mostly MG & FFA (47 mg/L) with a lesser amount of DG & TG (7 mg/L). In Experiment B-3 only MG & FFA were detected.

Table 7. Solvent phase lipid concentrations for dodecane biocompatibility experiments

[Concentration ± 95% confidence interval]

Lipid Group	Lipid Classes	Experiment	
		B-1	B-3
		Lipid concentration (mg/L)	
1	MG & FFA	47 ± 2.6	26 ± 4
2	FFA	--	53 ± 229
3	DG	--	--
4	DG & TG	7 ± 2.8	--
5	TG	--	--
--	Total Lipids	54 ± 4.4	79 ± 232

An analysis of the total lipids in solvent and cells is presented in Table 8 with a comparison to results from Zhang et al. (2011). The solvent phase total lipids concentration in Experiments B-1 and B-3 (54-79 mg/L) were somewhat larger than the total lipids concentration in the (dodecane) solvent phase (17 mg/L) reported by Zhang et al. (2011). Also, solvent and cellular lipids concentrations were similar for B-1 (54 mg/L solvent, 73 mg/L cellular) and B-3 (79 mg/L solvent, 63 mg/L cellular), whereas for the Zhang et al. (2011) study the solvent concentration was less than in the cells (17 mg/L solvent, 105 mg/L cellular). This indicates that the milking of lipids from algal cells was more effective in B-1 and B-3 than in Zhang et al. (2011) study. This may be attributable to the way in which solvent and algal suspensions were mixed; in the present study, the batch reactors were placed on the stirring plate so that the stir bar rotated vertically through both the solvent and the algal suspension. This caused effective dispersion of the solvent through the algal suspension and increased contact between the solvent and the cells, and may have contributed to the larger solvent concentrations of lipids milked from algae in B-1 and B-3. It is not known how exactly stirring was accomplished by Zhang et al. (2011), so further comparison between that study and the present study is not possible.

Table 8. Analysis of total lipids in solvent and aqueous phases with comparison to results from Zhang et al. (2011).

Experiment or study	Solvent	Solvent total lipids (mg/L)	Cellular lipids (mg/L)	TSS (mg/L)	Percent lipid content in cells after milking
B-1	dodecane	54	73	336	21.8%
B-3	dodecane	79	63	276	22.8%
Zhang et al. 2011	dodecane	17	105	~600	17.4%

3.2. Long Term Milking Experiments

Six long term milking experiments were performed in order to assess the extended biocompatibility of the solvents with the algae, the performance of milking lipids in a nitrogen-limiting state, and the retention of nutrients in the algal suspension. Experiments M-5 through M-8 used *Chlorella*, and Experiments M-9 and M-10 used *Ankistrodesmus*. The extended biocompatibility (up to 28 days) of the solvents was assessed using 3 indicators of cell viability: total cell counts, growth rate determinations and, for Experiment M-8, flow cytometry. The performance of lipid milking was assessed by analyzing the concentration and type of lipids in the solvent phase and in the cells. The retention of nutrients in the algal suspension was assessed by measuring the total nitrogen and total phosphorous concentrations and performing mass balance calculations to determine the percent of each nutrient retained at the end of the experiments.

3.2.1 Cell Viability

The total cell densities for *Chlorella* in Experiment M-5 declined from initial values but stabilized by 28 days (Figure 8A).When nutrients were added to the reactors (Experiment M-6, Figure 8B) the cell densities at 28 days were 6.5 x 10^8 cells/L (for 3.75 mg-N/L feeding rate) and 6.6 x 10^8 cells/L (for 0.75 mg-N/L feeding rate). Results of *t*-tests showed that these densities were not statistically different than the total cell density (6.3 x 10^8 cells/L) of the 0 mg-N/L control (3.75 mg-N/L, p=0.60; 0.75 mg-N/L, p=0.34) on the same day. The highest feeding rate (7.5 mg-N/L) did result in an increased cell density (9.7 x 10^8 cells/L) over the other treatments, which an ANOVA demonstrated was statistically significant (p=2.3 x 10^{-6}). The total cell densities for *Ankistrodesmus* declined in Experiment M-9 for the 100 and 250 mL/min pumping rates by day 12, and the 50 mL/min pumping rate resulted in a slight decline in total cell density by day 26 (Figure 8C). The total cell counts end at 12 days for the 100 mL/min and 250 mL/min pumping rates in Experiment M-9 because the treatments were stopped after visual inspection of the reactors indicated that cell populations had died. This was verified by growth rate analyses performed on day 12 (see Figure 12 below).

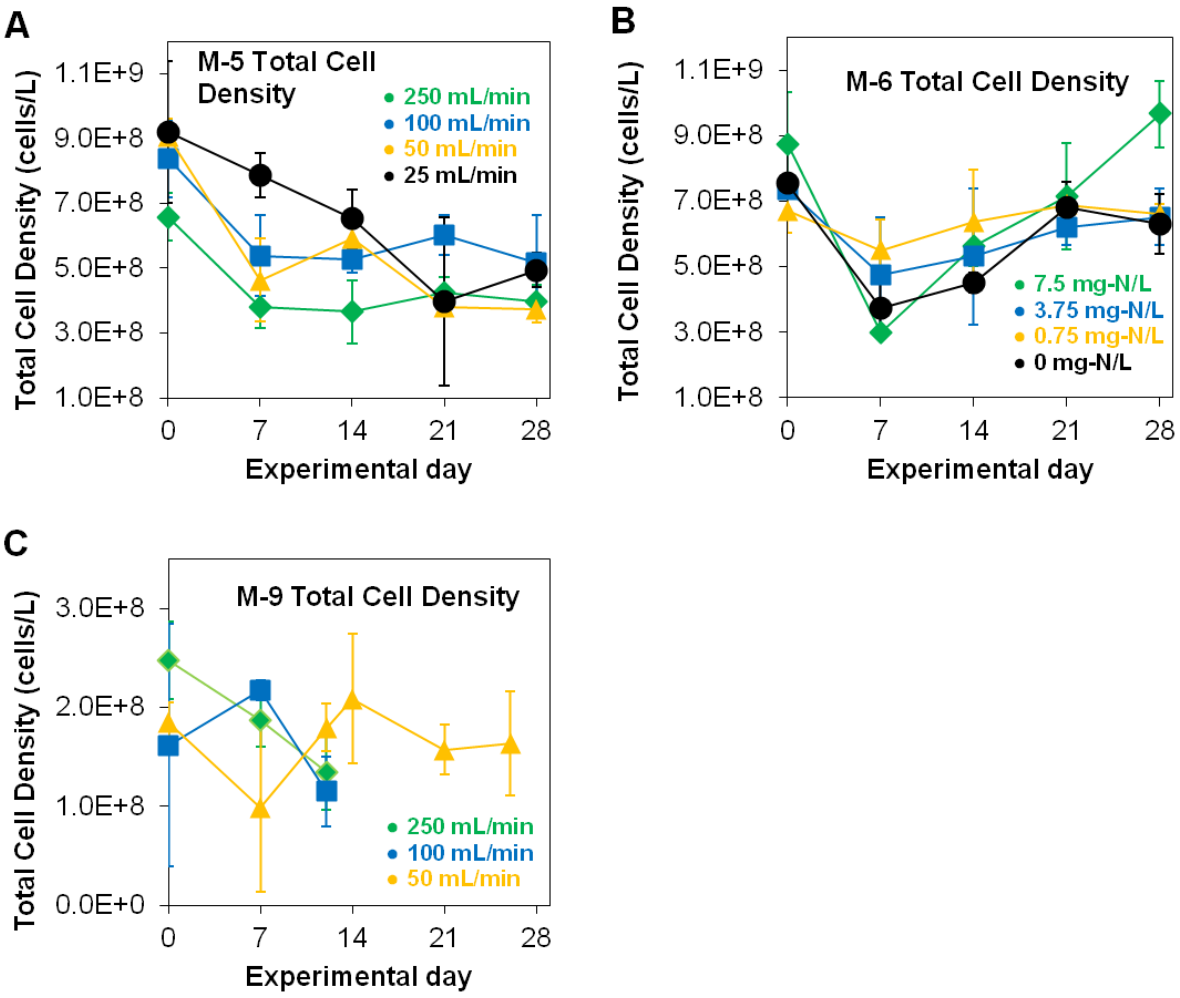

Figure 8. Total cell densities for long term milking experiments with *Chlorella* (A) M-5, (B) M-6, and *Ankistrodesmus* (C) M-9. Nutrient addition (B) only made a significant difference in final cell density at the maximum feeding rate.

For Experiment M-8, more frequent sampling allowed for a statistical analysis of trends in the total cell density. Figure 9 shows exponential decay curves from the mean total cell densities for 10 sampling points. The resulting decay rates ranged between -0.007 and -0.022 d^{-1}, with an average value of -0.0142 d^{-1}. The r^2 value for these curves was relatively low (r^2 = 0.333–0.757), but the average decay rate allows for estimates regarding the duration of effective milking of a batch population of cells. For example, the half-life of the population was estimated

by dividing ln(½) by the average decay rate of -0.0142 d^{-1}. The resulting half-life estimate is about 49 days, which gives an indication of the time frame in which batch operation could maintain a population of cells. Or, a stable cell density could be maintained in the reactor by supplying new cells continuously. For the average decay rate of -0.0142 d^{-1}, a stable population of cells could be maintained by continuous addition of about 1.4% per day of the total number of cells in the reactor at initial conditions. The total number of cells removed by sampling the reactors during the experiment was calculated as $\leq 2.2 \times 10^8$. The resulting percentage of the total cells removed per day of the experiment was ≤0.42% per day and demonstrated that sampling was (on average) not responsible for the majority of the decline of total cell densities in the reactors. For the 50 mL/min pumping rate the percentage of cells removed was 0.38% per day which was slightly larger than half of the value of the decay rate for that reactor (-0.007 d^{-1}, or 0.7% per day) obtained by regression.

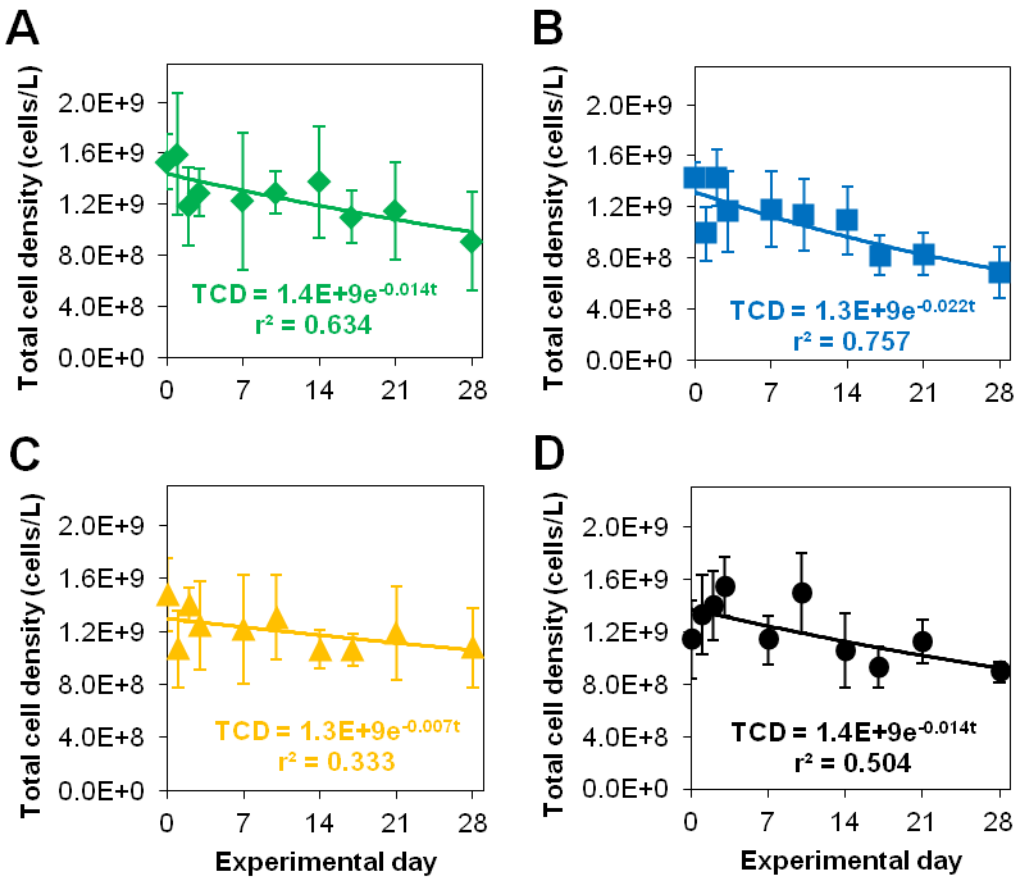

Figure 9. Total cell densities (TCD) in Experiment M-8 were regressed with an exponential decay model; (A) 250 mL/min, (B) 100 mL/min, (C) 50 mL/min and (D) 25 mL/min. The average decay rate of the 4 treatments, $\delta = -0.0142\ d^{-1}$, allows for estimations regarding the duration of effective milking of a batch population of cells, or new cell make-up rates for continuous milking.

Because total cell counts can include cells that are non-viable, growth rate analyses were also used to assess the viability of the algal populations. For experiment M-8, flow cytometry was also used to assess the percentage of live cells. The long term milking experiments with *Chlorella* (Experiments M-5 through M-7) had positive values of *k* throughout the 28 day duration of each experiment (Table 9). Experiment M-8 had some negative values of *k* for the 250 mL/min treatment between days 10 and 21 (Figure 10), but rebounded on day 28 to a

positive value (0.09 d^{-1}). This temporary drop into negative growth rates does not mean that the cell population was non-viable for the 11 day period, but is likely due to an emulsion which formed in the reactor by day 9. Suspended matter in the emulsion was carried over into the growth rate analysis and dissipated by the time of the second measurement. Thus, the second optical density measurement was lower in these cases, even though noticeable growth of algae was apparent during visual inspection of the test tube contents. Overall, these results demonstrated that a viable population of *Chlorella* was maintained, and dodecane was biocompatible with the alga for up to 4 weeks of contact.

Table 9. Summary of values for growth rate constants for milking experiments M-5 though M-8 with *Chlorella.*

[Min, minimum; Max, maximum]

Treatment	Growth rate, k (d^{-1})		
	Min	Average	Max
Experiment M-5			
250 mL/min	0.05	0.10	0.15
100 mL/min	0.05	0.09	0.11
50 mL/min	0.03	0.10	0.13
25 mL/min	0.07	0.09	0.10
Experiment M-6			
7.5 mg-N/L	0.07	0.09	0.11
3.75 mg-N/L	0.09	0.11	0.13
0.75 mg-N/L	0.09	0.10	0.12
0 mg-N/L	0.07	0.10	0.14
Experiment M-7			
control, no solvent	0.13	0.15	0.16
dark mixing period	0.10	0.11	0.12
light mixing period	0.11	0.13	0.17
Experiment M-8			
250 mL/min	-0.09	0.005	0.09
100 mL/min	0.03	0.06	0.12
50 mL/min	0.02	0.07	0.12
25 mL/min	0.03	0.07	0.10

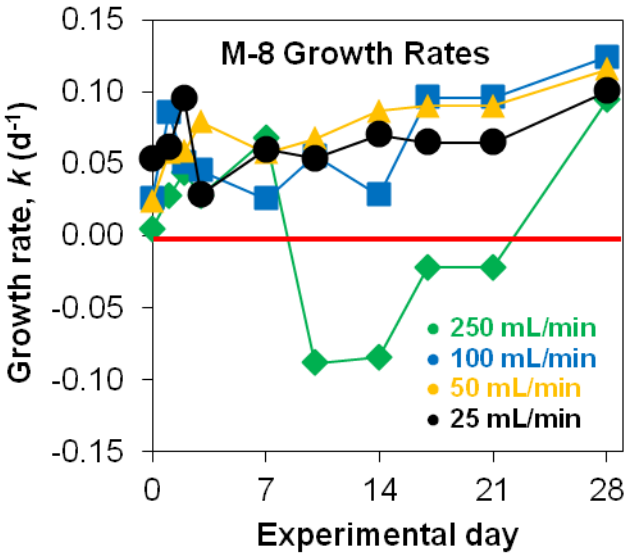

Figure 10. Growth rates for Experiment M-8. The negative values observed for the 250 mL/min treatment between days 10 and 21 were likely experimental artifacts due to the dissipation of an emulsion during the analyses. These values do not indicate the presence of a non-viable cell population in the reactor.

Results of flow cytometry for Experiment M-8 are shown in Figure 11A-D. The percentage of live cells was high (>95%) for all treatments through day 14 of the experiment. Beginning at day 18, however, the percentage of live cells declined sharply reaching a minimum at day 21 (as low as 28% live for the 100 mL/min pumping rate) then rebounding to between 52–78% live at day 28. This decline into low live cell percentages is again likely due to an emulsion which formed in the reactor with the 250 mL/min pumping rate; this disrupted the analysis of samples from that reactor and the control samples that were composited from samples from all treatments. The disruption of the control samples caused an obscuring of the distinction between signals for total and dead cells, so that the criteria used to classify cells as dead, likely also included a large proportion of cells that were actually alive. Therefore, the results shown for days

18–28 most likely underestimate the proportion of live cells. However, by day 28 at least 52% of the cells (and likely more) were alive for all treatments.

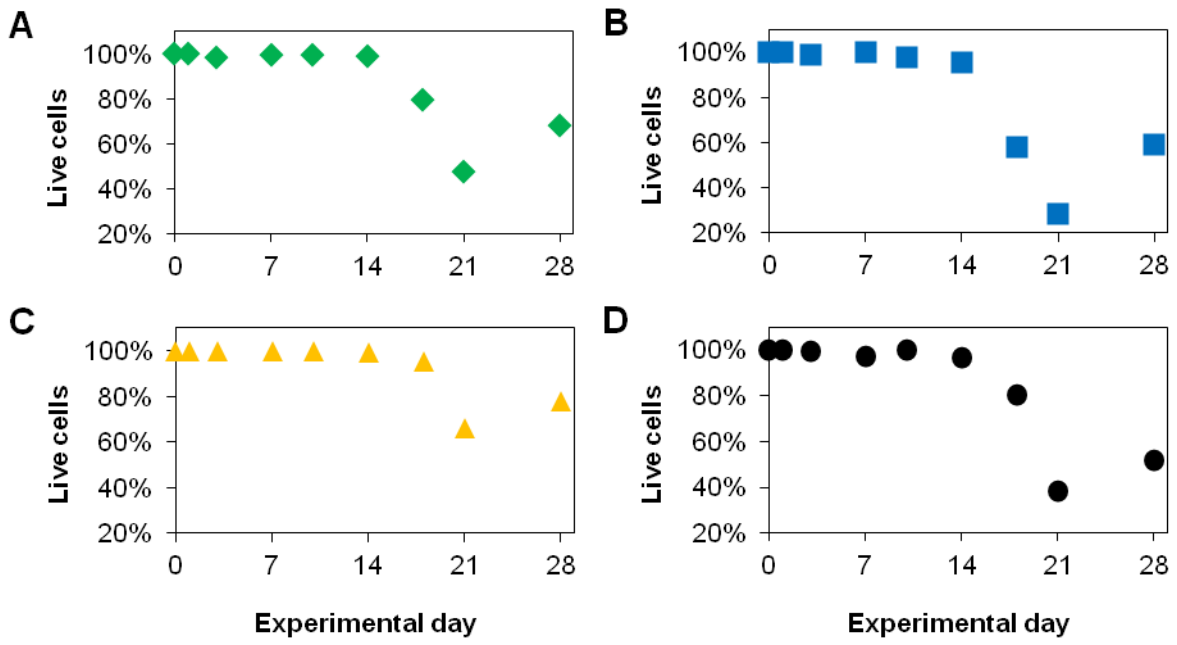

Figure 11. Results of flow cytometry for (A) 250 mL/min, (B) 100 mL/min, (C) 50 mL/min and (D) 25 mL/min treatments in Experiment M-8. The decline in live cell percentages which began at day 18 for all treatments and partially rebounded by day 28 is likely an experimental artifact due to the formation of an emulsion in the 250 mL/min reactor. The emulsion disrupted control samples which affected the distinction between total cell and dead cell signals during analysis.

Figure 12 shows the values of *k* for experiments with *Ankistrodesmus*. For Experiment M-9 (Figure 12A), *k* began to decline in the first week for the highest pumping rate (250 mL/min), and *k* for the other treatments started to drop in the second week. By 26 days, *k* had dropped into the negative range for all treatments, and increased pumping rate increased the rate of decline into negative values of *k*. This indicates that the viable cell population had collapsed for all pumping rates. In Experiment M-10, when solvent contact was reduced using intermittent mixing (Figure 12B), the decline of *k* into negative values was even more rapid. All solvent-

contacted treatments had large negative values of k ($\leq$ -0.25 d^{-1}) by day 6 of the experiment, whereas in the control treatment (no solvent contact) k was 0.12 d^{-1}. This increase in the rate of collapse of the viable cell population may be due to a lower initial total cell density, as indicated by an initial optical density of about 0.19 for M-9 vs. 0.04 for M-10. These results show that dodecane, even though biocompatible with *Ankistrodesmus* for 4 days, is not biocompatible with the alga for periods extensive enough for milking to be a feasible process.

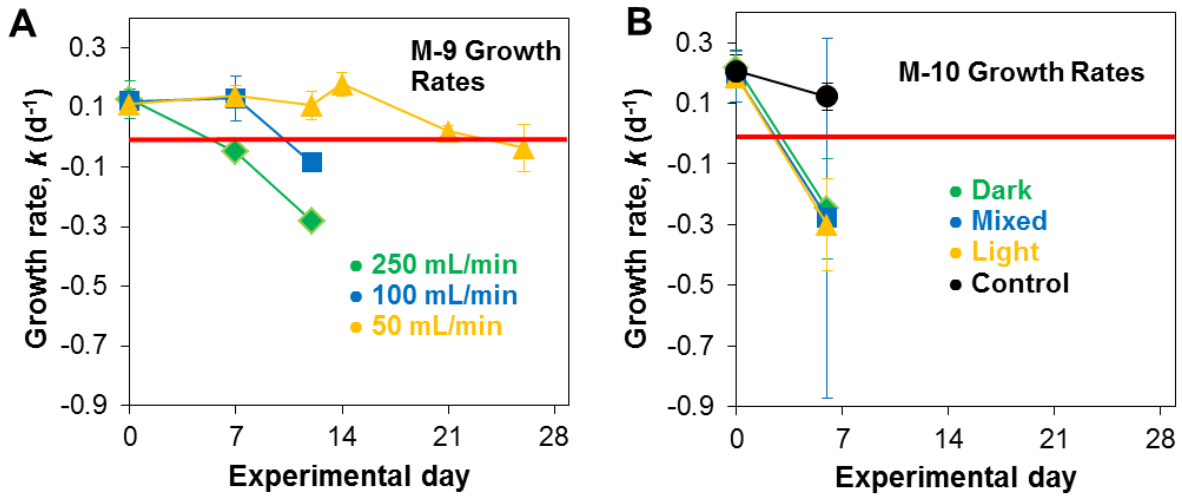

Figure 12. Growth rates (k) for long term milking experiments with *Ankistrodesmus*. Experiment M-9 (A) and Experiment M-10 (B) both had decline into negative values for all solvent-contacted treatments.

The reason for the decreased survivability of *Ankistrodesmus* relative to *Chlorella* is not known. However, it may be attributed to the morphologies of the cells. Figure 13 shows that *Chlorella kessleri* is a small spherical cell with a diameter of approximately 3 μm, *A. falcatus* and *A. braunii* are long, pin-shaped cells with a length of about 40 μm and a center diameter of about 5 μm. By modeling the geometry of *Ankistrodesmus* as two cones with the bases fused together, it can be seen that the approximate surface area of *Ankistrodesmus* (320 μm^2) is over 10

times larger than for *Chlorella* (30 μm^2). This would result in more potential contact between *Ankistrodesmus* cells and the solvent phase and may explain the reduced survivability of the algae. It is not likely that the decreased survivability of *Ankistrodesmus* is due to turbulence or mechanical abrasion in the static mixer because the control treatment (no solvent contact) in Experiment M-10 shows that the cells maintained viability for the 6 days of the experiment while the solvent-contacted samples resulted in cell death in the same time frame.

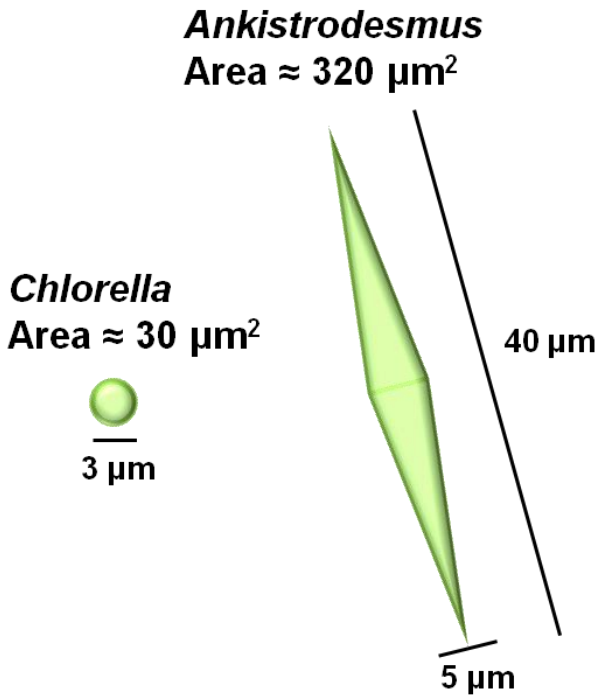

Figure 13. Approximate dimensions for *Chlorella* and *Ankistrodesmus* cells. *Ankistrodesmus* is modeled as two cones with contiguous bases.

For Experiment M-8 SEM imaging was performed to assess alterations in *Chlorella* cell morphology after solvent exposure. Figure 14 shows SEM images from the 100 mL/min treatment before solvent exposure (A), after 1 day of solvent contact (B) and at 28 days of contact (C). *Chlorella* was spherical with a rough surface both before solvent contact and after 1

day of exposure. At 28 days the cells were indented and had a raspberry-like appearance. The indented morphology was observed in all treatments from Experiment M-8 (See Figure 19, Appendix). Despite the deflated appearance of cells after 28 days of solvent contact, the cellular membranes were intact, which is consistent with flow cytometry data and positive growth rates observed in all treatments.

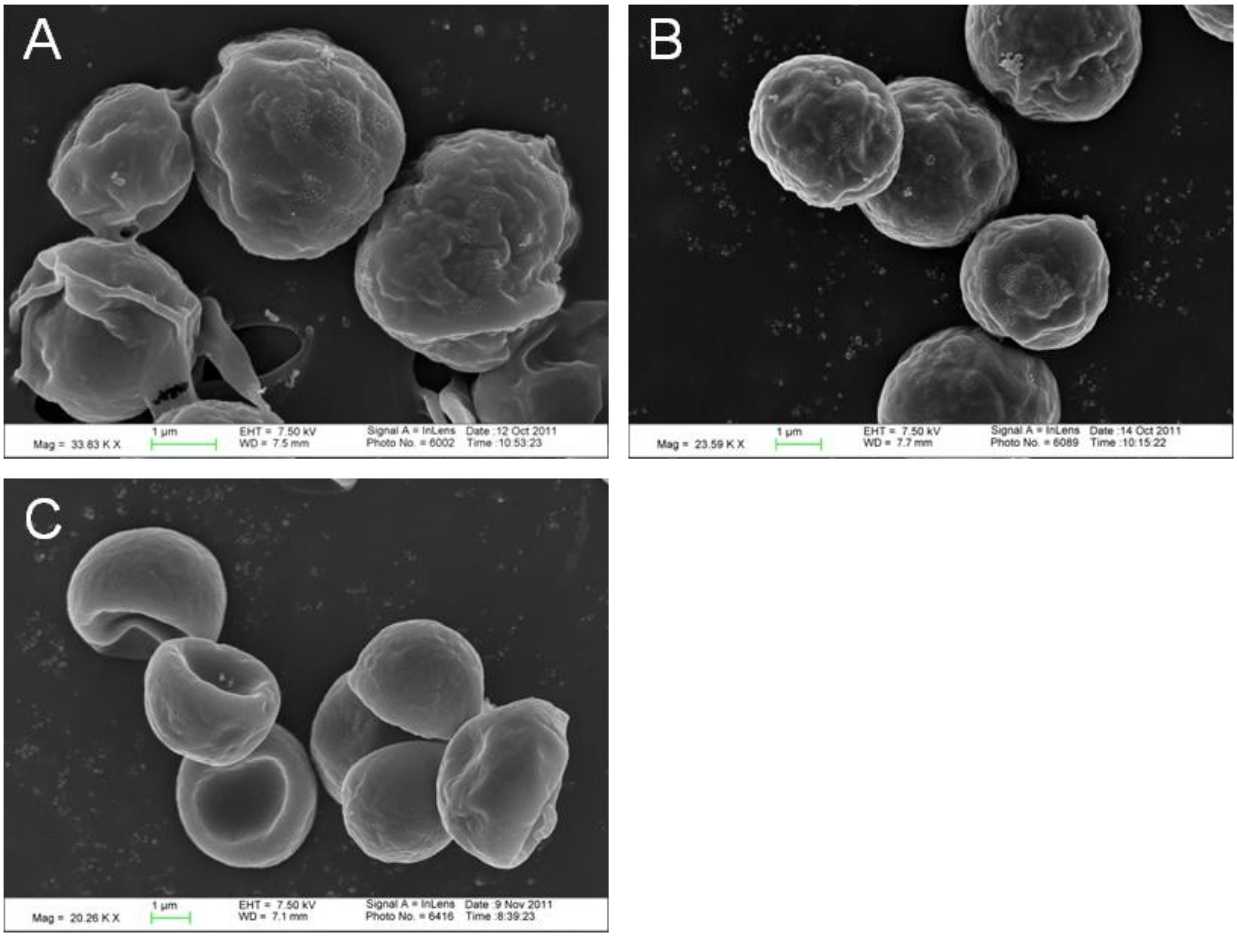

Figure 14. SEM images of *Chlorella* before exposure to solvent (A), and at 1 day contact (B) and 28 days contact (C) for the 100 mL/min treatment for Experiment M-8. Cells before solvent exposure and after 1 day of contact were rough and spherical, and at 28 days the cells assumed an indented, raspberry-like shape.

3.2.2 Analysis of Total Lipids

Table 10 shows the total lipids in the solvent and cells for the long term milking experiments. Lipids were extracted to the solvent phase in all treatments. For *Chlorella* the range of total solvent lipids concentrations was 1,133–4,720 mg/L; for *Ankistrodesmus* the range was 441–1,342 mg/L. The total cellular lipids concentrations did not vary as substantially as the solvent phase concentrations. For *Chlorella* cellular lipid concentrations ranged from 48–209 mg/L, and for *Ankistrodesmus* the range was 15-23 mg/L. In Experiment M-5, pumping rate had a large effect on the concentration of lipids in the solvent phase, and the highest pumping rate resulted in the largest solvent lipid concentration (4,720 mg/L). Interestingly, the next largest concentration (2,440 mg/L) in M-5 was from the lowest pumping rate, 25 mL/min. Feeding nitrogen and phosphorous at different rates (Experiment M-6) did not have a noticeable effect on solvent lipid concentrations, which varied between 1,940–2,042 mg/L in those experiments. TSS, however, was increased by feeding nitrogen at the maximum rate (7.5 mg/L), resulting in a TSS of 236 mg/L, about 20% larger than when no nutrients were added with make-up water. Intermittent mixing (Experiment M-7) did not result in large differences in solvent lipids concentrations for dark mixing period (1,302 mg/L) and light mixing period (1,670 mg/L) treatments. When the concentration of nitrogen and phosphorous was doubled in the media (Experiment M-8), the concentration of lipids in the solvent phase (1,133–3,100 mg/L) did not increase relative to those obtained in M-5, but the cellular lipids did increase (121–209 vs. 75–115 mg/L). The large percentage of lipids in algal solids (39–72% for *Chlorella*) indicates that the use of nitrogen-limiting conditions in the media was effective in inducing lipid accumulation in the cells.

These results indicate that the milking of *Chlorella* was successful. In contrast, even though lipids were extracted to the solvent phase in *Ankistrodesmus* experiments, this cannot be considered successful milking because the population of viable cells collapsed after solvent exposure, as shown above. Table 10 also presents the average lipid milking productivity over the course of the *Chlorella* experiments which ranged from 4.2–16.1 mg/L-d. These values of productivity were comparable to values obtained by (da Silva et al., 2009) who observed lipid productivities of 4 mg/L d for *Neochloris oleoabundans* and 30 mg/L d for *Scenedesmus obliquus* when grown in sleeve photobioreactors with illumination at 150 μmol/m^2 s. When *N. oleoabundans* was grown in an outdoor raceway pond, the lipid productivity was between 10–30 mg/L-d. It should be noted that the lipid productivity presented in this report represents the amount of lipids milked from the algae into the solvent phase, and does not include any lipids which were accumulated in the algae over the course of the experiment.

Table 10. Results of analyses of total lipids in solvent and cells, TSS and milking productivity for long term milking experiments.

[95% CI denoted with ± value]

Treatment	Solvent total lipids (mg/L)	Cellular total lipids (mg/L)	TSS (mg/L)	Percent lipid content in cells	Recovered Solvent (g)	Lipid milking productivity (mg/L d)
Experiment M-5						
250 mL/min	4,720	76	136	56%	77.92	16.1
100 mL/min	1,590	115 ± 30.4	178	65%	71.90	5.1
50 mL/min	1,432	75 ± 2.9	135	56%	75.97	4.8
25 mL/min	2,440	92 ± 5	160	58%	72.60	7.9
Experiment M-6						
7.5 mg-N/L	2,013	107 ± 12.5	236	45%	86.64	7.4
3.75 mg-N/L	1,940	97 ± 5.7	198	49%	82.52	6.9
0.75 mg-N/L	1,940	97 ± 7.6	188	51%	62.07	5.7
0 mg-N/L	2,042	98 ± 8.6	194	51%	85.45	7.5
Experiment M-7						
control, no solvent	--	50 ± 0	117	43%	--	--
dark mixing period	1,302	48 ± 5	124	39%	71.46	4.2
light mixing period	1,670	55 ± 18.8	111	49%	75.08	5.6
Experiment M-8						
250 mL/min	3,100	169 ± 101	234 ± 19.1	72%	80.77	10.9
100 mL/min	2,890	121 ± 37.3	218 ± 12.6	55%	92.09	11.2
50 mL/min	1,133	209 ± 69.6	346 ± 17.2	61%	91.36	4.4
25 mL/min	1,442	177 ± 25	300 ± 8.3	59%	91.95	5.6
Experiment M-9						
250 mL/min	1,342	15 ± 5.7	40	38%	64.39	--
100 mL/min	901	23 ± 15.2	66	34%	90.75	--
50 mL/min	1,172	22 ± 5	70	31%	82.58	--
Experiment M-10						
dark mixing period	612	--	12	--	90.46	--
dark+light mixing	672	--	4	--	90.43	--
light mixing period	441	--	10	--	90.77	--

The milking of *Chlorella* was able to extract and concentrate lipids in the solvent phase by a factor of 5–62 times the concentration in the algal suspension. A more in depth analysis of the extraction of lipids was performed to account for the total mass of lipids in the volumes of solvent and algal suspensions and more fully characterize the extraction efficiency of milking the cells. Figure 15 presents the total masses of lipids in the solvent and cells, with corresponding percentages of lipids extracted to the solvent phase for Experiments M-5 through M-8. A broad range in the fraction of lipids in the system were extracted to the solvent; 37–86%, but in most cases, the majority of lipids were extracted by the solvent. Figure 15C shows that the total mass of lipids in the cells was about the same for the control (no solvent; 0.075 g) and the solvent-contacted samples (0.072–0.082 g), but that an additional larger amount was present in the solvent phase (0.176–0.234 g). Thus, at least an additional 2.4 times more total lipids were present after solvent contact, indicating that milking stimulates the productivity of lipids in *Chlorella*.

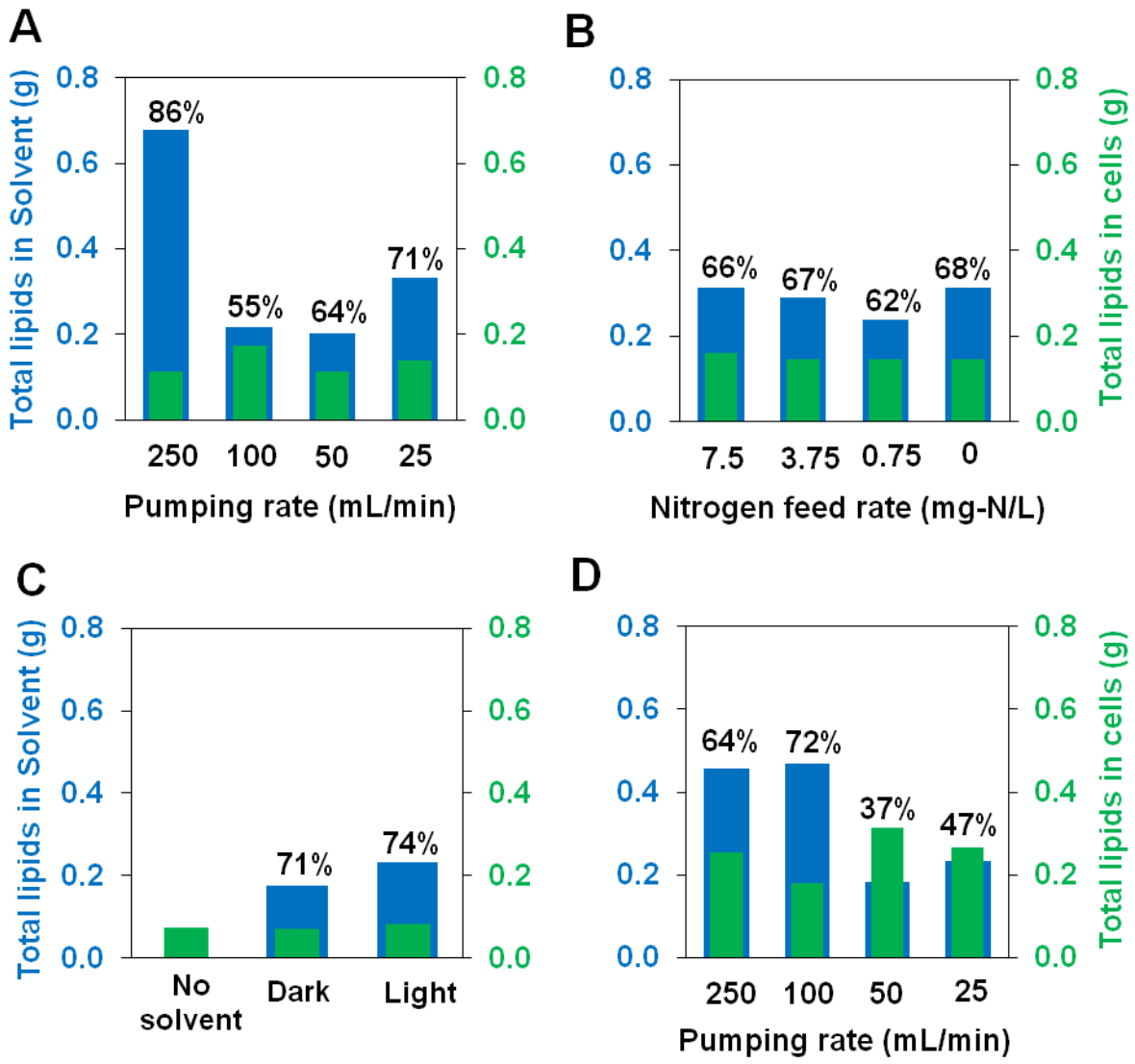

Figure 15. Total mass of lipids in the solvent and aqueous (cell) phases, with percentage extraction of lipids to solvent phases noted. Results from (A) M-5, (B) M-6 and(C) M-7 show that the majority (at least 55%) of the total lipids in the system were extracted to the solvent phase. In M-8 (D) the two lowest pumping rates resulted in less effective extraction (37–47%).

Figure 16A (and Table 19 and Table 20, Appendix) present the time series of total lipid concentrations in the solvent phase for each treatment in Experiment M-8. In general, there was a lag in the milking of lipids from the algae during the first three days of the experiment where the total lipids concentration was ≤3 mg/L, except for the 250 mL/min pumping rate which had

reached 242 mg/L by day 2. After the first 3 days, the lipid concentration increased and reached maxima at 28 days of the experiment. The temporary decline in the lipid concentrations between days 14–21 for the 250 and 50 mL/min pumping rates is likely due to analytical uncertainty associated with assignment of peaks to external standard correlations. Linear regression (intercept = 0) yielded solvent lipid accumulation rates shown in Table 11 and Figure 16B, which were 108.4 and 110.0 mg/L-d for 250 and 100 mL/min pumping rates, respectively, and 40.6 and 46.7 mg/L-d for 50 and 25 mL/min pumping rates, respectively. The results of *t*-tests (Table 12) showed that the two highest (250 and 100 mL/min, p=0.85) and two lowest pumping rates (50 and 25 mL/min, p=0.30) yielded statistically indistinguishable solvent lipid accumulation rates at a 95% confidence level. However, the solvent lipid accumulation rates for the 250 and 100 mL/min pumping rates were each significantly different from the solvent lipid accumulation rates for both the 50 and 25 mL/min pumping rates, as *t*-tests resulted in p-values $\leq 3.4 \times 10^{-7}$.

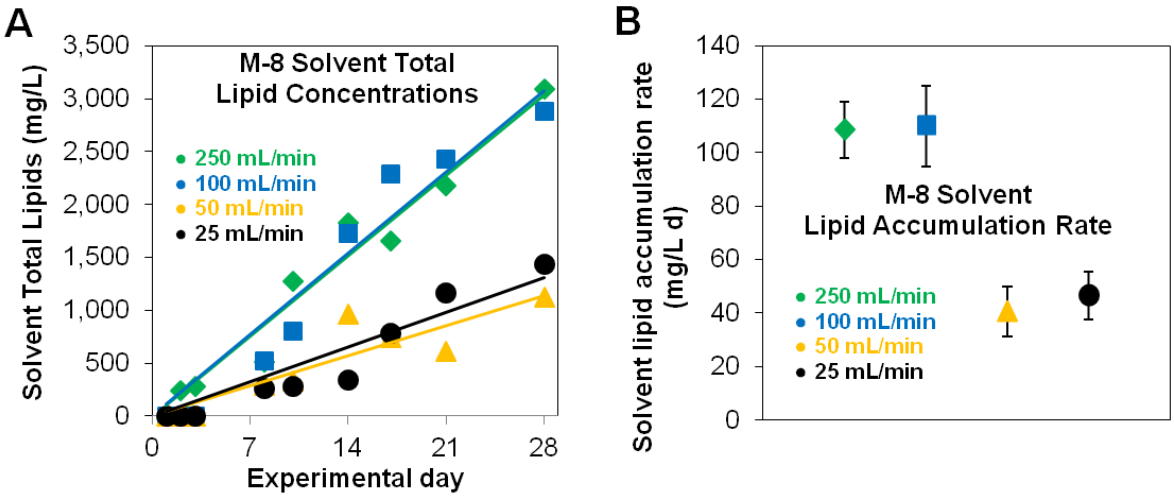

Figure 16. (A) The solvent lipid concentrations for Experiment M-8, and (B) the resulting solvent lipid accumulation rates. Error bars represent the 95% CI. The rates of accumulation were statistically indistinguishable between the 100 and 250 mL/min and the 25 and 50 mL/min treatments, but were significantly different in pair-wise comparisons between the two largest and two smallest pumping rates.

Table 11. Results of linear regression for the solvent lipid accumulation rates in Experiment M-8.

[Slope, solvent lipid accumulation rate; SE, standard error; DF, degrees of freedom]

Regression parameter estimate	Pumping rate (mL/min)			
	250	100	50	25
Slope (mg/L-d)	108.4	110.0	40.6	46.7
r^2	0.986	0.972	0.926	0.948
SE_{slope} (mg/L-d)	4.59	6.56	4.06	3.86
DF	8	8	8	8

Table 12. Matrix of *p*-values from *t*-tests comparing the solvent lipid accumulation rates for different pumping rates in Experiment M-8.

[Underlined *p*-values indicate statistical significance]

***p*-value by comparison of pumping rates (mL/min)**				
	250	**100**	**50**	**25**
250	--	0.85	6.6E-09	1.8E-08
100	--	--	1.2E-07	3.4E-07
50	--	--	--	0.30
25	--	--	--	--

3.2.3 Lipid Groups and Identified Lipids

The most prevalent class of lipids measured in solvent samples were free fatty acids (FFA, Lipid Group 2) (Table 17 and Table 18, Appendix) which comprised between 37.4–95.6% of the total lipids in the solvent phase. Reasonable fractions of the total lipid content also were typically found in Lipid Group 1 (MG & FFA; 3.1–27.5%) and Lipid Group 4 (DG & TG; 3.5–19.4%), except for experiments with *Ankistrodesmus* and in the 25 mL/min pumping rate of Experiment M-5. Large fractions (13.9–36.1%) of TG (Lipid Group 5) were only found in Experiments M-5, M-6 and M-8. The lipid samples extracted from the cells in the aqueous phase

were analyzed for composition following gravimetric analysis. For comparison of solvent and cellular lipids, the percent compositions of Lipid Groups 1 and 2 (MG & FFA) and Lipid Groups 3, 4 and 5 (DG & TG) were aggregated.

Table **13** presents the results of the aggregated lipid compositions. The MG & FFA aggregate values ranged from 44.4–99.9% of lipids in the solvent phase and comprised the majority of solvent lipids for all but one treatment (44.4%, 25 mL/min, M-8). In contrast, MG & FFA were a minority of cellular lipids and ranged from 0–44.1% of lipids in cells.

Table 13. Comparison of aggregated lipid class compositions of cellular and solvent phase lipids

[MG, monoglycerides; FFA, free fatty acids; DG, diglycerides; TG, triglycerides; S/C ratio, ratio of MG & FFA in solvent phase to MG & FFA in cells]

Treatment	Cellular lipids		Solvent lipids		S/C Ratio
	MG & FFA	DG & TG	MG & FFA	DG & TG	
Experiment M-5					
250 mL/min	44.1%	55.9%	64.0%	36.0%	1.45
100 mL/min	28.0%	72.0%	64.8%	35.2%	2.31
50 mL/min	24.7%	75.3%	70.5%	29.5%	2.85
25 mL/min	22.9%	77.1%	99.5%	0.5%	4.35
Experiment M-6					
7.5 mg-N/L	25.1%	74.9%	76.0%	24.0%	3.03
3.75 mg-N/L	32.4%	67.6%	73.7%	26.3%	2.28
0.75 mg-N/L	30.0%	70.0%	69.6%	30.4%	2.32
0 mg-N/L	29.5%	70.5%	74.4%	25.6%	2.52
Experiment M-7					
dark mixing period	17.1%	82.9%	89.9%	10.1%	5.26
light mixing period	20.8%	79.2%	85.0%	15.0%	4.09
Experiment M-8					
250 mL/min	6.5%	93.5%	65.8%	34.2%	10.0
100 mL/min	12.2%	87.8%	54.7%	45.3%	4.48
50 mL/min	9.0%	91.0%	54.7%	45.3%	6.09
25 mL/min	9.9%	90.1%	44.4%	55.6%	4.46
Experiment M-9					
250 mL/min	0.0%	100.0%	99.9%	0.1%	--
100 mL/min	0.0%	100.0%	99.9%	0.1%	--
50 mL/min	0.0%	100.0%	99.8%	0.2%	--

The unequal distribution of the lipid group compositions between the cells and solvent phases can be quantified by computing the ratio between the solvent phase MG & FFA composition and the cellular MG & FFA composition (S/C ratio). Values of S/C ratio greater than 1 mean that MG & FFAs tend to be extracted to the solvent phase at a greater rate than DG & TG.

Table **13** shows that the values of the S/C ratio ranged from 1.45 (M-5, 250 mL/min) to 10.0 (M-8, 250 mL/min), and Figure 17 shows the aggregate cellular and solvent lipid compositions corresponding to these cases. Experiment M-8 used media that had twice the concentration of N and P so it is likely that, after inoculation, cells approached stationary phase at a later time in the experiment relative to the cells in Experiment M-5. This may explain the much larger S/C ratio observed for the 250 mL/min pumping rate in M-8 (Figure 17C-D) when compared to the same pumping rate in M-5 (Figure 17A-B). Overall, these results indicate that MG & FFAs are preferentially extracted from cells during the milking process.

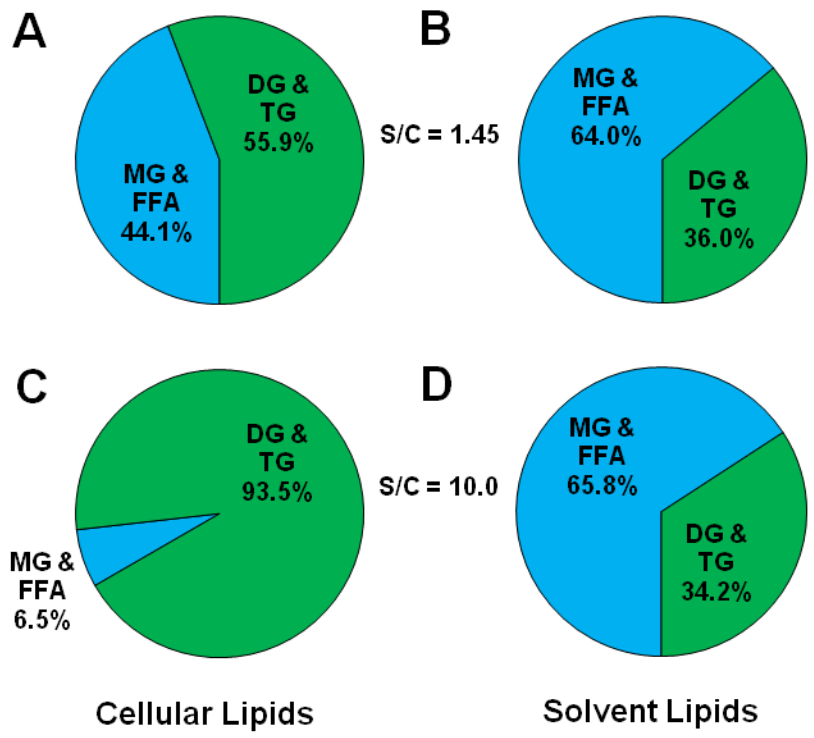

Figure 17. Aggregated lipid compositions for *Chlorella* treatments with 250 mL/min pumping rates. In Experiment M-5 the cellular lipids (A) and solvent phase lipids (B) had aggregate percent composition of MG & FFAs that displayed the minimum S/C ratio determined in all experiments (S/C = 1.45). The cellular composition (C) and solvent composition (D) in Experiment M-8 (when the concentration of N and P in the media was doubled) resulted in the maximum S/C ratio (S/C = 10.0).

Table 14 presents a summary of the lipid compounds identified in 52 analyses of the solvent phase. Of the 13 compounds identified, 4 different compounds with the linolenic fatty acid group (18:3) were found, and altogether were detected most frequently. Other compounds frequently detected with the number of detections noted included: monopalmitin (21), oleic acid (8), stearic acid (13), trilinolein (9) and tripalmitolein (6). With the exception of trilinolein, these compounds were also detected at the highest concentrations in the set of identified compounds. The maximum detected concentrations were 570 mg/L for monopalmitin, 280 mg/L for oleic

acid, 1,830 mg/L for stearic acid and 99 mg/L for tripalmitolein. The concentration of compounds of linolenic acid were detected at relatively small concentrations, ≤45 mg/L.

Table 14. Summary of compounds identified in 52 lipid analyses of solvent phase in long term milking experiments.

[ND, number of detections; Min, minimum concentration; Med, median concentration; Max, maximum concentration; --, not applicable]

Compound	ND	Min (mg/L)	Med (mg/L)	Max (mg/L)
Monolinolenin	46	<1	4	45
Linolenic acid	13	17	20	42
Monopalmitin	21	<1	74	570
Oleic acid	8	46	114	280
Palmitic acid	1	--	--	240
Stearic acid	12	430	1,075	1,830
Dilinolenin	14	<1	1	9
Dipalmitin	1	--	--	75
Trilinolenin	13	1	1	3
Distearin	3	43	74	85
Trilinolein	9	1	6	12
Triolein	1	--	--	6
Tripalmitolein	6	16	69	99
Totals	148	--	--	--

3.2.4 Retention of Nutrients

The nutrients nitrogen (N), phosphorous (P), total dissolved inorganic carbon, and total dissolved organic carbon were analyzed for Experiments M-5 through M-8 (Table 21, Appendix) and the percent retention of total-N and total-P were characterized for the algal suspension. Figure 18A-D presents the percent retention of total-N and total-P at 28 days for Experiments M-

5 through M-8. Between 55–85% of total-N was retained, and between 66–98% of total-P was retained. Figure 18A shows that the higher pumping rates (250–100 mL/min) in Experiment M-5 resulted in larger retention of N and P in comparison to the lower pumping rates, 25–50 mL/min. The increased pumping likely increased the amount of algae that remained suspended as opposed to forming biofilms on reactor system surfaces. In Experiment M-8 (Figure 18D) the 25, 50 and 250 mL/min treatments had relatively similar retention of nutrients, and the 100 mL/min treatment had a slightly decreased retention of both N and P. This is again likely due to biofilm development, as portions of the reactor surface had attached algal growth for the 100 mL/min treatment, which was noticeably larger than those observed in the other treatments. Figure 18B shows increased feeding of nutrients tended to decrease the amount of nutrients retained in the algal suspension, which may largely be due to increased formation of biofilms. The 100 mL/min and 0 mg-N/L treatments in Figure 18A and B, respectively, are temporal replicates, and these had similar retention of nutrients (77–80% N-retention, 98% P-retention). Experiment M-7 (Figure 18C) had similar retention of N and P for all treatments. The control treatment for this experiment was an algal suspension without solvent contact and the fact that the retention of N (59%) and P (78%) for this treatment was similar to the solvent-contacted treatments (N, 58-61%; P, 69-76%) indicates that little of the total mass of these nutrients leave the algal suspension through extraction of N- or P-containing compounds. Overall, the majority of nutrients were retained in the algal suspensions by the end of the experiments, and nutrient addition during milking did not enhance the proportion of total-N or P that were retained in algal suspensions.

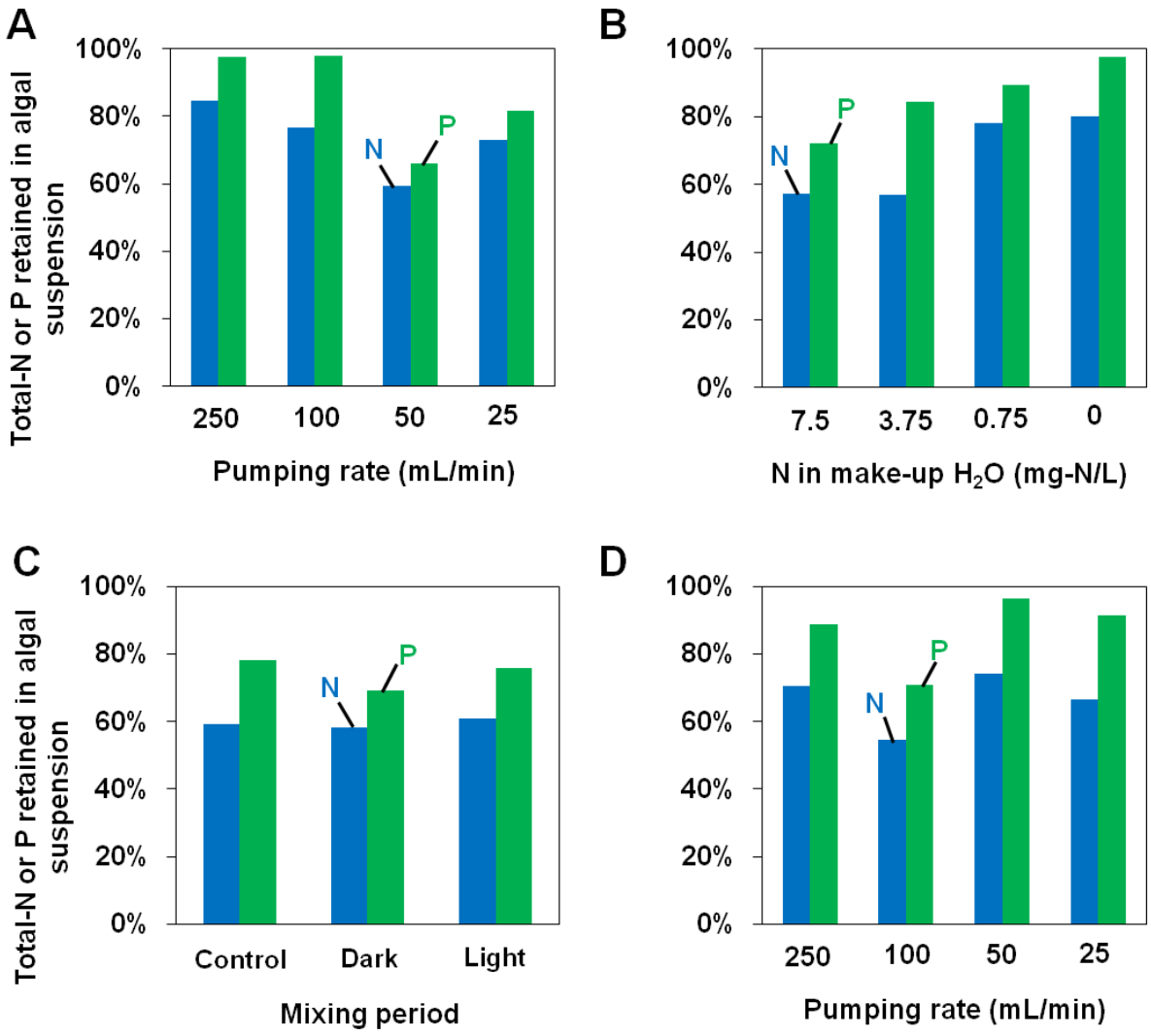

Figure 18. Retention of total nitrogen (N) and total phosphorous (P) in algal suspensions at day 28 for Experiments (A) M-5, (B) M-6, (C) M-7 and (D) M-8.

4. CONCLUSIONS

The results of this study demonstrate the feasibility of milking the green alga *Chlorella kessleri* for production of lipids for biofuels production. *Chlorella* maintained viable cell populations throughout 28 days of solvent (n-dodecane) exposure, and lipids were extracted and concentrated into the solvent phase at concentrations between 1,113–4,720 mg/L. The productivity of lipid milking was between 4.2–16.1 mg/L-d. Nutrients were largely retained in the algal suspensions, and the nitrogen-limiting conditions of the media induced a large percentage of lipid accumulation (39–72%) in the algae. Even though *Ankistrodesmus* was viable in the presence of n-dodecane and biodiesel solvents for 4 days, the long term exposure of the algae to dodecane in the milking bioreactor resulted in the collapse of viable cell populations.

Milking with n-dodecane preferentially extracts monoglycerides and free fatty acid lipid compounds. This has implications for downstream processing of lipid products as free fatty acids require catalytic methods different from those typically used for conversion of vegetable oils to biodiesel. Because milking of *Chlorella* for biodiesel production was demonstrated to be feasible, it is recommended that further investigation be performed. Possibilities for scale up of the process, critical analysis of energy balances, and lipid purification methods should be evaluated.

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APPENDIX

Table 15. Concentration of nutrient compounds in WC media

Compound	Chemical formula	Concentration	
		mg/L	µM
N:P-5 media (nitrogen-limiting)			
Sodium Nitrate	$NaNO_3$	21.25	250
Potassium Phosphate Dibasic	K_2HPO_4	8.71	50
2x [N:P-5] media (nitrogen-limiting)			
Sodium Nitrate	$NaNO_3$	42.51	500
Potassium Phosphate Dibasic	K_2HPO_4	17.42	100
N:P-50 media (nitrogen sufficient)			
Sodium Nitrate	$NaNO_3$	212.53	2500
Potassium Phosphate Dibasic	K_2HPO_4	8.71	50
Other nutrients, all media			
Calcium Chloride Dihydrate	$CaCl_2 \cdot 2H_2O$	36.76	250
Magnesium Sulfate Heptahydrate	$MgSO_4 \cdot 7H_2O$	36.97	150
Sodium Bicarbonate	$NaHCO_3$	12.60	150
Sodium meta-Silicate Nonahydrate	$Na_2SiO_3 \cdot 9H_2O$	28.42	100
Boric Acid	H_3BO_3	6.00	97
Cupric (II) Sulfate Pentahydrate	$CuSO_4 \cdot 5H_2O$	9.8E-03	3.9E-02
Zinc Sulfate Heptahydrate	$ZnSO_4 \cdot 7H_2O$	2.2E-02	7.7E-02
Cobalt Chloride Hexahydrate	$CoCl_2 \cdot 6H_2O$	1.0E-02	4.2E-02
Manganese (II) Chloride Tetrachloride	$MnCl_2 \cdot 4H_2O$	1.8E-01	9.1E-01
Sodium Molybdate Dihydrate	$Na_2MoO_4 \cdot 2H_2O$	6.3E-03	2.6E-02
Iron (III) Chloride Hexahydrate	$FeCl_3 \cdot 6H_2O$	2.7E-01	1.0E+00

Table 16. Cellular lipids, total suspended solids, and percent lipid content of algal suspension for biocompatibility experiments

[95% CI denoted by ± value]

Experiment	Exp. ID	Treatment	Cellular lipids (mg/L)	TSS (mg/L)	Percent lipid content in cells
Ankistrodesmus +dodecane	B-1	Feed	19.5 ± 4.3	91 ± 2.2	21.4%
		Solvent	73.2 ± 108.5	336 ± 171	21.8%
		Control	46.2 ± 57.8	207 ± 56.2	22.3%
Ankistrodesmus +biodiesel	B-2	Feed	28 ± 34.4	74.3 ± 7.6	37.7%
		Solvent	303 ± 418	1750 ± 926	17.3%
		Control	86.5 ± 42.1	246 ± 21.1	35.2%
Chlorella +dodecane	B-3	Feed	21.2 ± 5.6	95.8 ± 20.5	22.1%
		Solvent	63 ± 59.3	276 ± 113	22.8%
		Control	54.3 ± 22.4	320 ± 84.3	17.0%
Chlorella +biodiesel	B-4	Feed	22.7 ± 6.1	111 ± 5	20.4%
		Solvent	457 ± 943	2530 ± 2180	18.0%
		Control	55.4 ± 24.5	399 ± 116	13.9%

Table 17. Concentration of lipids in the solvent phase by lipid group and class for long term milking experiments with *Chlorella*.

[MG, monoglycerides; FFA, free fatty acids; DG, diglycerides; TG, triglycerides; Conc., concentration]

Treatment	Solvent parameter	Lipid Group or Class					Total lipids
		1	2	3	4	5	
		MG & FFA	FFA	DG	DG & TG	TG	
Experiment M-5							
250 mL/min	Conc. (mg/L)	710	2,310	190	500	1,010	4,720
	% of total lipids	15.0%	48.9%	4.0%	10.6%	21.4%	
100 mL/min	Conc. (mg/L)	430	600	30	180	350	1,590
	% of total lipids	27.0%	37.7%	1.9%	11.3%	22.0%	
50 mL/min	Conc. (mg/L)	200	810	2	100	320	1,432
	% of total lipids	14.0%	56.6%	0.1%	7.0%	22.3%	
25 mL/min	Conc. (mg/L)	150	2,280	2	0	10	2,440
	% of total lipids	6.1%	93.4%	0.1%	0.0%	0.4%	
Experiment M-6							
7.5 mg-N/L	Conc. (mg/L)	100	1,430	3	200	280	2,013
	% of total lipids	5.0%	71.0%	0.1%	9.9%	13.9%	
3.75 mg-N/L	Conc. (mg/L)	60	1,370	30	190	290	1,940
	% of total lipids	3.1%	70.6%	1.5%	9.8%	14.9%	
0.75 mg-N/L	Conc. (mg/L)	100	1,250	50	200	340	1,940
	% of total lipids	5.2%	64.4%	2.6%	10.3%	17.5%	
0 mg-N/L	Conc. (mg/L)	80	1,440	2	180	340	2,042
	% of total lipids	3.9%	70.5%	0.1%	8.8%	16.7%	
Experiment M-7							
dark mixing period	Conc. (mg/L)	40	1,130	2	100	30	1,302
	% of total lipids	3.1%	86.8%	0.2%	7.7%	2.3%	
light mixing period	Conc. (mg/L)	460	960	40	140	70	1,670
	% of total lipids	27.5%	57.5%	2.4%	8.4%	4.2%	
Experiment M-8							
250 mL/min	Conc. (mg/L)	260	1,780	210	110	740	3,100
	% of total lipids	8.4%	57.4%	6.8%	3.5%	23.9%	
100 mL/min	Conc. (mg/L)	460	1,120	40	390	880	2,890
	% of total lipids	15.9%	38.8%	1.4%	13.5%	30.4%	
50 mL/min	Conc. (mg/L)	110	510	3	120	390	1,133
	% of total lipids	9.7%	45.0%	0.3%	10.6%	34.4%	
25 mL/min	Conc. (mg/L)	100	540	2	280	520	1,442
	% of total lipids	6.9%	37.4%	0.1%	19.4%	36.1%	

Table 18. Concentration of lipids in the solvent phase by lipid group and class for long term milking experiments with *Ankistrodesmus*.

[MG, monoglycerides; FFA, free fatty acids; DG, diglycerides; TG, triglycerides; Conc., concentration]

Treatment	Solvent parameter	Lipid Group or Class					Total lipids
		1	2	3	4	5	
		MG & FFA	FFA	DG	DG & TG	TG	
Experiment M-9							
250 mL/min	Conc. (mg/L)	120	1,220	2	0	0	1,342
	% of total lipids	8.9%	90.9%	0.1%	0.0%	0.0%	
100 mL/min	Conc. (mg/L)	40	860	1	0	0	901
	% of total lipids	4.4%	95.4%	0.1%	0.0%	0.0%	
50 mL/min	Conc. (mg/L)	50	1,120	2	0	0	1,172
	% of total lipids	4.3%	95.6%	0.2%	0.0%	0.0%	
Experiment M-10							
dark mixing	Conc. (mg/L)	30	580	1	0	1	612
period	% of total lipids	4.9%	94.8%	0.2%	0.0%	0.2%	
dark+light	Conc. (mg/L)	30	640	1	0	1	672
mixing	% of total lipids	4.5%	95.2%	0.1%	0.0%	0.1%	
light mixing	Conc. (mg/L)	30	410	0	0	1	441
period	% of total lipids	6.8%	93.0%	0.0%	0.0%	0.2%	

Table 19. Solvent concentrations of lipids for periodic sampling of 250 and 100 mL/min treatments in Experiment M-8.

Exp. Day	Treatment											
	250 mL/min						100 mL/min					
	Lipid Group					Total	Lipid Group					Total
	1	2	3	4	5		1	2	3	4	5	
	Solvent lipid concentration (mg/L)						Solvent lipid concentration (mg/L)					
1	1	0	0	0	0	1	1	0	0	0	0	1
2	2	240	0	0	0	242	1	0	0	0	0	1
3	2	280	0	0	0	282	1	0	0	0	0	1
8	3	390	0	5	120	518	2	330	0	7	190	529
10	270	510	0	90	410	1,280	2	260	0	150	390	802
14	270	1,250	6	3	300	1,829	570	410	3	240	510	1,733
17	170	960	1	120	410	1,661	740	690	6	200	660	2,296
21	240	1,240	80	60	560	2,180	90	1,280	5	230	830	2,435
28	260	1,780	210	110	740	3,100	460	1,120	40	390	880	2,890

Table 20. Solvent concentrations of lipids for periodic sampling of 50 and 25 mL/min treatments in Experiment M-8.

Exp. Day	Treatment											
	50 mL/min						25 mL/min					
	Lipid Group					Total	Lipid Group					Total
	1	2	3	4	5		1	2	3	4	5	
	Solvent lipid concentration (mg/L)						Solvent lipid concentration (mg/L)					
1	2	0	0	0	0	2	1	0	0	0	0	1
2	2	0	0	0	0	2	1	0	0	0	0	1
3	3	0	0	0	0	3	1	0	0	0	0	1
8	6	290	0	0	0	296	2	260	0	0	0	262
10	7	330	0	0	0	337	2	280	0	0	0	282
14	610	360	0	0	0	970	3	320	0	5	20	348
17	680	70	0	0	0	750	380	60	0	3	340	783
21	100	400	0	5	110	615	490	60	3	250	370	1,173
28	110	510	3	120	390	1,133	100	540	2	280	520	1,442

Table 21. Concentrations of nutrients analyzed in long term milking experiments.

[TN, total nitrogent; TDN, total dissolved nitrogent; TP, total phosphorous; TDP, total dissolved phosphorous; TDIC, total dissolved inorganic carbon; TDOC, total dissolved organic carbon; Inoculum, reserve culture used to inoculate experimental media; --, not analyzed or not available]

Treatment or sample	TN (mg/L)	TDN (mg/L)	TP (mg/L)	TDP (mg/L)	TDIC (ppm)	TDOC (ppm)
Experiment M-5						
250 mL/min	2.72	0.53	1.38	0.27	0.38	105
100 mL/min	2.46	0.35	1.39	0.07	3.86	62
50 mL/min	1.90	<0.2	0.94	0.03	6.60	41
25 mL/min	2.34	0.33	1.16	0.02	5.81	37
Incoculum	4.39	--	1.31	--	--	--
Experiment M-6						
7.5 mg-N/L	3.70	<0.2	2.07	0.09	5.91	68
3.75 mg-N/L	2.60	<0.2	1.81	0.12	5.17	64
0.75 mg-N/L	2.77	<0.2	1.50	0.06	4.45	63
0 mg-N/L	2.56	<0.2	1.39	0.05	4.38	85
Incoculum	2.85	--	1.72	--	--	--
Experiment M-7						
control-no solvent	1.90	0.21	1.11	0.31	--	--
dark mixing period	1.87	0.14	0.98	0.04	--	--
light mixing period	1.95	0.08	1.08	0.20	--	--
Incoculum	3.33	--	1.75	--	--	--
Experiment M-8						
250 mL/min	4.18	0.07	2.33	0.36	0.04	130
100 mL/min	3.21	--	1.85	0.07	5.60	57
50 mL/min	4.43	<0.1	2.55	0.03	8.00	39
25 mL/min	3.98	--	2.42	0.04	7.90	40
Incoculum	1.98	--	1.65	--	--	--

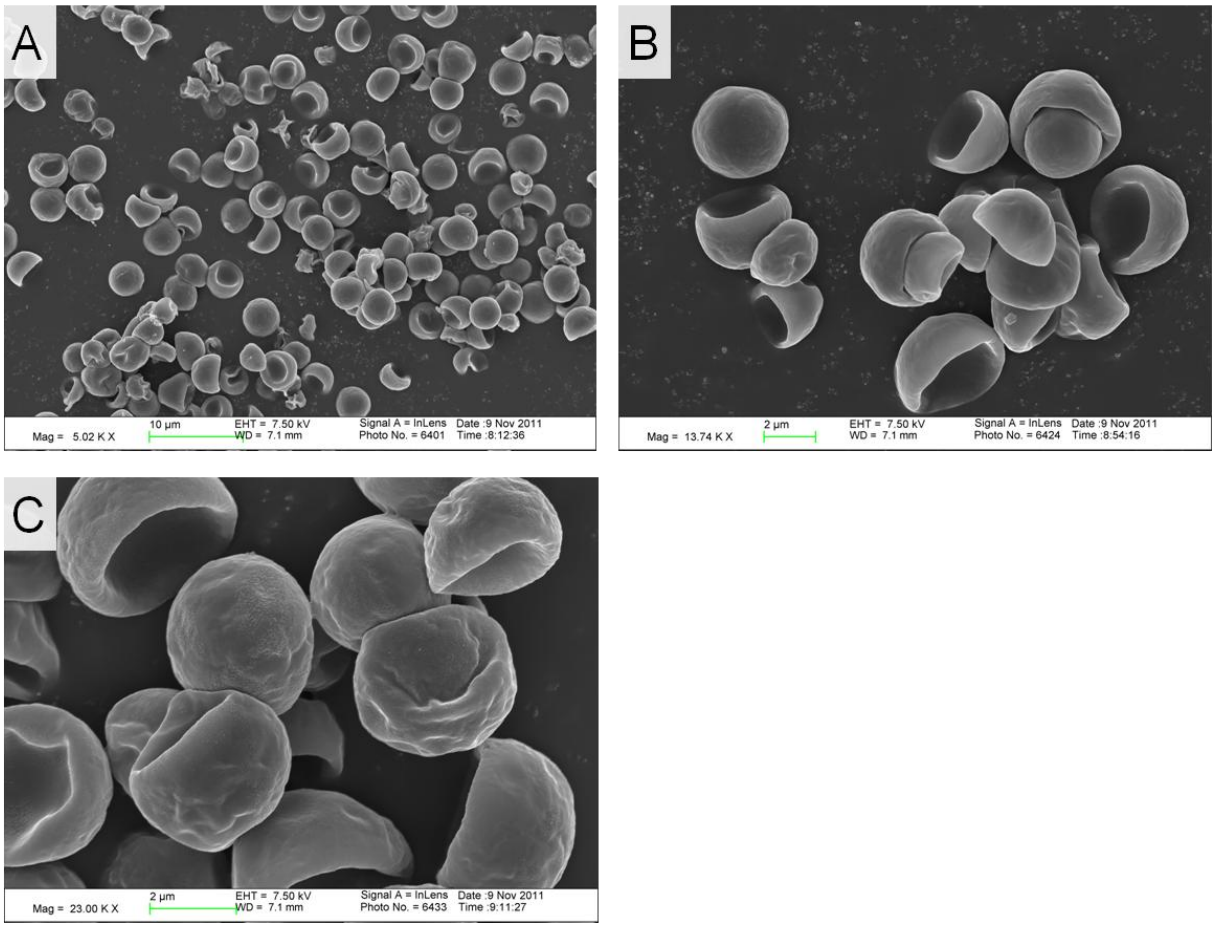

Figure 19. SEM images of *Chlorella* cells in Experiment M-8 after 28 days of solvent contact. Increasing magnification is presented for the 250 mL/min (A), 50 mL/min (B), and 25 mL/min (C) pumping rates. The typical cell morphology after 4 weeks of solvent contact was an indented, raspberry-like shape.