Promoting Lipid Accumulation in *Chlorella vulgaris* UTEX395 Using Nitrogen Limitation and Bicarbonate Amendment

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Introduction/Rationale

- The sufficient supply of water and nutrients have been identified as potentially limiting factors in the large scale production of algal biofuels.
- Nitrogen fertilizer production is energy intensive and may compromise the energy balance for biofuel production.
- The use of low quality or recycled water and nutrient sources for algae cultivation is of interest.
- The primary inorganic nitrogen forms of interest are nitrate, ammonium, and urea.
  - Nitrate has received the most attention in algal biofuel research, but is likely the most cost prohibitive of the three.
  - Ammonium and urea are also of interest as they are the dominant forms of inorganic nitrogen in many waste streams (municipal wastewater, piggery wastewater).

Objectives

- Assess the growth and lipid accumulation of *Chlorella vulgaris* UTEX 395 when cultured using different nitrogen sources (nitrate, urea, ammonium, and a combination of all three).
- Evaluate effect of nitrogen stress and bicarbonate amendment at the time of nitrogen stress on lipid accumulation of C. *vulgaris* UTEX 395 under different nitrogen growth conditions.

Methods

- *C. vulgaris* UTEX 395 was cultured under eight conditions (Table 1) using Bold's Basal Medium modified with three different nitrogen sources.
- The initial nitrogen concentration for all conditions was approximately 40mg-N/L.
- The cultures were grown in 1.25L tube reactors (Figure 1) with a 14h:10h light:dark cycle.
- All reactor systems were aerated at 0.4LPM. During the light period, prior to nitrate depletion, the air being supplied to all conditions was supplemented continuously with 5%(v/v) CO_2.
- The ammonium control and bicarbonate conditions were pH controlled at pH 6.8 using KOH.

Condition	Bicarbonate Amendment
Nitrate Control	None
Nitrate Bicarbonate	50mmol at N depletion
Urea Control	None
Urea Bicarbonate	50mmol at N depletion
Ammonium Control	None
Ammonium Bicarbonate	50mmol at N depletion
Mixed Nitrogen Control	None
Mixed Nitrogen Bicarbonate	50mmol at N depletion

Table 1. Eight experimental conditions.

Figure 1. Experimental reactor setup.

Parameter	Frequency	Method	Source
Cell concentration	2x daily	Direct cell counts	
Optical density (440nm, 700nm)	2x daily	Spectrophotometry	
Nitrate concentration	2x daily	NAS Schzechrome	Polysciences, Inc.
Ammonium concentration	2x daily	2-phenylphenol	(Rhine & Mulvaney
Urea concentration	2x daily	Modified Jung	(Zawada et al., 2009)
Total nitrogen concentration	Every 3 days	Hach Test'N'Tube Total Nitrogen Kit	Hach Company
Chlorophyll	2x daily	Hot methanol extraction	(Ördög et al., 2012)
Lipid type and concentration	Before bicarbonate amendment and at time of harvest.	Transesterification followed by GC-MS analysis	(Lohman et al., 2013); Agilent 6890N and Agilent 5973N

Table 2. Parameters evaluated during this study.

Results

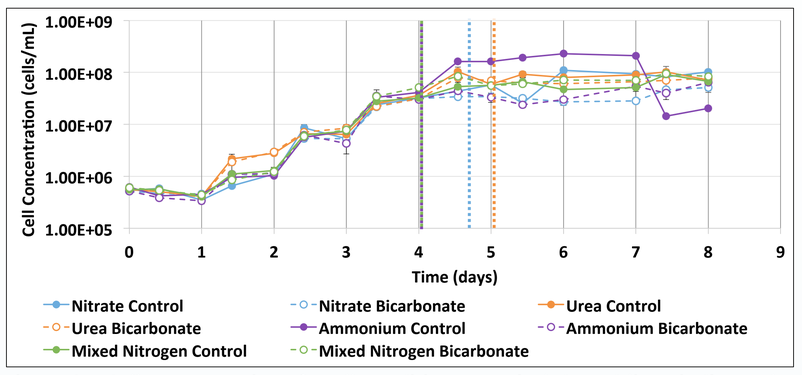

Figure 2. Cell concentrations for all conditions. Vertical dotted lines indicate the time of the bicarbonate amendment for each of the conditions. Blue=nitrate; orange=urea; purple=ammonium; green=mixed nitrogen (The purple and green dashed lines are overlapping). Error bars represent data range for each of the conditions.

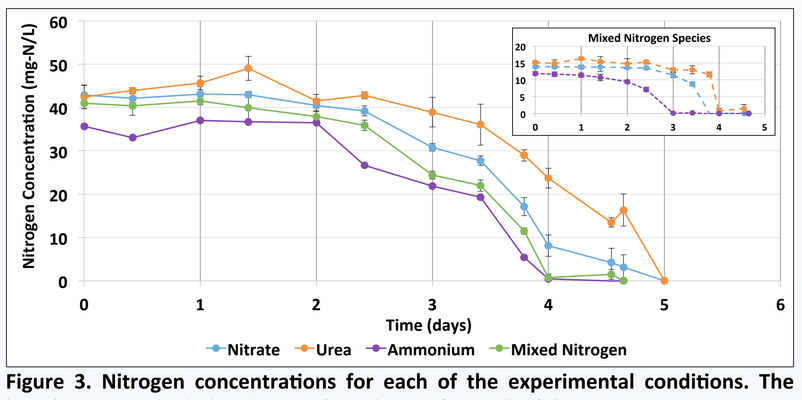

Figure 3. Nitrogen concentrations for each of the experimental conditions. The bicarbonate amended and control conditions for each of the nitrogen scenarios are plotted together. Error bars represent the range of experimental data for each of the nitrogen conditions. The inset graph presents a breakdown of nitrogen speciation for the mixed nitrogen conditions.

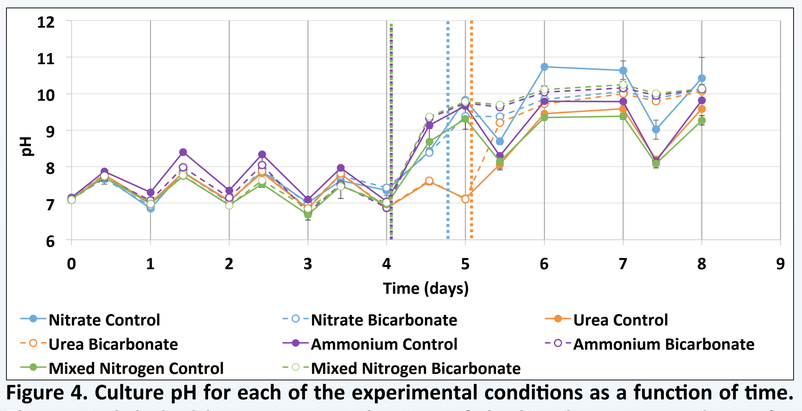

Figure 4. Culture pH for each of the experimental conditions as a function of time. The vertical dashed lines represent the time of the bicarbonate amendment for each of the bicarbonate amended conditions. Blue=nitrate; orange=urea; purple=ammonium; green=mixed nitrogen (The purple and green dashed lines are overlapping). Error bars represent data range for each of the conditions.

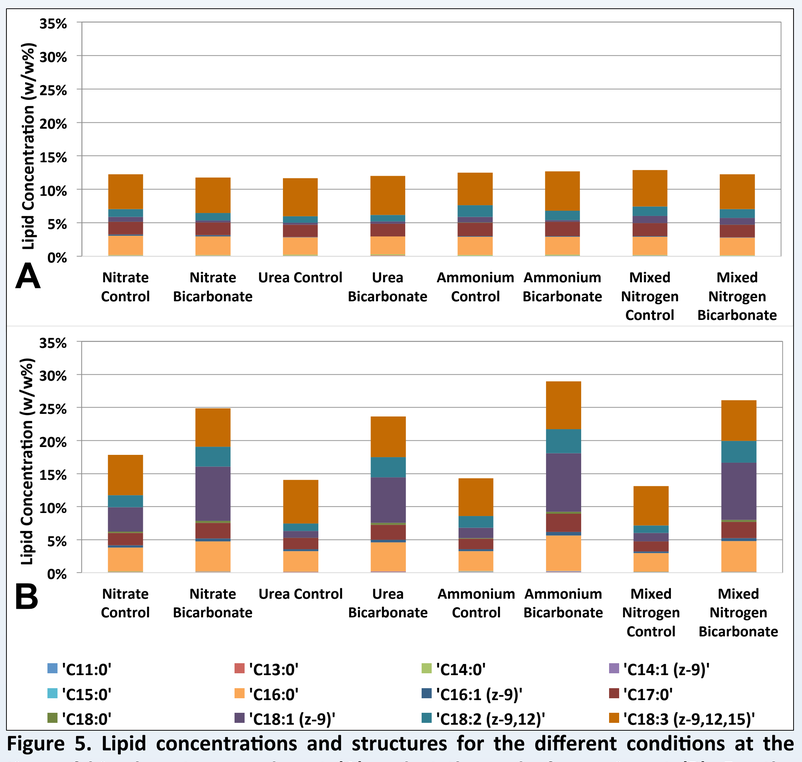

Figure 5. Lipid concentrations and structures for the different conditions at the time of bicarbonate amendment (A) and at the end of experiment (B). For the ammonium and mixed nitrogen conditions the data are for 4d 2h (A) and 7d 2h(B) after the inoculation of the reactors. For the nitrate condition the data are for 5d 2h (A) and 8d 2h(B) after inoculation. For the urea condition the data are for 4d 17h (A) 9d 20h. Lipid concentrations are in weight lipid per weight dry biomass percentages.

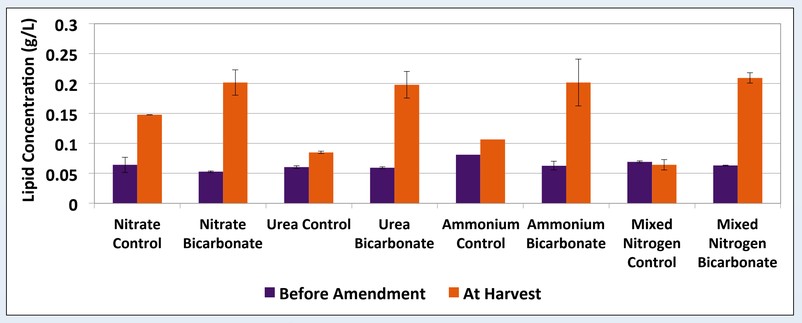

Figure 6. Lipid productivity (g/L) for each of the experimental conditions before the addition of bicarbonate and 3d after the amendment with the exception of the urea culture. The urea conditions' second lipid data set is for 5d 3h after the addition of bicarbonate. The reason for this variance is that additional lipid samples are still being analyzed and urea lipid data from earlier time points following the bicarbonate amendment are not yet available.

Discussion

Growth:
The growth of *C. vulgaris* UTEX 395 was similar under all conditions (Figure 2). All of the conditions, with the exception of the ammonium control condition, reached stationary phase just prior to or at nitrogen depletion. The ammonium control condition continued to grow exponentially for one day beyond nitrogen depletion.

Nitrogen Uptake:
Ammonium was the most readily utilized nitrogen species evaluated, followed by nitrate and then urea (Figure 3). This was observed in the mixed nitrogen conditions and when comparing the single nitrogen species conditions.

Lipid Accumulation:
The addition of bicarbonate just prior to nitrogen depletion resulted in an increase in lipid production for all of the bicarbonate amended cultures (Table 3).

Condition	Increase in lipid production relative to control (g/L)
Nitrate Bicarbonate	0.05
Urea Bicarbonate	0.11
Ammonium Bicarbonate	0.10
Mixed Nitrogen Bicarbonate	0.15

Table 3. Increase in lipid productivity (g/L) resulting from the addition of bicarbonate just prior to nitrogen depletion. Data presented are relative to each of the conditions respective controls.

Experimental Issues/Concerns:

(1) A malfunction with the pH control system for one of the ammonium cultures resulted in the loss of an experimental replicate. Additional replicate data will be collected in a subsequent experiment.

(2) Lipid sample analysis is currently still in progress.

Conclusions

- *C. vulgaris* UTEX 395 was able to grow comparably under all of the experimental conditions.
- The combination of nitrogen stress and a sodium bicarbonate amendment resulted in an increase in lipid accumulation under all of the experimental conditions.

Future Work

The current study serves as a foundation for continuing to investigate the effects of using different nitrogen species for the cultivation of microalgae. Future research will:

(1) Reevaluate the current experimental conditions in order to increase confidence in the current findings.

(2) Evaluate the growth and lipid accumulation of *C. vulgaris* UTEX 395 using primary clarifier effluent collected from the Bozeman Wastewater Reclamation Facility.

(3) Evaluate the effect of nitrogen stress and bicarbonate amendment at the time of nitrogen stress on lipid accumulation of C. *vulgaris* UTEX 395 using primary clarifier effluent as a growth medium.

Acknowledgements

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