

**BIOREMEDIATION OF INDUSTRIAL EMISSIONS AND DAIRY  
WASTEWATER USING THE MARINE MICROALGA,  
*HETEROSIGMA AKASHIWO*, COUPLED TO BIOFUEL PRODUCTION**

by

Anna Schutschkow

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Marine Studies

Summer 2016

© 2016 Anna Schutschkow  
All Rights Reserved

ProQuest Number: 10191139

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



ProQuest 10191139

Published by ProQuest LLC (2016). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code  
Microform Edition © ProQuest LLC.

ProQuest LLC.  
789 East Eisenhower Parkway  
P.O. Box 1346  
Ann Arbor, MI 48106 - 1346

**BIOREMEDIATION OF INDUSTRIAL EMISSIONS AND DAIRY  
WASTEWATER USING THE MARINE MICROALGA,  
*HETEROSIGMA AKASHIWO*, COUPLED TO BIOFUEL PRODUCTION**

by

Anna Schutschkow

Approved: \_\_\_\_\_  
Kathryn Coyne, Ph.D.  
Professor in charge of thesis on behalf of the Advisory Committee

Approved: \_\_\_\_\_  
Mark A. Moline, Ph.D.  
Director of the School of Marine Science and Policy

Approved: \_\_\_\_\_  
Mohsen Badiey, Ph.D.  
Acting Dean of the College of Earth, Ocean, and Environment

Approved: \_\_\_\_\_  
Ann L. Ardis, Ph.D.  
Senior Vice Provost for Graduate and Professional Education

## **ACKNOWLEDGMENTS**

I would like to thank Kathy Coyne and Jennifer Stewart for everything they have taught me over the past two years. I would also like to thank Mark Warner for his guidance and Katherine Miller for her help and support with the fatty acid methyl ester analysis.

Thank you to my wonderful lab mates: Yanfei Wang, Jenna Schambach, Eva Ternon, Deepak Nanjappa, and Chris Main. Your help and advice have meant so much during my time here.

## TABLE OF CONTENTS

LIST OF TABLES .....	vi
LIST OF FIGURES .....	viii
ABSTRACT .....	xi

### Chapter

1	INTRODUCTION .....	1
1.1	Industrial Flue Gas .....	1
1.2	<i>Heterosigma akashiwo</i> .....	2
1.3	Dairy Waste .....	5
1.4	Simultaneous Bioremediation of Flue Gas and Dairy Waste .....	6
1.5	Goals of my Research.....	6
	REFERENCES.....	8
2	NITROGEN RATIO AND SOURCE FOR OPTIMAL GROWTH AND LIPID CONTENT IN <i>HETEROSIGMA AKASHIWO</i> .....	11
2.1	Abstract.....	11
2.2	Introduction .....	12
2.3	Materials and Methods .....	15
2.3.1	Experimental Design .....	15
2.3.1.1	Stock culture.....	15
2.3.1.2	Growth rate and lipid content at varying N:P ratios.....	15
2.3.1.3	Determining the optimal nitrogen source for growth and lipid content .....	18
2.3.2	Statistical Analysis .....	20
2.4	Results .....	20
2.4.1	Growth rate and lipid content at varying N:P ratios.....	20
2.4.2	Growth and lipid content when cultured with nitrate vs. ammonium.....	21

2.5	Discussion.....	21
2.6	Conclusion.....	24
	REFERENCES.....	37
3	BIOREMEDIATION OF INDUSTRIAL FLUE GAS AND BIOFUEL PRODUCTION USING <i>HETEROSIGMA AKASHIWO</i> GROWN ON DAIRY WASTE.....	40
3.1	Abstract.....	40
3.2	Introduction .....	41
3.3	Materials and Methods .....	44
3.3.1	Nitrate reductase activity under varying nitrate to ammonium ratios .....	44
3.3.2	Evaluation of growth and productivity on flue gas and dairy wastewater .....	45
3.3.3	Evaluation of bioremediation potential for flue gas .....	48
3.3.4	Statistical Analysis .....	48
3.4	Results .....	49
3.4.1	Nitrate reductase activity under varying nitrate to ammonium ratios .....	49
3.4.2	Bioremediation of flue gas and dairy waste .....	49
3.4.2.1	Growth.....	49
3.4.2.2	Nitrate reductase activity.....	49
3.4.2.3	Bioremediation potential of flue gas .....	50
3.4.2.4	Biochemical profile and biodiesel potential .....	50
3.5	Discussion.....	52
3.6	Conclusion.....	58
	REFERENCES.....	70

## LIST OF TABLES

Table 1	The percentage of lipids, monomeric sugars, and protein relative to the dry weight of <i>H. akashiwo</i> cultured at nitrate to phosphate ratios of 16:1, 10:1, and 7:1 (total P=36.2 $\mu$ M). Data are expressed as means (n=4). Different letters denote significant difference between groups using a one-way ANOVA and Tukey-Kramer HSD ( $p < 0.05$ ).....	26
Table 2	The growth rate ( $d^{-1}$ ), dry weight (g/L), and chlorophyll <i>a</i> content ( $\mu$ g/L) of <i>H. akashiwo</i> cultured semi-continuously with nitrate or ammonium as the nitrogen source, after cultures reached a constant growth rate (n=4). Data are expressed as means $\pm$ standard deviations..	30
Table 3	The dissolved inorganic carbon ( $\mu$ M), total percent carbon, nitrogen, and protein relative to the dry weight of <i>H. akashiwo</i> cultured semi-continuously with nitrate or ammonium as the nitrogen source, after cultures reached a constant growth rate (n=4). Data are expressed as means $\pm$ standard deviations. Different letters denote significant differences between groups using a t-test ( $p < 0.05$ ).....	31
Table 4	The total lipids as a percentage of the dry weight, fatty acid profile, and biodiesel parameters for <i>H. akashiwo</i> cultured semi-continuously with nitrate or ammonium as the nitrogen source, after cultures reached a constant growth rate (n=4). Data are expressed as means $\pm$ standard deviations. ....	32
Table 5	Gas constituents, nitrogen, and phosphate concentrations in each treatment group where AD and FG refer to anaerobically digested dairy wastewater and flue gas, respectively. ....	47
Table 6	The growth rate ( $d^{-1}$ ), cell density (cells/mL), dry weight (g/L), and chlorophyll <i>a</i> content ( $\mu$ g/L) of <i>H. akashiwo</i> cultured semi-continuously on ammonium and carbon dioxide, dairy waste and carbon dioxide, ammonium and flue gas, and dairy waste and flue gas. Data are expressed as means $\pm$ standard deviations (n=4) and an asterisk denotes significant interactions. Values are the average over days 2-14. ....	60

Table 7	The percentage of carbon (% C) and nitrogen (% N) relative to the total biomass in <i>H. akashiwo</i> cultures supplemented with ammonium and carbon dioxide, dairy waste and carbon dioxide, ammonium and flue gas, and dairy waste and flue gas. Data are expressed as means $\pm$ standard deviations (n=4). Samples were taken once cultures reached a constant growth rate. Asterisks denote significant interaction effect... 65
Table 8	The saponification value (SV), iodine value (IV), and cetane number (CN) for the fatty acid profile of <i>H. akashiwo</i> cultures supplemented with ammonium and carbon dioxide, dairy waste and carbon dioxide, ammonium and flue gas, and dairy waste and flue gas. Data are expressed as means $\pm$ standard deviations (n=4). Samples were taken once cultures reached a constant growth rate. Letters denote significant differences. .... 66

## LIST OF FIGURES

- Figure 1: The growth rate of *Heterosigma akashiwo* cultured at nitrate to phosphate ratios of 16:1, 10:1, and 7:1 (total P= 36.2  $\mu$ M). Data are expressed as means  $\pm$  standard deviations (n=4). Different letters denote significant differences between groups using a one-way ANOVA and Tukey-Kramer HSD post-hoc testing (p<0.05)..... 25
- Figure 2: The percentage of lipids, monomeric sugars, and protein relative to the dry weight of *H. akashiwo* cultured at nitrate to phosphate ratios of 16:1, 10:1, and 7:1 (total P= 36.2  $\mu$ M). Data are expressed as means (n=4). Different letters denote significant differences between groups using a one-way ANOVA and Tukey-Kramer HSD (p<0.05)..... 27
- Figure 3: The percentage of lipids, monomeric sugars, and protein relative to the dry weight, and corrected to 100%, for *H. akashiwo* cultured at nitrate to phosphate ratios of 16:1, 10:1, and 7:1 (total P= 36.2  $\mu$ M). Data are expressed as means (n=4). Different letters denote significant differences between groups using a one-way ANOVA and Tukey-Kramer HSD (p<0.05)..... 28
- Figure 4: The percentage of particulate carbon and nitrogen relative to the dry weight of *H. akashiwo* cultured at nitrate to phosphate ratios of 16:1, 10:1, and 7:1 (total P= 36.2  $\mu$ M). Data are expressed as means (n=4). Different letters denote significant differences between groups using a one-way ANOVA and Tukey-Kramer HSD (p<0.05). ..... 29
- Figure 5: The percentage of lipids and protein relative to the dry weight of *H. akashiwo* cultured semi-continuously with nitrate or ammonium as the nitrogen source, after cultures reached a constant growth rate (n=4). Data are expressed as means. Different letters denote significant differences between groups using a t-test (p<0.05)..... 33
- Figure 6: The percentage of particulate carbon and nitrogen relative to the dry weight of *H. akashiwo* cultured semi-continuously with nitrate or ammonium as the nitrogen source, after cultures reached a constant growth rate. Data are expressed as means (n=4). Different letters denote significant differences between groups using a t-test (p<0.05)... 34

Figure 7:	The percentage of saturated, monounsaturated, and polyunsaturated fatty acids relative to the total fatty acid content of <i>H. akashiwo</i> cultured semi-continuously with nitrate or ammonium as the nitrogen source, after cultures reached a constant growth rate. Data are expressed as means (n=4).....	35
Figure 8:	The fatty acid methyl ester (FAME) profile of <i>H. akashiwo</i> cultured semi-continuously with nitrate or ammonium as the nitrogen source, after cultures reached a constant growth rate. Data are expressed as means $\pm$ standard deviations (n=4).....	36
Figure 9:	The nitrate reductase activity (pmoles of nitrate reduced/ $\mu$ g protein/min) in <i>H. akashiwo</i> following a three-week acclimation to culture conditions (total nitrogen=200 $\mu$ M). Data are expressed as means $\pm$ standard deviation (n=3). Different letters denote significant differences between groups using a one-way ANOVA and Tukey-Kramer HSD (p<0.05).....	59
Figure 10:	The growth rate ( $d^{-1}$ ) of <i>H. akashiwo</i> cultured semi-continuously on ammonium and carbon dioxide, dairy waste and carbon dioxide, ammonium and flue gas, and dairy waste and flue gas. Data are expressed as means $\pm$ standard deviations (n=4). Values are an average over days 2-14.....	61
Figure 11:	Nitrate reductase activity (pmole nitrate reduced/ $\mu$ g protein/min) for <i>H. akashiwo</i> cultures grown on ammonium and carbon dioxide, dairy waste and carbon dioxide, ammonium and flue gas, and dairy waste and flue gas. Data are expressed as means $\pm$ standard deviations (n=4). Samples were taken once cultures reached a constant growth rate.....	62
Figure 12:	Aqueous nitric oxide concentration (nM) for <i>H. akashiwo</i> cultures grown on ammonium and carbon dioxide, dairy waste and carbon dioxide, ammonium and flue gas, and dairy waste and flue gas. Data are expressed as means $\pm$ standard deviations (n=3). Values are averages over days 2-14. Samples without algae are technical replicates.....	63

Figure 13: Dissolved inorganic carbon (DIC) concentration ( $\mu\text{M}$ ) for *H. akashiwo* cultures supplemented with ammonium and carbon dioxide, dairy waste and carbon dioxide, ammonium and flue gas, and dairy waste and flue gas. Data are expressed as means  $\pm$  standard deviations ( $n=3$ ). Values are averages over the final two sampling days for each treatment. Samples without algae are technical replicates. There were no significant interaction effects or differences. .... 64

Figure 14: The percentage of carbon (% C) and nitrogen (% N) relative to the total biomass in *H. akashiwo* cultures supplemented with ammonium and carbon dioxide, dairy waste and carbon dioxide, ammonium and flue gas, and dairy waste and flue gas. Data are expressed as means ( $n=4$ ). Samples were taken once cultures reached a constant growth rate. Asterisks indicates a significant interaction effect. .... 67

Figure 15: The percentage of monomeric sugars, protein, and lipids relative to the total biomass in *H. akashiwo* cultures supplemented with ammonium and carbon dioxide, dairy waste and carbon dioxide, ammonium and flue gas, and dairy waste and flue gas. Data are expressed as means ( $n=4$ ). Samples were taken once cultures reached a constant growth rate. Asterisks indicate a significant interaction effect. .... 68

Figure 16: The percentage of fatty acid methyl esters (FAME) relative to the total amount of FAMEs in *H. akashiwo* cultures supplemented with ammonium and carbon dioxide, and ammonium and flue gas. Data are expressed as means  $\pm$  standard deviations ( $n=4$ ). Samples were taken once cultures reached a constant growth rate. Asterisks denote FAMEs that are significantly different. .... 69

## ABSTRACT

Photosynthetic microalgae have the ability to convert solar energy and carbon dioxide into algal biomass that can be used as a feedstock for biofuel. The carbon dioxide and nitric oxide in industrial emissions, and the ammonium and phosphate in dairy waste, can be used as sustainable nutrient sources for micro-algal biomass production. Previous studies have shown that the marine microalga, *Heterosigma akashiwo*, is well suited for biofuel production due to high lipid content and productivity. *H. akashiwo* may be able to metabolize the nitric oxide in flue gas due to a novel nitrate reductase enzyme, NR2-2/2HbN, however, nitrate reductase (NR) activity may be inhibited by the ammonium present in dairy waste. The goal of this study was to determine if *H. akashiwo* is suitable for the simultaneous bioremediation of industrial emissions and dairy waste during the production of quality biodiesel. First, the effects of nitrogen limitation on growth and lipid content of *H. akashiwo* was evaluated at varying nitrogen to phosphate ratios (16:1, 10:1, and 7:1) and nitrogen sources (nitrate and ammonium). Biofuel production was highest under N-replete conditions. The nitrogen source had no effect on the growth rate, biochemical profile, or fatty acid profile of *H. akashiwo*, indicating that the quality of the biodiesel will not be affected by the nitrogen source. Then, the effects of varying nitrate to ammonium ratios (1:0, 1:1, 1:10, 1:40) on NR activity in *H. akashiwo* was evaluated. NR activity was detected in cultures with 10x and 40x more ammonium than nitrate, although NR activity was significantly reduced compared to cultures supplemented with only nitrate. This reduction in activity is most likely due to the lower concentrations of

nitrate and not the presence of ammonium. NR activity was detected in all cultures, indicating that ammonium does not completely inhibit NR activity. These results suggest that bioremediation of the ammonium in the dairy wastewater and the nitric oxide in the flue gas may be able to occur simultaneously. Finally, the potential for biodiesel production coupled with bioremediation of flue gas when *H. akashiwo* was grown on dairy waste was evaluated. Here, *H. akashiwo* was cultured on a factorial combination of CO<sub>2</sub> or flue gas containing CO<sub>2</sub> and nitric oxide, with either ammonium or dairy waste. It was found that *H. akashiwo* could sustain growth on dairy waste and industrial flue gas, but was unable to bio-remediate the CO<sub>2</sub> and nitric oxide in the flue gas under these experimental conditions. Additionally, low lipid contents indicated that the treatments used here are not suitable for biofuel production from *H. akashiwo*. However, high protein content supports the use of this alga for animal feed and/or bioplastics.

## **Chapter 1**

### **INTRODUCTION**

#### **1.1 Industrial Flue Gas**

Flue gas is the exhaust gas that is generated by combustion processes. The composition of flue gas from each individual point source depends on the type of fuel, as well as the combustion conditions. Two main toxic components of flue gas are carbon dioxide and nitric oxide (Kuckshinrichs and Hake 2015). Both contribute greatly to climate change and the greenhouse effect by filtering heat radiation and forming ozone (EPA, 2014). Carbon dioxide derived from flue gas and other anthropogenic sources accounts for 81% of total greenhouse gas emissions in the United States (EPA, 2014). It is important to develop methods to remediate carbon dioxide and other harmful gases that are entering the atmosphere. Current methods of capturing and storing carbon emissions include carbon sequestration, where carbon is stored underground in saline aquifers, unmineable coal fields, and oil/gas reservoirs, and bioremediation using photosynthetic organisms, such as microalgae (Kuckshinrichs and Hake 2015). Bioremediation of industrial flue gas produces algal biomass that may be used for biofuel, plastics, animal feeds, and other bio-products. Currently, there are many commercial companies that use waste streams to produce algal products. For example, Algenol (Fort Myers, FL) uses carbon dioxide from industrial resources as a carbon source for the production of ethanol from cyanobacteria (Algenol 2016). LanzaTech (Auckland, NZ) uses flue gas from steel manufacturing as a source of nutrients for a microbe isolated from hydrothermal vents,

*Clostridium autoethanogenum*, to produce chemicals such as butanediol, butadiene, and isopropanol, as well as ethanol for fuel (LanzaTech 2016).

Microalgae, such as *Chlorella* sp., *Scenedesmus* sp., *Nannochloropsis* sp., *Boytryococcus braunii*, and *Heterosigma akashiwo*, can withstand the high CO<sub>2</sub> concentrations within flue gas (Huang et al. 2016, Stewart et al. 2015). *Chlorella* sp. MFT-15 cultured on flue gases from coke ovens, hot stoves, and power plants can use the carbon dioxide, nitric oxide, and sulfur oxide present in the flue gas with average growth rates and lipid production of 0.77/day and 12 g/m<sup>2</sup>/day, respectively (Kao et al. 2014). Pilot-scale tests of *Scenedesmus acutus* grown on flue gas at a sulfur coal-fired power plant demonstrated average productivities of 10-39 g/m<sup>2</sup>/day. Additionally, the high levels of C16:0 and C18:0 fatty acid chains indicated that the biomass produced would be suitable for biofuels (Wilson et al. 2014). Some microalgae, such as the previously mentioned *Chlorella* strain, are also able to withstand and/or utilize the nitric oxide present in the flue gas. *Dunaliella tertiolecta* has been shown to remove about 65% of nitric oxide present in the growth medium when nitric oxide concentrations range from 25 to 500 ppm (Nagase et al. 1997).

## 1.2 *Heterosigma akashiwo*

*Heterosigma akashiwo* (Hada) Hada ex Y. Hara et Chihara (Hara and Chihara 1987) is a globally distributed marine raphidophyte. The strain that was used for this study, isolated from the Delaware Inland Bays, Delaware, USA, forms dense blooms greater than 10<sup>7</sup> cells/L and can tolerate temperatures and salinities between 16-30°C and 10-30 ppt, respectively (Zhang et al. 2006). Previous studies by Fuentes-Grünewald et al. (2013) showed that *H. akashiwo* cultures grown outdoors in bubble column photobioreactors had a maximum lipid content of 23%, with lipid

productivities reaching as high as 56 mg/L/day. The fatty acids produced in their study were dominated by C16:0, C18:0, and C14:0, indicating that the high percentage of lipids produced would be suitable for biodiesel production. (Fuentes-Grünewald et al. 2013).

*H. akashiwo* can use ammonium, nitrate, nitrite, and urea as a nitrogen source (Zhang et al. 2006). Nitrate is converted to nitrite through nitrate reductase (NR) (Falkowski and Raven 2013). NR catalyzes the rate-limiting step in nitrate assimilation and is an important checkpoint in both C and N assimilation pathways. Nitrite is then reduced to ammonium by nitrite reductase in the chloroplasts where ammonium is converted to cellular nitrogen through the GS-GOGAT pathway. Ammonium and glutamic acid are reacted to form glutamine, which combines with 2-oxoglutarate to produce two glutamate molecules, one of which is transported to the cytoplasm to be converted to amino acids, while the other glutamate is recycled as a substrate for GS-GOGAT (Falkowski and Raven 2013).

*H. akashiwo* may also have the ability to metabolize nitric oxide as a nitrogen source due to the presence of a unique assimilatory nitrate reductase (NR2-2/2HbN) reported by Stewart and Coyne (2011). It was found that a 2/2HbN domain homologous to a mycobacterial 2/2HbN was likely inserted by horizontal gene transfer into the existing hinge 2 region of the nitrate reductase of *H. akashiwo* (Stewart and Coyne 2011). In mycobacteria, the 2/2HbN reacts nitric oxide and heme-bound oxygen to produce nitrate. This serves as protection from mammalian host cells that produce nitric oxide as an immune response to mycobacteria (Poole and Hughes 2000, Ouellet et al. 2002, Lama et al. 2006). A proposed model for NR2-2/2HbN (Stewart and Coyne, 2011) suggests that electrons from the FAD/NADH reductase domain are

transferred to the 2/2HbN domain, allowing for the conversion of nitric oxide to nitrate. The nitrate may then be converted to nitrite by the Mo-MPT site of NR2-2/2HbN and assimilated as cellular nitrogen. This proposed mechanism is supported by the fact that the 2/2HbN domain in *H. akashiwo* shares conserved active site residues with flavohemoglobins (flavoHbs) that are involved in nitric oxide reactivity, and that it is situated within the enzyme so that it can participate in electron transfers (Workun et al. 2008). Stewart and Coyne (2011) found that the addition of the chemical nitric oxide donor, sodium nitroprusside, to *H. akashiwo* cultures significantly increased the expression of NR2-2/2HbN compared to cultures without added nitric oxide. Simultaneously, the amount of nitric oxide in *H. akashiwo* cultures decreased as compared to cell-free medium with nitric oxide added (Stewart and Coyne 2011). In another experiment, addition of <sup>15</sup>N-labeled nitric oxide resulted in a significant increase of the labeled nitrogen in cellular biomass (Coyne, unpublished). Further studies showed that when the growth medium of *H. akashiwo* was supplemented with simulated industrial flue gas (150 ppm nitric oxide and 12% carbon dioxide), the transcript abundance of *NR2-2/2HbN* significantly increased compared to cultures supplemented with air (Stewart et al. 2015).

These experiments provide evidence that *H. akashiwo* can use nitric oxide as a nitrogen source, and other data indicates that this species may be useful for bioremediation of nitric oxide and carbon dioxide in industrial emissions. The addition of flue gas did not inhibit photosynthesis, and doubled the biomass productivity of *H. akashiwo* (Stewart et al. 2015). Growth on flue gas also significantly increased carbohydrate and protein productivity, suggesting that this species is effective at fixing the excess carbon dioxide present in the simulated flue gas (Stewart et al. 2015).

### 1.3 Dairy Waste

Many dairy farms use anaerobic digestion to process dairy waste. Anaerobic digestion is the microbially-mediated biochemical degradation of complex organic materials into simple organic molecules and dissolved nutrients (Lansing et al. 2008). This process is beneficial for farmers because it can produce methane gas that can then be used as an energy source. In a study analyzing anaerobic digesters, 66% of the biogas produced was methane gas (Lansing et al. 2008). The methane gas from the anaerobically digested dairy waste collected for my research is used to generate electricity on site for the milk processing facility. However, the digestion process concentrates the ammonium and phosphorus by an average of 73.8% and 15.8%, respectively (Lansing et al. 2008). These high nitrogen and phosphorus levels in dairy waste can lead to the eutrophication of aquatic ecosystems and groundwater pollution (Razzak et al. 2013) and must be removed. Microalgae may be useful in the bioremediation of wastewater, as they have the ability to metabolize inorganic nitrogen and phosphorus (Woertz et al. 2009, Razzak et al. 2013).

In a study using *Chlorella* sp. for the bioremediation of anaerobic digest from a dairy farm, the algae reduced ammonia, total nitrogen, and total phosphorus by 100%, 75-82%, and 62-74%, respectively (Wang et al. 2010). In another study, using a locally collected mixture of green algae and diatoms, ammonium and phosphate were reduced in dairy waste by 96% and 99%, respectively, after only 12 days (Woertz et al. 2009). Both of these studies showed that the biomass produced had the potential to be used for biofuels due to high total fatty acid contents (Wang et al. 2009, Woertz et al. 2009). As other algae have been shown to be useful in the biological treatment of dairy wastewater, it seems likely that *H. akashiwo* would be suitable as well, due to its ability to metabolize ammonium.

#### **1.4 Simultaneous Bioremediation of Flue Gas and Dairy Waste**

*Heterosigma akashiwo* may not be suitable for the simultaneous bioremediation of dairy wastewater and industrial flue gas, however. Studies suggest that NR, which may be involved in nitric oxide metabolism, may be inhibited by ammonium present in dairy waste. For example, addition of ammonium to cultures of the cyanobacteria *Spirulina* sp., growing on nitrate resulted in a decrease in nitrate reductase activity. This decrease was concentration dependent; more ammonium led to a larger decrease in nitrate reductase activity (Jha et al., 2007). Other studies showed that transcription of *nitrate reductase* mRNA was completely inhibited by the presence of ammonium for the microalgae, *Cylindrotheca fusiformis* and *Dunaliella tertiolecta*, even when nitrate was present (Poulsen and Kroger 2005, Song and Ward 2004). Other studies have shown that nitrate reductase activity in most microalgae will return after ammonium is depleted, and that it is common for microalgae to repress nitrate reductase in the presence of ammonium (Syrett, 1981; Berges et al. 1995). NR in *H. akashiwo* is constitutively transcribed even in the presence of ammonium (Coyne 2010). It is also known that *H. akashiwo* will grow on nitrate and ammonium when both are present in equal concentrations with no significant decrease in nitrate reductase activity (Coyne, unpublished data), but higher ammonium to nitrate ratios have not been tested.

#### **1.5 Goals of my Research**

In this study, I wanted to determine if *H. akashiwo* is suitable for the simultaneous bioremediation of dairy wastewater and industrial flue gas. The specific goals of my research were to:

1. Determine the effects of nitrogen to phosphate ratios, as well as nitrogen source (nitrate vs. ammonium), on growth and lipid content in *H. akashiwo*.
2. Evaluate the effects of nitrate to ammonium ratios on nitrate reductase activity in *H. akashiwo*.
3. Compare growth rates, biomass yields, and productivity yields of *H. akashiwo* cultured on industrial flue gas, dairy wastewater, or a combination of industrial flue gas and dairy wastewater.

## REFERENCES

- Berges, J. A., Cochlan, W. P., & Harrison, P. J. (1995). Laboratory and field responses of algal nitrate reductase to diel periodicity in irradiance, nitrate exhaustion, and the presence of ammonium. *Marine Ecology Progress Series*. Oldendorf, 124(1), 259-269.
- Coyne, K. J. (2010). Nitrate reductase (nr1) sequence and expression in the harmful alga *Heterosigma akashiwo* (Raphidophyceae) 1. *Journal of Phycology*, 46(1), 135-142.
- Direct to ethanol (2016). Algenol Retrieved from <http://www.algenol.com/direct-to-ethanol/the-technology>
- EPA. (2014). *Overview of Greenhouse Gases*. Available at: <https://www3.epa.gov/climatechange/ghgemissions/gases.html>
- Falkowski, P. G., & Raven, J. A. (2013). *Aquatic photosynthesis* Princeton University Press.
- Fuentes-Grünwald, C., Garcés, E., Alacid, E., Rossi, S., & Camp, J. (2013). Biomass and lipid production of dinoflagellates and raphidophytes in indoor and outdoor photobioreactors. *Marine Biotechnology*, 15(1), 37-47.
- Hara, Y., & Chihara, M. (1987). Morphology, ultrastructure and taxonomy of the Raphidophycean alga *Heterosigma akashiwo*. *The Botanical Magazine*, 100(2), 151-163.
- Huang, G., Chen, F., Kuang, Y., He, H., & Qin, A. (2016). Current techniques of growing algae using flue gas from exhaust gas industry: A review. *Applied Biochemistry and Biotechnology*, 178(6), 1220-1238. doi:10.1007/s12010-015-1940-4 [doi]
- Jha, P., Ali, A., & Raghuram, N. (2007). Nitrate-induction of nitrate reductase and its inhibition by nitrite and ammonium ions in *Spirulina platensis*. *Physiology and Molecular Biology of Plants*, 13(2), 163.
- Kao, C., Chen, T., Chang, Y., Chiu, T., Lin, H., Chen, C., . . . Lin, C. (2014). Utilization of carbon dioxide in industrial flue gases for the cultivation of microalga *Chlorella* sp. *Bioresource Technology*, 166, 485-493.
- Kuckshinrichs, W., & Hake, J. (2015). *Carbon capture, storage and use* Springer.

- Lama, A., Pawaria, S., & Dikshit, K. L. (2006). Oxygen binding and NO scavenging properties of truncated hemoglobin, HbN, of mycobacterium *Smegmatis*. *FEBS Letters*, 580(17), 4031-4041.
- Lansing, S., Botero, R. B., & Martin, J. F. (2008). Waste treatment and biogas quality in small-scale agricultural digesters. *Bioresource Technology*, 99(13), 5881-5890.
- Nagase, H., Yoshihara, K., Eguchi, K., Yokota, Y., Matsui, R., Hirata, K., & Miyamoto, K. (1997). Characteristics of biological NO<sub>x</sub> removal from flue gas in a *Dunaliella tertiolecta* culture system. *Journal of Fermentation and Bioengineering*, 83(5), 461-465.
- Ouellet, H., Ouellet, Y., Richard, C., Labarre, M., Wittenberg, B., Wittenberg, J., & Guertin, M. (2002). Truncated hemoglobin HbN protects mycobacterium *Bovis* from nitric oxide. *Proceedings of the National Academy of Sciences of the United States of America*, 99(9), 5902-5907. doi:10.1073/pnas.092017799 [doi]
- Poole, R. K., & Hughes, M. N. (2000). New functions for the ancient globin family: Bacterial responses to nitric oxide and nitrosative stress. *Molecular Microbiology*, 36(4), 775-783.
- Poulsen, N., & Kröger, N. (2005). A new molecular tool for transgenic diatoms. *Febs Journal*, 272(13), 3413-3423.
- Razzak, S. A., Hossain, M. M., Lucky, R. A., Bassi, A. S., & de Lasa, H. (2013). Integrated CO<sub>2</sub> capture, wastewater treatment and biofuel production by microalgae culturing—a review. *Renewable and Sustainable Energy Reviews*, 27, 622-653.
- Song, B., & Ward, B. B. (2004). Molecular characterization of the assimilatory nitrate reductase gene and its expression in the marine green alga *Dunaliella tertiolecta* (chlorophyceae) 1. *Journal of Phycology*, 40(4), 721-731.
- Stewart, J. J., Bianco, C. M., Miller, K. R., & Coyne, K. J. (2015). The marine microalga, *Heterosigma akashiwo*, converts industrial waste gases into valuable biomass. *Frontiers in Energy Research*, 3, 12.
- Stewart, J. J., & Coyne, K. J. (2011). Analysis of raphidophyte assimilatory nitrate reductase reveals unique domain architecture incorporating a 2/2 hemoglobin. *Plant Molecular Biology*, 77(6), 565-575.

- Syrett, P. (1981). Nitrogen metabolism of microalgae. *Canadian Bulletin of Fisheries and Aquatic Sciences*,
- Technical overview (2016). LanzaTech Retrieved from <http://www.lanzatech.com/innovation/technical-overview/>
- Wang, L., Li, Y., Chen, P., Min, M., Chen, Y., Zhu, J., & Ruan, R. R. (2010). Anaerobic digested dairy manure as a nutrient supplement for cultivation of oil-rich green microalgae *Chlorella* sp. *Bioresource Technology*, 101(8), 2623-2628.
- Wilson, M., Groppo, J., Placido, A., Graham, S., Morton III, S., Santillan-Jimenez, E., . . . Andrews, R. (2014). CO<sub>2</sub> recycling using microalgae for the production of fuels. *Applied Petrochemical Research*, 4(1), 41-53.
- Woertz, I., Feffer, A., Lundquist, T., & Nelson, Y. (2009). Algae grown on dairy and municipal wastewater for simultaneous nutrient removal and lipid production for biofuel feedstock. *Journal of Environmental Engineering*, 135(11), 1115-1122.
- Workun, G. J., Moquin, K., Rothery, R. A., & Weiner, J. H. (2008). Evolutionary persistence of the molybdopyranopterin-containing sulfite oxidase protein fold. *Microbiology and Molecular Biology Reviews : MMBR*, 72(2), 228-48, table of contents. doi:10.1128/MMBR.00041-07 [doi]
- Zhang, Y., Fu, F., Whereat, E., Coyne, K. J., & Hutchins, D. A. (2006). Bottom-up controls on a mixed-species HAB assemblage: A comparison of sympatric *Chattonella subsalsa* and *Heterosigma akashiwo* (Raphidophyceae) isolates from the Delaware inland bays, USA. *Harmful Algae*, 5(3), 310-320.

## Chapter 2

### NITROGEN RATIO AND SOURCE FOR OPTIMAL GROWTH AND LIPID CONTENT IN *HETEROSIGMA AKASHIWO*

#### 2.1 Abstract

The aim of this study was to determine the nitrogen to phosphate ratio and nitrogen source for optimal growth and lipid content of *Heterosigma akashiwo* CCMP 2393. Microalgae are suitable feedstocks for the production of biofuels due to their ability to produce and store neutral lipids. Enhanced production of neutral storage lipids in microalgae can be stimulated through nitrogen limitation. *H. akashiwo* was cultured semi-continuously on media containing 16:1, 10:1 and 7:1 nitrate to phosphate ratios, keeping total P constant (36.2  $\mu\text{M}$ ) and supplemented with 9%  $\text{CO}_2$  until a stable growth rate was achieved. The 16:1 cultures showed optimal growth and lipid content as compared to the other treatments due to an overall higher algal biomass at higher N:P ratios. Then, *H. akashiwo* was cultured semi-continuously on media containing a 16:1 nitrogen to phosphate ratio with either nitrate or ammonium as the nitrogen source. Cultures were supplemented with 9%  $\text{CO}_2$  and harvested once a stable growth rate was achieved. There were no differences in growth rate, lipid content, or fatty acid profile between the two treatments as the nitrogen supply in the media was sufficient to support growth and necessary cellular reactions. The finding that *H. akashiwo* can achieve optimal growth and lipid productivities on ammonium indicates that it may be useful for the bioremediation of ammonium in dairy wastewater.

## 2.2 Introduction

Autotrophic microalgae have the ability to convert solar energy and carbon dioxide into biomass that can be used as a sustainable source of biofuel (Wilson et al. 2014, Schlagermann et al. 2012, Davis et al. 2011). Compared to other sources of biodiesel, such as corn, soybean, and canola, microalgae are the only source that can produce enough biofuel to reduce the usage of fossil fuels by 50%, using only 1.1-2.2% of cropping area in the US (Chisti 2007). Mixtures of petroleum diesel and up to 50% microalgae-derived biodiesel were shown to work in conventional diesel engines and to reduce harmful exhaust emissions (Tüccar and Aydin, 2013).

The most desirable algal lipids for biodiesel production are triacylglycerols (TAGs) which function as neutral storage lipids for algal cells (Cagliari et al. 2011). TAGs are composed of three fatty acids that are bound to a glycerol molecule. Fatty acids in algae are synthesized using acetyl-CoA. This is then converted to malonyl-CoA, transferred to an acyl carrier protein, and then the consecutive addition of two carbons occurs until C16:0 is produced. Fatty acids of varying chain lengths and degrees of saturation are produced from C16:0. Three separate fatty acids are then transferred from CoA to glycerol to form TAGs (Cagliari et al. 2011). Nutrient starvation, pH, temperature, light intensity and aeration are major chemical and physical stimuli for TAG synthesis and accumulation in microalgae to ensure there is a sufficient reserve of cellular energy during stressful conditions (Cagliari et al. 2011, Hu et al. 2008). Many strains of microalgae, such as *Nannochloropsis* sp., *Scenedesmus* sp., *Chlorella* sp., and *Botryococcus braunii*, have been identified as high lipid producers and extensively studied to determine their viability as a source of biofuel (National Research Council 2012). *Heterosigma akashiwo* is also of interest

due to its ability to form dense blooms in the environment and to adapt to a wide range of temperatures, salinities, and nutrient conditions (Zhang et al. 2006).

Previous studies using *H. akashiwo* have found that altering abiotic parameters such as aeration, temperature, and nitrogen stress can lead to an enhancement of cellular oil concentration (Fuentes-Grunewald et al. 2012). A gentle aeration of 0.1 v/v min<sup>-1</sup> of *H. akashiwo* cultures yielded an increase in growth and productivity due to the carbon dioxide present in the air and the mixing of nutrients (Fuentes-Grunewald et al. 2012). Similarly, increasing the temperature from 15°C to 20°C and 25°C significantly increased growth rates and productivity (Fuentes-Grunewald et al. 2012). Once cultures reached stationary phase, the limited nitrogen in the growth medium induced lipid accumulation, specifically TAGs, in *H. akashiwo*. Specifically, when the concentration of nitrate was 9 times more than the phosphate in the growth media, TAG accumulation was increased by 30% when compared to cultures grown on a 24:1 nitrate to phosphate ratio (Fuentes-Grunewald et al. 2012). It was proposed that for optimal commercial biofuel production using *H. akashiwo*, a combined approach of aeration, increased temperature, and limited nitrogen be implemented using recycled heated seawater from power plants (Fuentes-Grunewald et al. 2012). *H. akashiwo* can also grow semi-continuously outside in photobioreactors at pilot-scale for 4 months (Fuentes-Grunewald et al. 2013). These outdoor cultures accumulated 23% of total biomass as lipids, with a total lipid productivity of  $56.1 \pm 9.7$  mg/L/day, similar to other high oil producing marine microalgae (Fuentes-Grunewald et al. 2013).

Nitrogen preference varies among strains of *Heterosigma*. Ammonium supported the fastest growth of *H. akashiwo* CCMP 1912 (Kalaloch, WA isolate) (Herndon and Cochlan 2007), while multiple Japanese strains have shown no nitrogen

preference in terms of overall growth (Iwasaki and Sasada 1969, Watanabe et al. 1982, Hosaka 1992). Previous studies on *H. akashiwo* CCMP 2393, the Delaware Inland Bay isolate used here, identify nitrate as the preferred inorganic nitrogen source. *H. akashiwo* was able to sustain growth at nitrate concentrations from 5 to 200  $\mu\text{M}$ , with saturation occurring at 5  $\mu\text{M}$  (Zhang et al. 2006). Differences in nitrogen preference among strains of *Heterosigma* may be due to physiological differences among strains that are geographically isolated, or by differences in culturing and analytical methods among researchers (Herndon and Cochlan 2007).

Nitrate is the preferred nitrogen source for biofuel production of *D. tertiolecta*, as growth of the alga is significantly higher when supplemented with nitrate as compared to ammonium (Chen et al. 2011). Other algal strains, *Scenedesmus* sp. 131 and *Monoraphidium* sp. 92, showed a higher growth rate and biodiesel potential when cultured on nitrate compared to ammonium (Eustance et al. 2013). Buffering of the cultures supplemented with ammonium was necessary as the pH dropped too low to support algal growth without pH control. Results of this study also indicated that it is more financially feasible to supplement these two strains of algae with nitrate rather than ammonium (Eustance et al. 2013).

The goal of this study was to determine the nitrogen to phosphate ratio for optimal growth and productivity of *H. akashiwo* CCMP 2393 as a biofuel feedstock, and to evaluate productivity when cultured in nitrate versus ammonium as a nitrogen source.

## 2.3 Materials and Methods

### 2.3.1 Experimental Design

#### 2.3.1.1 Stock culture

*Heterosigma akashiwo* (Delaware Inland Bays isolate, CCMP 2393) stock cultures were maintained at 25°C in 20 ppt f/2 medium (-Si) (Guillard 1975). Stock cultures were maintained on a 12:12 light dark cycle with an irradiance of 100  $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ .

#### 2.3.1.2 Growth rate and lipid content at varying N:P ratios

The nitrate to phosphate ratios used here were 0.579 mM NaNO<sub>3</sub> to 0.0362 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.362 mM NaNO<sub>3</sub> to 0.0362 mM NaH<sub>2</sub>PO<sub>4</sub>, and 0.253 mM NaNO<sub>3</sub> to 0.0362 mM NaH<sub>2</sub>PO<sub>4</sub> to achieve N:P ratios of 16:1, 10:1, and 7:1, respectively. Additionally, 5:1 and 1:1 were also tested, however, all cultures grown at these ratios had a negative growth rate; therefore, they are not included in this study. All cultures were supplemented with 9% CO<sub>2</sub> with light levels at 150  $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$  on a 12-hour light and dark cycle. Growth media was prepared with 20 ppt artificial seawater buffered with 20 mM HEPES (pH=7.4) amended with nitrate and f/2 nutrients (-Si) (Guillard, 1975). Stock cultures (250 mL) were maintained in 500 mL clear polycarbonate bottles for 18 days to ensure cultures were nutrient deplete and that there were no nutrients transferred into the experiment.

Nutrient deplete cultures were then inoculated into media containing the proper nitrate and phosphate concentrations (n=4). The cultures were maintained semi-continuously where every other day the cultures were diluted back to 180,000 cells/mL. Cells were counted every other day using the Countess II FL Automated

Cell Counter (ThermoFisher Scientific, Waltham, MA) to monitor the growth rate.

Growth rates were calculated as:

$$\mu = \ln(N_2/N_1) / (t_2 - t_1)$$

where N is the cell number (cells/mL) and t is the time in days.

Each treatment was harvested once the growth rates of the algae were consistent between sampling days. Consistent growth rates were achieved on days 13, 15, and 17 for the 16:1, 10:1, and 7:1 treatments, respectively. Samples were taken for dry weights, colorimetric lipid analysis, carbohydrate analysis, and total carbon and nitrogen content.

Oven dry weights were determined by filtering a known volume of algal culture onto a pre-weighed, pre-combusted GF/C filter. The filtered sample was washed three times with 20 mL of 0.5 M ammonium formate for 20 second intervals to remove residual salts. The filters were dried overnight at 105°C and re-weighed to determine the oven dry weight in grams per liter.

Lipid content was determined colorimetrically using corn oil dissolved in chloroform as the standard (Cheng et al. 2011). Lipids were extracted from a freeze dried cell pellet using the Folch method (1957). Methanol was added back to the lipids extracted in chloroform and the solvents were then evaporated in a hot water bath for 3 hours. The lipids were re-suspended in concentrated sulfuric acid and heated to 90°C for 20 minutes. Background absorbance was read at 540 nm in an Omega Star Microplate (BMG LABTECH, Germany), and then 0.2 mg/mL vanillin in 17% phosphoric acid was added. The absorbance was measured at 540 nm again and the total oil content within the algal sample was determined by linear regression analysis.

Carbohydrate concentrations were determined by using a two-step sulfuric acid hydrolysis to produce monomeric sugars (Van Wychen and Laurens, 2013). Freeze-dried algal biomass was weighed into 10 mL glass vials and 250  $\mu$ L of 72% sulfuric acid was added to each vial. Tubes were vortexed and incubated at 30°C for 1 hour. Seven mL of 18.2 MegaOhm water was added to each vial, a rubber stopper and crimp cap was secured on each vial, and the samples were autoclaved for 1 hour at 121°C. The tubes were cooled to room temperature for 1 hour and then neutralized to pH 6-8 using solid calcium carbonate. Once neutralized, the solids were removed by centrifugation and the supernatant was used for colorimetric analysis.

For the colorimetric assay, 500  $\mu$ L of the neutralized sample was added to glass test tubes. An additional 500  $\mu$ L of 0.5 M NaOH and 500  $\mu$ L of MBTH working solution (50:50 v/v mixture of 3 mg/mL 3-Methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate and 1 mg/mL DL-Dithiothreitol) were added to the tubes. Tubes were incubated on a heat block at 80°C for 15 minutes. One mL of ferric solution (0.5% ferric ammonium sulfate dodecahydrate, 0.5% sulfamic acid, and 0.25 M HCl) was added and reacted at room temperature for 15 minutes. 2.5 mL of 18.2 MegaOhm water was added to the tubes and 200  $\mu$ L of each tube was transferred to a 96-well clear-bottomed plate in triplicate. The absorbance was measured at 620 nm using an Omega Star Microplate reader (BMG LABTECH, Germany). Glucose was used as the standard (Van Wychen and Laurens, 2013) and the concentration of monomeric sugars in each sample was calculated by linear regression analysis.

Carbon and nitrogen concentrations within samples filtered on pre-combusted GF/F Whatman glass-fiber filters (25 mm) were determined using the Costech elemental analyzer (Costech Analytical Technologies, Valencia, CA) as described in

Hutchins et al. (2002). EDTA and phenylalanine were used as standards. Protein content was determined by multiplying the total percent of nitrogen in a sample by 4.78, the specific conversion factor for algae (Laurens 2013).

### **2.3.1.3 Determining the optimal nitrogen source for growth and lipid content**

Growth rate and lipid content of *H. akashiwo* was compared for cultures maintained in 16:1 N:P (0.579 mM nitrogen to 0.0362 mM phosphate) with nitrate or ammonium as the nitrogen source. This N:P ratio was selected based on the results from the experiment described above. Cultures were transitioned to growth on ammonium over an 18-day period and acclimated to these growth conditions for an additional 30 days, with fresh media added every 7 days. Cultures previously acclimated to growth in nitrate were also maintained with periodic additions of growth media for the acclimation period. All acclimating cultures were supplemented with 9% CO<sub>2</sub> with light levels at 150  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  on a 12-hour light and dark cycle. Growth media was prepared with 20 ppt artificial seawater buffered with 20 mM HEPES (pH=7.4) amended with nitrate or ammonium and f/2 nutrients (-Si) (Guillard, 1975).

After acclimation, experimental cultures (300 mL, n=4) were grown semi-continuously, being diluted back to 180,000 cell/mL every other day. Growth was monitored by cell counts using the Countess II FL Automated Cell Counter (ThermoFisher Scientific, Waltham, MA) until consistent growth was achieved. At this point, samples were collected for colorimetric lipid analysis, dissolved inorganic carbon, dry weight, chlorophyll a content, and total carbon and nitrogen content as described above.

To analyze the fatty acid methyl ester (FAME) content, freeze-dried biomass was weighed into a 1.5 mL GC vial and dried overnight at 60°C. Vials were reweighed, and 25 µL of 5 mg/mL C13:0ME (Sigma-Aldrich, St. Louis, MO) internal standard, 200 µL of chloroform:methanol (2:1), and 300 µL of 0.6 M HCl in methanol was added to each vial. Samples were heated to 85°C on a heat block for 1 hour then cooled to room temperature for 15 minutes. One mL of HPLC grade hexane was added, the vials were vortexed vigorously for 30 seconds, and stood undisturbed for 3 to 4 hours at room temperature to allow the lipids to separate into the upper hexane phase. The upper hexane phase was transferred to a new vial using a gas-tight syringe and stored at -20°C until analysis. Supelco 37 Component FAME mix (Sigma Aldrich, St. Louis, MO) was used as a standard. FAMES were analyzed by gas chromatography on a Hewlett Packard HP 5890 Series (Palo Alto, CA) equipped with a flame ionization detector and a Zebron ZB-1 wax column (Phenomenex, Torrance, CA). FAMES were separated using splitless injection and heating the column to an initial temperature of 190°C, then increasing the temperature by 15°C/min to 250°C, and holding at 250°C for 25 minutes (Van Wychen and Laurens 2013).

For dissolved inorganic carbon analysis, samples were collected using a syringe one hour before the lights turned on. Samples were stored in airtight scintillation vials with 200 µL of 5% mercuric chloride. Dissolved inorganic carbon was obtained using a CO<sub>2</sub> analyzer model LI-6252 (LI-COR Biosciences, Lincoln, NE) as described in Sharp et al. (2009).

For chlorophyll analysis, samples were filtered onto 25 mm GF/F glass filters and stored at -20°C until extraction. Chlorophyll *a* was extracted in the dark in 10 mL

of 90% acetone at -20°C overnight. Chlorophyll *a* concentrations were measured with a Turner 10 AU fluorometer (Turner Designs, San Jose, CA).

### **2.3.2 Statistical Analysis**

The experiments were performed in four replicates. Data represent the mean  $\pm$  standard deviation. The data were checked for normality and equal variances using the Shapiro-Wilk and Levene tests, respectively. For the nitrogen limitation experiment, a one-way ANOVA was performed to determine significant differences between treatments. Once a statistical difference was detected by the one-way ANOVA, a Tukey's HSD post hoc test was performed. For the nitrate vs. ammonium experiment, a student's t-test was performed to check for statistical differences. The standard for statistical significance was a p-value  $< 0.05$ . All statistical tests were performed using JMP Pro 12.1.0 (SAS, Cary, NC).

## **2.4 Results**

### **2.4.1 Growth rate and lipid content at varying N:P ratios**

Growth rates for *H. akashiwo* grown at 16:1 ( $0.645 \pm 0.07 \text{ day}^{-1}$ ) nitrate to phosphate were significantly higher than growth rates at 10:1 ( $0.469 \pm 0.09 \text{ day}^{-1}$ ) and 7:1 ( $0.169 \pm 0.08 \text{ day}^{-1}$ ) (Figure 1,  $p < 0.001$ ). Similarly, when *H. akashiwo* was cultured at 10:1 and 7:1 nitrate to phosphate ratios, the monomeric sugar and protein contents were not significantly different from the control (16:1) (Table 1, Figure 2). The percentage of total lipids in the 16:1 treatment ( $23.5 \pm 3.0$ ) was significantly higher than the 7:1 treatment ( $9.07 \pm 4.3$ ) ( $p = 0.0040$ ); however, it is important to note that the biochemical constituents for the 10:1 and 7:1 treatments only summed to 76% and 73%, respectively. For analysis, biochemical constituent percentages were

normalized within each treatment by setting the sum of lipids, proteins, and monomeric sugars recovered for each treatment, individually, to 100% (Figure 3). Here lipids, monomeric sugars, and proteins account for 12-24%, 45-53%, and 25-34% of the total biomass, respectively. The percentage of carbon within the total biomass of the 16:1 treatment ( $43.97 \pm 4.4$ ) was also significantly higher ( $p=0.003$ ) than the 10:1 ( $28.62 \pm 1.3$ ) and 7:1 treatments ( $34.04 \pm 2.3$ ), while there was no significant difference in the percentage of total nitrogen (Figure 4).

#### **2.4.2 Growth and lipid content when cultured with nitrate vs. ammonium**

While growth rates ( $d^{-1}$ ), dry weights (g/L), and chlorophyll *a* concentration ( $\mu\text{g/L}$ ) were slightly higher for *H. akashiwo* cultured with ammonium as the nitrogen source, there was no significant difference between the two treatments in any growth parameters measured (Table 3). Similarly, there was no difference in dissolved inorganic carbon or the percentage of particulate carbon between ammonium and nitrate treatments (Table 4). Nitrogen (and protein content) was significantly higher in *H. akashiwo* cultured with nitrate compared to ammonium (Table 4,  $p<0.05$ ). There was no significant difference in lipid content, or the fatty acid profile between the two nitrogen sources (Table 5, Figures 7 and 8). The predominant fatty acids in the FAME profiles for both treatments were C14:0, C16:0, and C16:1 (Figure 8). There were also no significant differences in the cetane number, iodine value, or saponification value between the two treatments (Table 5).

### **2.5 Discussion**

The two biggest cost drivers for large-scale commercially produced algal biofuel feedstocks are lipid content and growth rate (Davis et al. 2011). Ideally, both

should be high in order to reduce consumer costs of biofuels per gallon, however, due to the physiological limitations of algae it is difficult to maximize both parameters (Davis et al. 2011). Through techno-economic analysis it was determined that maximizing the lipid content of algae as a biofuel feedstock would offer more substantial cost reductions than enhancing growth rates (Davis et al. 2011). It is widely known that nitrogen limitation induces lipid accumulation in many species of microalgae (Falkowski and Raven 2013, Falkowski et al. 1989), however, nitrogen starvation can have an adverse effect on algal growth.

Here, growth rate and biochemical composition of *H. akashiwo* cultured on nitrogen-deficient growth media was evaluated. The most common culturing technique for biofuel production from microalgae is to grow cultures in batch with sufficient nutrients supplied at the start (Davis et al. 2011). This allows the algae to initially achieve high growth rates. As the nutrient supply is depleted over time, the algae become nitrogen limited and lipids begin to accumulate. These oleaginous algae can then be harvested and converted to biofuels. In this experiment, nitrogen depletion was simulated by culturing *H. akashiwo* at two nitrogen deplete ratios (10:1 and 7:1) and lipid content was evaluated. The lower growth rates of N-limited cultures compared to N-sufficient cultures confirmed that *H. akashiwo* was nitrogen deficient.

As expected, the growth rate of *H. akashiwo* was significantly higher when the algae were cultured on a nitrate to phosphate ratio of 16:1, compared to the 10:1 and 7:1 treatments, since nutrient availability directly affects how fast an alga can grow. Interestingly, however, the 16:1 treatment also had the highest lipid content, with 23% of its total biomass as lipids, as compared to 18% and 9% in the 10:1 and 7:1 treatments, respectively. Lipid content for the 16:1 treatment was equal to previously

reported values for *H. akashiwo* cultured at large scale in photobioreactors (Fuentes-Grunewald et al. 2013). A similar experiment comparing growth rate, lipid productivity, and lipid content for *Scenedesmus* sp. LX1 cultured with nitrate to phosphate ratios ranging from 2:1 to 20:1 showed similar results (Xin et al. 2010), with higher growth rates and cell densities, as well as lipid contents of about 25% in nitrogen sufficient growth media (Xin et al. 2010). Higher lipid content is most likely due to the up-regulation of RuBisCO under higher nitrate concentrations, which allows *H. akashiwo* to synthesize more storage molecules (Glibert et al. 2016).

While there was a difference in the total lipid content between treatments, there was no difference in the monomeric sugar or protein content. Similarly, there was no significant difference in the C/N ratio or the particulate nitrogen content between treatments. This is a surprising result, as nitrogen limitation is thought to limit protein synthesis due to a decrease in free amino acids (Falkowski et al. 1989). There was, however, an increase in the particulate carbon in the 16:1 treatment compared to the 10:1 and 7:1 treatments. This may also be due to higher levels of nitrate in the media, which may stimulate RuBisCO activity (Glibert et al. 2016). The total sum of lipids, monomeric sugars, and proteins for the 16:1, 10:1, and 7:1 treatments accounted for 97%, 77%, and 74% of the total biomass, respectively. This supports the idea that more carbon may have been assimilated into biomass in the 16:1 cultures, but also indicates that some cellular constituents are unaccounted for in the 10:1 and 7:1 treatments. Analytical errors could be the cause of the lower total mass balances in the 10:1 and 7:1 treatments, as the lipid, sugar, and protein assays are all sensitive to low biomass concentrations (Van Wychen and Laurens 2013, Laurens 2013).

Little is known about the effects of nitrogen source on biofuel production in microalgae. It takes less energy to assimilate ammonium into cellular nitrogen compared to nitrate (Falkowski and Raven 2013), suggesting that nitrogen source may impact productivity. Results of my experiments, however, showed that nitrogen source did not have an effect on growth, lipid content, or the fatty acid profile of *H. akashiwo*. Similarly, there was no difference in biodiesel quality for the FAMES produced by each treatment. This is most likely because, regardless of the nitrogen source, there was still a sufficient supply of nitrogen to support growth and necessary cellular reactions. This may have not been the case if light levels were lowered due to the energy demands of nitrate reduction; eight electrons, generated from photosynthesis, are required to reduce nitrate to ammonium (Glibert et al. 2016). However, the protein and nitrogen content for *H. akashiwo* were significantly higher for cultures supplemented with nitrate compared to ammonium. The protein demand for cells supplemented with nitrate may be higher as more enzymes are necessary to assimilate nitrogen compared to ammonium (Falkowski and Raven 2013, Glibert et al. 2016). The higher levels of particulate nitrogen may be due to the ability of *H. akashiwo* to store intracellular nitrogen (Kok et al. 2015). Protein content was calculated from the particulate nitrogen levels so it is difficult to determine how much of the intracellular nitrogen is stored nitrogen versus protein.

## **2.6 Conclusion**

Here, it was determined that growth and lipid content in *H. akashiwo* was highest with a nitrate to phosphate ratio of 16:1 compared to 10:1 or 7:1. However, there were no differences in growth, lipid content, or fatty acid profile of *H. akashiwo* when cultured on ammonium compared to nitrate. These findings indicate that biofuel

production by *H. akashiwo* is highest under N-replete media, and that reduced nitrogen sources from dairy waste, for example, may be used as a cost-effective alternative to nitrate.

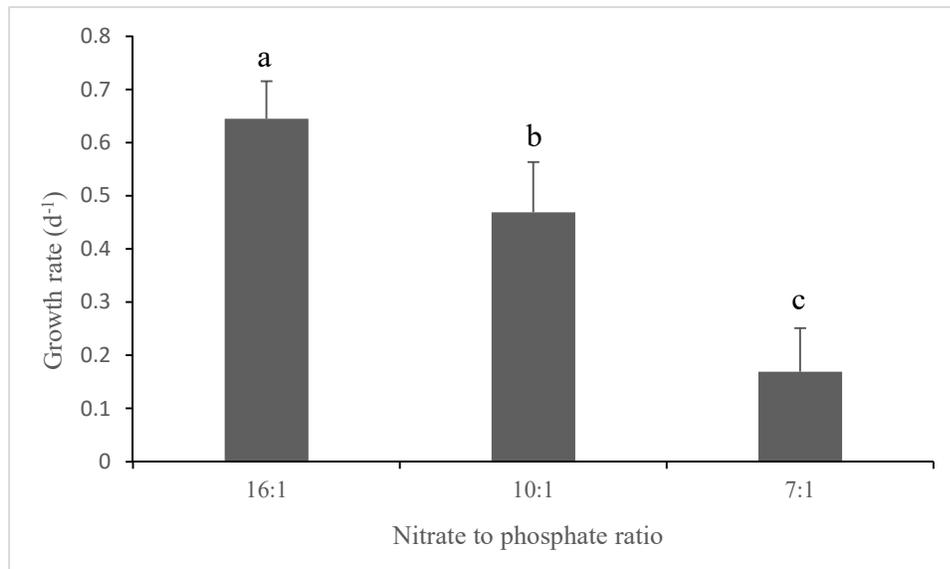


Figure 1: The growth rates of *Ta* cultured at nitrate to phosphate ratios of 16:1, 10:1, and 7:1 (total P= 36.2  $\mu$ M). Data are expressed as means  $\pm$  standard deviations (n=4). Different letters denote significant differences between groups using a one-way ANOVA and Tukey-Kramer HSD post-hoc testing ( $p < 0.05$ ).

Table 1 The percentage of lipids, monomeric sugars, and protein relative to the dry weight of *H. akashiwo* cultured at nitrate to phosphate ratios of 16:1, 10:1, and 7:1 (total P=36.2  $\mu$ M). Data are expressed as means (n=4). Different letters denote significant difference between groups using a one-way ANOVA and Tukey-Kramer HSD (p<0.05).

	<b>16:1</b>	<b>10:1</b>	<b>7:1</b>
% Lipid	23.5 $\pm$ 3.0 (a)	18.4 $\pm$ 6.0 (a)	9.07 $\pm$ 4.3 (b)
% Monomeric Sugar	44.7 $\pm$ 10.8 (a)	38.4 $\pm$ 7.0 (a)	39.4 $\pm$ 10.4 (a)
% Protein	29.2 $\pm$ 6.6 (a)	19.9 $\pm$ 8.3 (a)	25.5 $\pm$ 3.9 (a)

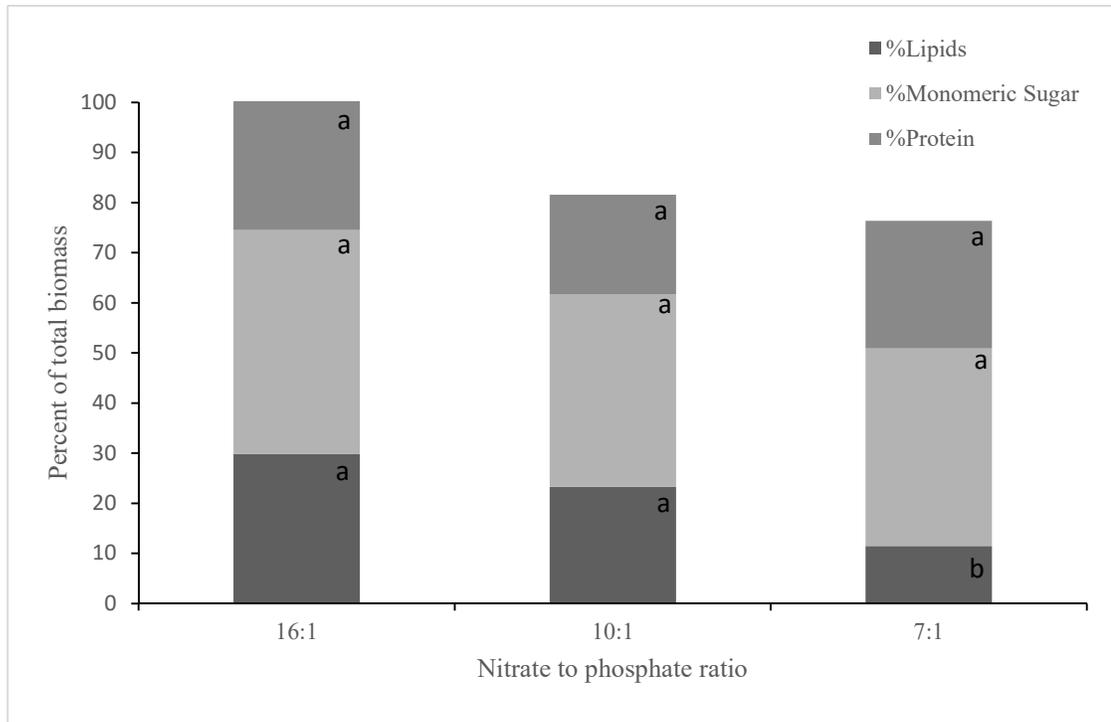


Figure 2: The percentage of lipids, monomeric sugars, and protein relative to the dry weight of *H. akashiwo* cultured at nitrate to phosphate ratios of 16:1, 10:1, and 7:1 (total P= 36.2  $\mu$ M). Data are expressed as means (n=4). Different letters denote significant differences between groups using a one-way ANOVA and Tukey-Kramer HSD ( $p < 0.05$ ).

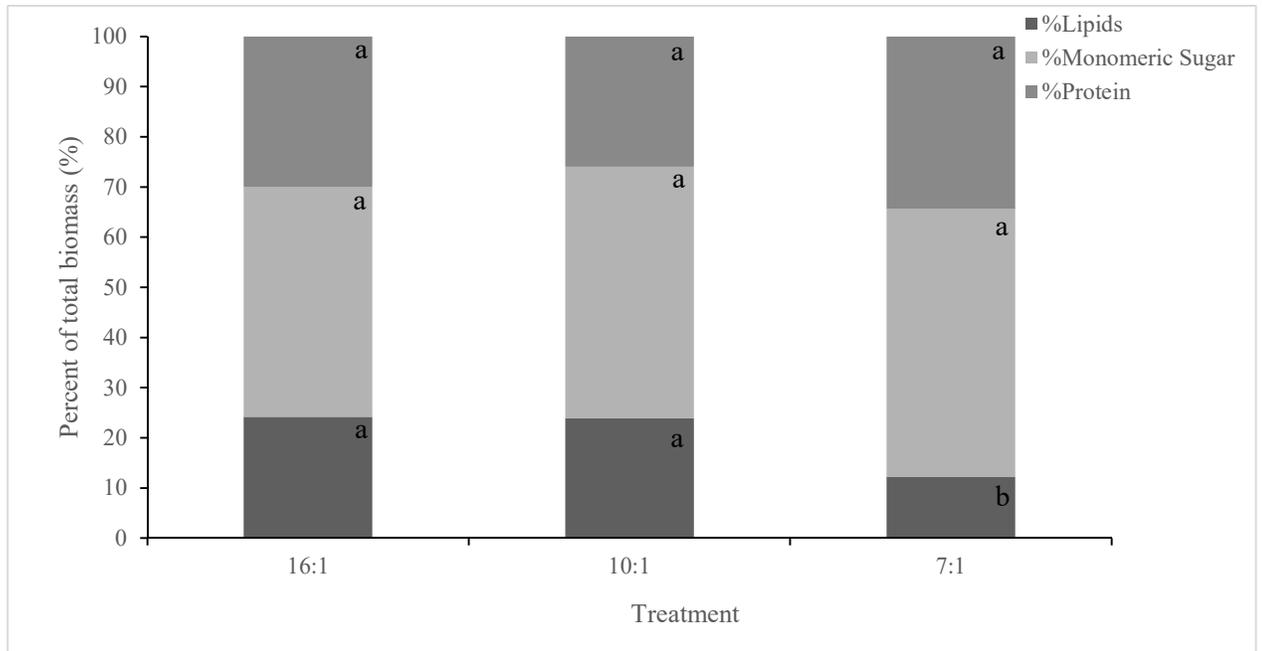


Figure 3: The percentage of lipids, monomeric sugars, and protein relative to the dry weight, and corrected to 100%, for *H. akashiwo* cultured at nitrate to phosphate ratios of 16:1, 10:1, and 7:1 (total P= 36.2  $\mu$ M). Data are expressed as means (n=4). Different letters denote significant differences between groups using a one-way ANOVA and Tukey-Kramer HSD ( $p < 0.05$ ).

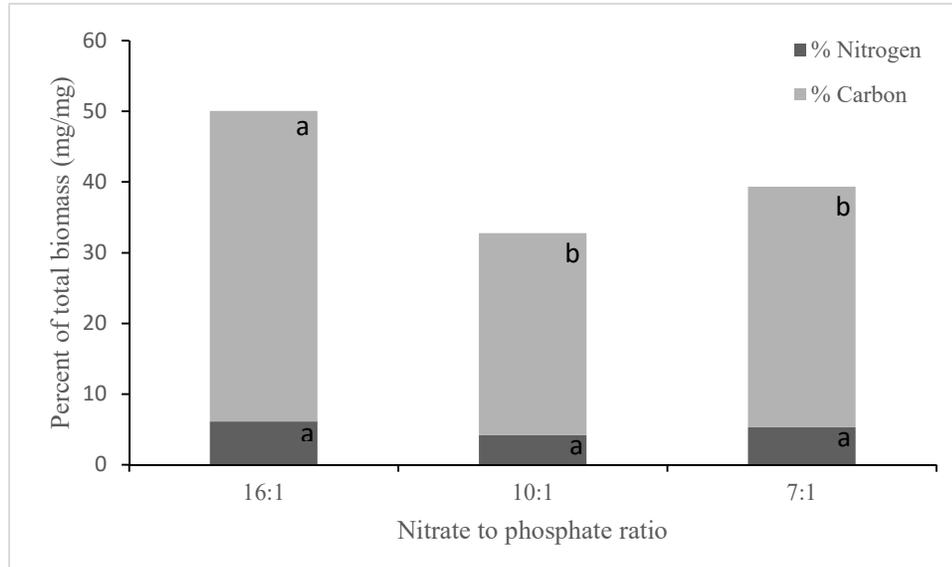


Figure 4: The percentage of particulate carbon and nitrogen relative to the dry weight of *H. akashiwo* cultured at nitrate to phosphate ratios of 16:1, 10:1, and 7:1 (total P= 36.2  $\mu$ M). Data are expressed as means (n=4). Different letters denote significant differences between groups using a one-way ANOVA and Tukey-Kramer HSD ( $p < 0.05$ ).

Table 2 The growth rate ( $d^{-1}$ ), dry weight (g/L), and chlorophyll *a* content ( $\mu\text{g/L}$ ) of *H. akashiwo* cultured semi-continuously with nitrate or ammonium as the nitrogen source, after cultures reached a constant growth rate (n=4). Data are expressed as means  $\pm$  standard deviations.

	Nitrogen Source	
	Nitrate	Ammonium
Growth rate ( $d^{-1}$ )	0.563 $\pm$ 0.057	0.592 $\pm$ 0.061
Dry weight (g/L)	0.1028 $\pm$ 0.002	0.1353 $\pm$ 0.084
Chlorophyll <i>a</i> ( $\mu\text{g/L}$ )	63.32 $\pm$ 15.22	78.13 $\pm$ 21.92

Table 3 The dissolved inorganic carbon ( $\mu\text{M}$ ), total percent carbon, nitrogen, and protein relative to the dry weight of *H. akashiwo* cultured semi-continuously with nitrate or ammonium as the nitrogen source, after cultures reached a constant growth rate (n=4). Data are expressed as means  $\pm$  standard deviations. Different letters denote significant differences between groups using a t-test ( $p < 0.05$ ).

	Nitrogen Source	
	Nitrate	Ammonium
Dissolved inorganic carbon ( $\mu\text{M}$ )	6965.13 $\pm$ 1473.0	7053.595 $\pm$ 1149.1
Particulate carbon (%)	58.15 $\pm$ 3.7	62.17 $\pm$ 17.2
Particulate nitrogen (%)	13.14 $\pm$ 0.4 (a)	11.45 $\pm$ 0.7(b)
Protein (%)	62.82 $\pm$ 1.8 (a)	54.71 $\pm$ 3.1 (b)

Table 4 The total lipids as a percentage of the dry weight, fatty acid profile, and biodiesel parameters for *H. akashiwo* cultured semi-continuously with nitrate or ammonium as the nitrogen source, after cultures reached a constant growth rate (n=4). Data are expressed as means  $\pm$  standard deviations.

	Nitrogen Source	
	Nitrate	Ammonium
Lipids (%)	10.18 $\pm$ 2.7	6.99 $\pm$ 1.7
Saturated FA (%)	66.13 $\pm$ 5.2	59.81 $\pm$ 4.6
Monounsaturated FA (%)	27.23 $\pm$ 3.4	28.95 $\pm$ 5.9
Polyunsaturated FA (%)	6.63 $\pm$ 3.6	11.24 $\pm$ 7.1
Cetane Number	61.92 $\pm$ 2.4	58.75 $\pm$ 5.1
Iodine Value	43.41 $\pm$ 13.5	60.28 $\pm$ 21.8
Saponification Value	215.10 $\pm$ 6.4	210.07 $\pm$ 7.9

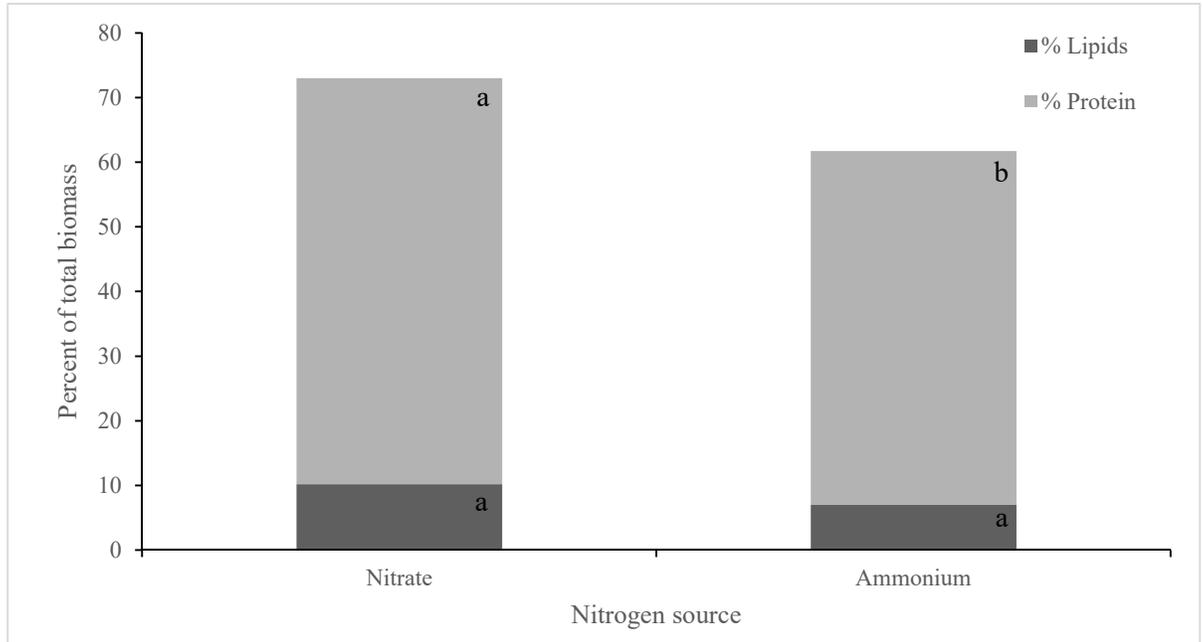


Figure 5: The percentage of lipids and protein relative to the dry weight of *H. akashiwo* cultured semi-continuously with nitrate or ammonium as the nitrogen source, after cultures reached a constant growth rate (n=4). Data are expressed as means. Different letters denote significant differences between groups using a t-test ( $p < 0.05$ ).

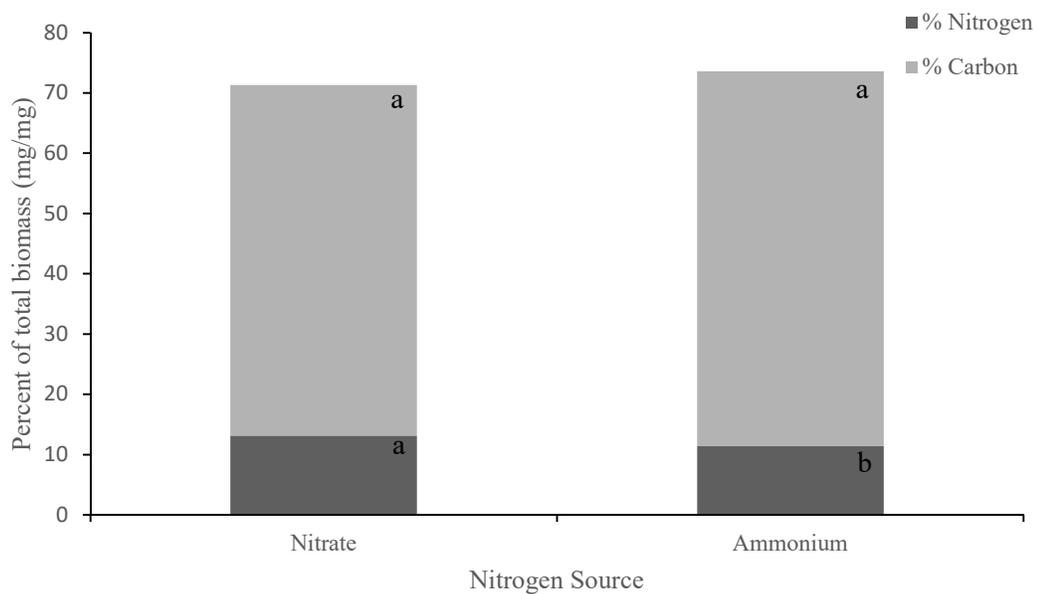


Figure 6: The percentage of particulate carbon and nitrogen relative to the dry weight of *H. akashiwo* cultured semi-continuously with nitrate or ammonium as the nitrogen source, after cultures reached a constant growth rate. Data are expressed as means (n=4). Different letters denote significant differences between groups using a t-test ( $p < 0.05$ ).

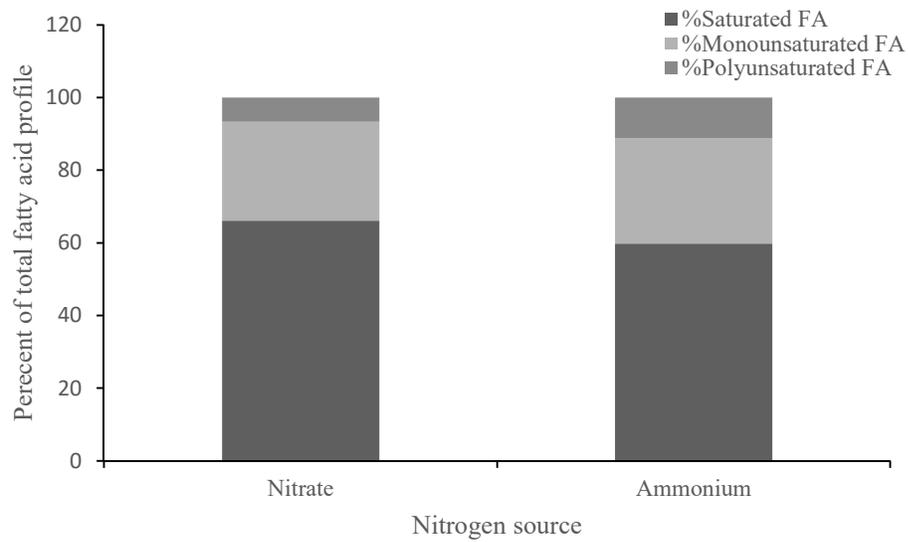


Figure 7: The percentage of saturated, monounsaturated, and polyunsaturated fatty acids relative to the total fatty acid content of *H. akashiwo* cultured semi-continuously with nitrate or ammonium as the nitrogen source, after cultures reached a constant growth rate. Data are expressed as means (n=4).

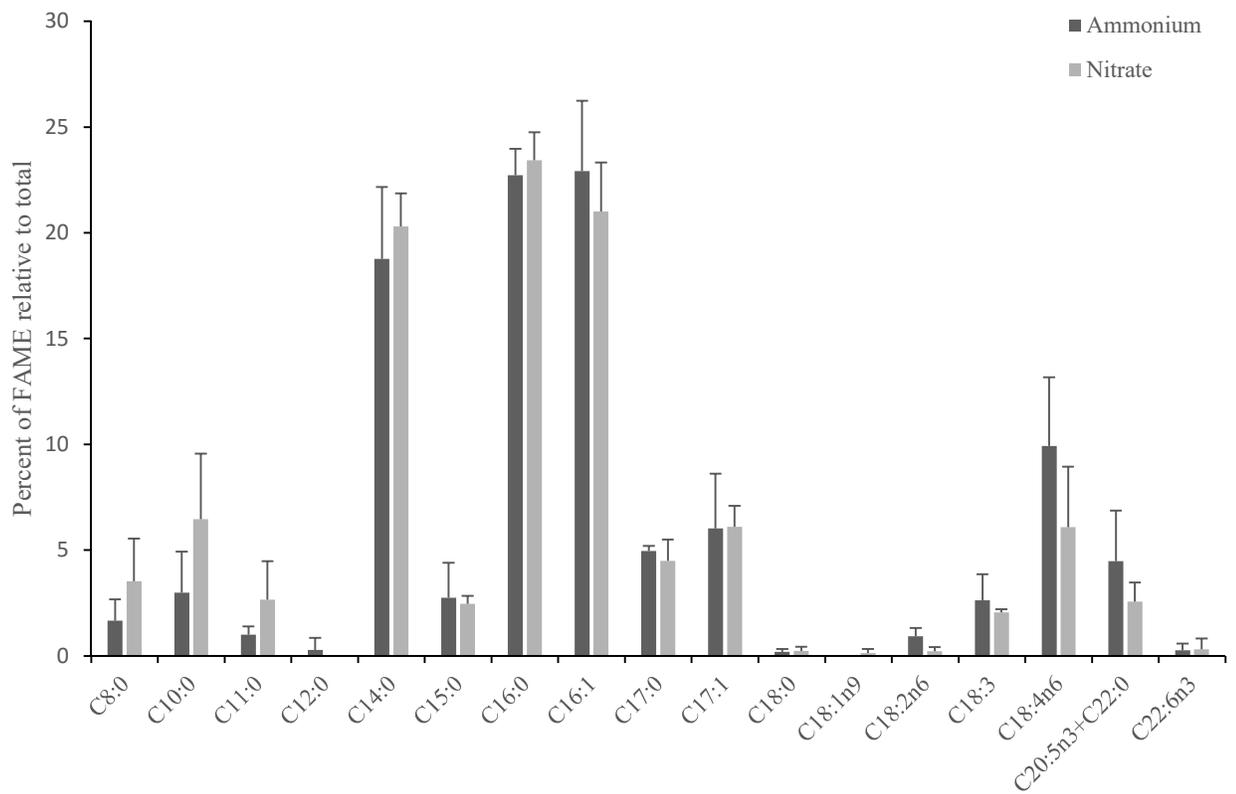


Figure 8: The fatty acid methyl ester (FAME) profile of *H. akashiwo* cultured semi-continuously with nitrate or ammonium as the nitrogen source, after cultures reached a constant growth rate. Data are expressed as means  $\pm$  standard deviations (n=4).

## REFERENCES

- Cagliari, A., Margis, R., dos Santos Maraschin, F., Turchetto-Zolet, A. C., Loss, G., & Margis-Pinheiro, M. (2011). Biosynthesis of triacylglycerols (TAGs) in plants and algae. *International Journal of Plant Biology*, 2(1), 10.
- Chen, M., Tang, H., Ma, H., Holland, T. C., Ng, K. S., & Salley, S. O. (2011). Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. *Bioresource Technology*, 102(2), 1649-1655.
- Cheng, Y., Zheng, Y., & VanderGheynst, J. S. (2011). Rapid quantitative analysis of lipids using a colorimetric method in a microplate format. *Lipids*, 46(1), 95-103.
- Chisti, Y. (2007). Biodiesel from microalgae. *Biotechnology Advances*, 25(3), 294-306.
- Davis, R., Aden, A., & Pienkos, P. T. (2011). Techno-economic analysis of autotrophic microalgae for fuel production. *Applied Energy*, 88(10), 3524-3531.
- Eustance, E., Gardner, R. D., Moll, K. M., Menicucci, J., Gerlach, R., & Peyton, B. M. (2013). Growth, nitrogen utilization and biodiesel potential for two chlorophytes grown on ammonium, nitrate or urea. *Journal of Applied Phycology*, 25(6), 1663-1677.
- Falkowski, P. G., & Raven, J. A. (2013). *Aquatic photosynthesis* Princeton University Press.
- Falkowski, P. G., Sukenik, A., & Herzig, R. (1989). Nitrogen limitation in *Isochrysis galbana* (Haptophyceae). ii. relative abundance of chloroplast proteins. *Journal of Phycology*, 25(3), 471-478.
- Folch, J., Lees, M., & Sloane-Stanley, G. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem*, 226(1), 497-509.
- Fuentes-Grünewald, C., Garcés, E., Alacid, E., Rossi, S., & Camp, J. (2013). Biomass and lipid production of dinoflagellates and raphidophytes in indoor and outdoor photobioreactors. *Marine Biotechnology*, 15(1), 37-47.

- Fuentes-Grünewald, C., Garcés, E., Alacid, E., Sampedro, N., Rossi, S., & Camp, J. (2012). Improvement of lipid production in the marine strains *Alexandrium minutum* and *Heterosigma akashiwo* by utilizing abiotic parameters. *Journal of Industrial Microbiology & Biotechnology*, 39(1), 207-216.
- Glibert, P. M., Wilkerson, F. P., Dugdale, R. C., Raven, J. A., Dupont, C. L., Leavitt, P. R., . . . Kana, T. M. (2016). Pluses and minuses of ammonium and nitrate uptake and assimilation by phytoplankton and implications for productivity and community composition, with emphasis on nitrogen-enriched conditions. *Limnology and Oceanography*, 61(1), 165-197.
- Guillard, R. R. (1975). Culture of phytoplankton for feeding marine invertebrates. *Culture of marine invertebrate animals* (pp. 29-60) Springer.
- Herndon, J., & Cochlan, W. P. (2007). Nitrogen utilization by the raphidophyte *Heterosigma akashiwo*: Growth and uptake kinetics in laboratory cultures. *Harmful Algae*, 6(2), 260-270.
- Hosaka, M. (1992). Growth characteristics of a strain of *Heterosigma akashiwo* (Hada) isolated from Tokyo bay, Japan. *Bulletin of Plankton Society of Japan (Japan)*,
- Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., & Darzins, A. (2008). Microalgal triacylglycerols as feedstocks for biofuel production: Perspectives and advances. *The Plant Journal*, 54(4), 621-639.
- Hutchins, D., Hare, C., Weaver, R., Zhang, Y., Firme, G., DiTullio, G., . . . Geesey, M. (2002). Phytoplankton iron limitation in the Humboldt current and Peru upwelling. *Limnology and Oceanography*, 47(4), 997-1011.
- Iwasaki, H., & Sasada, K. (1969). Studies on the red tide dinoflagellates-II. on *Heterosigma inlandica* appeared in Gokasho bay, Shima peninsula. *Bull.Jpn.Soc.Sci.Fish*, 35, 943-947.
- Kok, J. W. K., Yeo, D. C. J., & Leong, S. C. Y. (2015). Growth and physiological responses of a tropical toxic marine microalga *Heterosigma akashiwo* (Heterokontophyta: Raphidophyceae) from Singapore waters to varying nitrogen sources and light conditions. *Ocean Science Journal*, 50(3), 491-508.
- Laurens, L. M. (2013). Summative mass analysis of algal Biomass–Integration of analytical procedures. *Contract*, 303, 275-3000.

- National Research Council (U.S.), Committee on the Sustainable Development of Algal Biofuels., National Research Council (U.S.), Board on Agriculture and Natural Resources., National Research Council (U.S.), Board on Energy and Environmental Systems., National Academies Press (U.S.). (2012). *Sustainable development of algal biofuels in the united states*. Washington, D.C.: National Academies Press.
- Razzak, S. A., Hossain, M. M., Lucky, R. A., Bassi, A. S., & de Lasa, H. (2013). Integrated CO<sub>2</sub> capture, wastewater treatment and biofuel production by microalgae culturing—a review. *Renewable and Sustainable Energy Reviews*, *27*, 622-653.
- Sharp, J. H., Yoshiyama, K., Parker, A. E., Schwartz, M. C., Curless, S. E., Beaugard, A. Y., . . . Davis, A. R. (2009). A biogeochemical view of estuarine eutrophication: Seasonal and spatial trends and correlations in the Delaware estuary. *Estuaries and Coasts*, *32*(6), 1023-1043.
- Tüccar, G., & Aydın, K. (2013). Evaluation of methyl ester of microalgae oil as fuel in a diesel engine. *Fuel*, *112*, 203-207.
- Van Wychen, S., & Laurens, L. M. (2013). Determination of total carbohydrates in algal biomass. *Contract*, *303*, 275-3000.
- Van Wychen, S., & Laurens, L. (2013). Determination of total lipids as fatty acid methyl esters (FAME) by in situ transesterification. *Contract*, *303*, 375-300.
- Watanabe, M. (1982). Effects of physico-chemical factors and nutrients on the growth of *Heterosigma akashiwo* (Hada) from Osaka bay, Japan. *Jap.J.Phycol.(Sorui)*, *30*, 279-287.
- Xin, L., Hong-ying, H., Ke, G., & Ying-xue, S. (2010). Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus* sp. *Bioresource Technology*, *101*(14), 5494-5500.
- Zhang, Y., Fu, F., Whereat, E., Coyne, K. J., & Hutchins, D. A. (2006). Bottom-up controls on a mixed-species HAB assemblage: A comparison of sympatric *Chattonella subsalsa* and *Heterosigma akashiwo* (Raphidophyceae) isolates from the Delaware inland bays, USA. *Harmful Algae*, *5*(3), 310-320.

## Chapter 3

### BIOREMEDIATION OF INDUSTRIAL FLUE GAS AND BIOFUEL PRODUCTION USING *HETEROSIGMA AKASHIWO* GROWN ON DAIRY WASTE

#### 3.1 Abstract

Harmful emissions from industrial flue gas can be reduced by photosynthetic microalgae due to their ability to fix carbon dioxide using solar-derived energy. Microalgae have also been used to remove high levels of nutrients in dairy wastewater that can contribute to eutrophication and ground water pollution. These remediation processes result in algal biomass that can be used for biofuels and other bio-products. Here, the potential for biofuel production coupled with bioremediation of flue gas using the microalga, *Heterosigma akashiwo*, grown on dairy waste was evaluated. This alga may be able to metabolize the nitric oxide present in flue gas using a novel nitrate reductase enzyme, NR2-2/2HbN, to convert nitric oxide to nitrate. However, nitrate reductase (NR) activity may be inhibited by ammonium in dairy waste; therefore, NR activity was evaluated at varying nitrate to ammonium ratios of 1:0, 1:1, 1:10, and 1:40. NR activity was detected at all of the treatments, indicating that bioremediation of the ammonium in the dairy wastewater and the nitric oxide in flue gas may be able to occur simultaneously. Then, *H. akashiwo* was cultured on a factorial combination of 9% CO<sub>2</sub> or flue gas (9% CO<sub>2</sub> and 55 ppm nitric oxide), with ammonium or dairy waste, and the potential of *H. akashiwo* for biofuel production was evaluated. *H. akashiwo* was able to sustain growth on dairy waste and flue gas,

although growth rates were lower than the control treatment (ammonium and 9% CO<sub>2</sub>). NR activity was highest for cultures supplemented with flue gas and dairy waste (NH<sub>4</sub><sup>+</sup>=0.491 mM) together, confirming that nitrate reductase in *H. akashiwo* is not completely inhibited by ammonium. *H. akashiwo* was unable to significantly reduce the CO<sub>2</sub> or nitric oxide in the flue gas. *H. akashiwo* accumulated 2-5% of its total biomass as lipids indicating that when cultured on dairy waste and flue gas this alga is not suitable for biofuels. Interestingly, C20:5n3 (EPA) represented up to 20% of the total fatty acid methyl esters. *H. akashiwo* also produced C13:0, an uncommon fatty acid in algae, only when grown on flue gas.

### 3.2 Introduction

Carbon emissions from industrial flue gases are contributing to global climate change (EPA, 2014). Under the EPA Clean Power Plan, carbon emissions generated from power plants need to be reduced to 32% below 2005 levels by 2030 (EPA, 2015). Industrial flue gases also contain nitric oxide, another harmful greenhouse gas (EPA, 2014). Microalgae offer a sustainable method to reduce harmful emissions, as microalgae capture carbon through photosynthesis (Wilson et al. 2014, Huang et al. 2016). In this process, solar energy is converted to chemical energy and used to build carbon chains from carbon dioxide (Falkowski and Raven 2013). Algal biomass can then be converted to biofuels, bioplastics, animal feeds, pharmaceuticals, and other commercial products (National Research Council 2012). Microalgae, like *Scenedesmus acutus*, *Boytryococcus braunii*, and *Chlorella* sp., are suitable biofuel feedstocks when grown on industrial flue gas (Wilson et al. 2014, Huang et al. 2016, Kao et al. 2014). This process essentially turns harmful emissions into useful products.

Excess nutrients from agricultural waste also have negative effects on the environment by contributing to eutrophication and ground water pollution (Razzak et al. 2013). Microalgae have been used to remove high levels of nitrogen and phosphorus in dairy and piggery effluent (An et al. 2003, Mulbry et al. 2008, Woertz et al. 2009). Woertz et al. (2009) demonstrated the removal of 96 and 99% of ammonium and orthophosphate in dairy wastewater, respectively, after just 12 days of algal growth. Algal biomass cultured with dairy wastewater could also be used as a biofuel feedstock due to high fatty acid contents (Wang et al. 2009, Woertz et al. 2009). However, algal growth on dairy wastewater can be limited by low C:N and C:P ratios, so supplementation with CO<sub>2</sub>, specifically from flue gas, may increase productivity (Burlew 1953, Straka et al. 2000, Metcalf and Eddy 2003). Here, I evaluated the use of algae for the simultaneous bio-remediation of carbon and nitrogen emissions in industrial flue gas and nutrients in dairy wastewater.

Previous studies have demonstrated that the marine microalga, *Heterosigma akashiwo*, can utilize both carbon and nitric oxide present in flue gas (Stewart et al. 2015). The ability of this alga to assimilate nitric oxide may be due to the presence of a novel nitrate reductase, NR2-2/2HbN (Stewart and Coyne 2011). A proposed model of NR2-2/2HbN suggests that it may catalyze the conversion of nitric oxide to nitrate, followed by reduction of nitrate to nitrite. Previous studies showed that *H. akashiwo* can use a variety of reduced nitrogen sources such as ammonium and urea (Chapter 2, Zhang et al. 2006, Stewart and Coyne 2011), indicating that it would be suitable for growth on dairy wastewater. It is unknown however, if NR2-2/2HbN in *H. akashiwo* will be inhibited by the ammonium present in dairy waste as this is common in other algae (Jha et al. 2007, Poulsen and Kroger 2005, Song and Ward 2004). Ammonium

can inhibit nitrate reductase at the transcriptional level by changing the glutamine to glutamate ratio, where an increase in glutamine decreases NR activity (Vergara et al. 1998, Glibert et al. 2016). The inhibition of NR by ammonium is often concentration dependent, where an increase in nitrate can relieve the inhibition (Jha et al. 2007, Glibert et al. 2016). *H. akashiwo* will grow on nitrate and ammonium when both are present in equal concentrations with no significant decrease in nitrate reductase activity (Coyne, unpublished data), but higher ammonium to nitrate ratios have not been tested.

*H. akashiwo* has also demonstrated potential as a biofuel feedstock (Chapter 2, this thesis, Fuentes-Grunewald et al. 2012, Fuentes-Grunewald et al. 2013, Stewart et al. 2015). Outdoor cultures of *H. akashiwo* have reached lipid productivities of 56 mg/L/day with a maximum lipid content of 23%, and had fatty acid profiles desirable for biodiesel production (Fuentes-Grunewald et al. 2013). Previous studies using the Delaware Inland Bays strain (CCMP 2393) demonstrated total lipid contents of 26% under medium light conditions ( $560 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ). The algae were rich in palmitic acid and had the potential to create high quality biodiesel (Bianco 2013). In Chapter 2 of this thesis, *H. akashiwo* CCMP 2393 accumulated 23% of total biomass as lipids under N-replete conditions. The nitrogen source (ammonium vs. nitrate) did not have an effect on growth, lipid content, or fatty acid profile indicating that *H. akashiwo* may be able to use sustainable sources of inorganic nitrogen, such as dairy waste, as a growth medium for biofuel production.

In this study, I first investigated nitrate reductase activity at varying nitrate to ammonium concentrations to identify the potential of *H. akashiwo* for bioremediation of NO from flue gas when cultured with ammonium from dairy waste. As a second

objective, *H. akashiwo* was cultured on a factorial combination of CO<sub>2</sub> or flue gas containing CO<sub>2</sub> and NO, with ammonium or dairy wastewater. Here, I evaluated the potential for biofuel production coupled with bioremediation of flue gas when *H. akashiwo* was grown on dairy waste.

### 3.3 Materials and Methods

#### 3.3.1 Nitrate reductase activity under varying nitrate to ammonium ratios

*Heterosigma akashiwo* CCMP 2393 (Delaware Inland Bays isolate) cultures (n=3) were acclimated to varying nitrate to ammonium ratios of 1:0, 1:1, 1:10, and 1:40, with a total nitrogen concentration of 200  $\mu\text{M}$ , for 18 days at 25°C. Growth media was prepared using 20 ppt artificial seawater and amended with nitrate and/or ammonium, as well as f/2 nutrients (trace metals, phosphate, and vitamins, no silicate) (Guillard, 1975). Cultures were maintained semi-continuously, with dilutions every other day, at an irradiance of 150  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  on a 12-hour light dark cycle. The acclimated cultures within each treatment were then inoculated into experimental cultures at 42,000 cells/mL (n=3) at the appropriate nitrate to ammonium ratio. After two days, the experimental cultures were diluted back to 42,000 cells/mL. On day four, nitrate and/or ammonium were spiked into each 100 mL culture to ensure 200  $\mu\text{M}$  total nitrogen at the correct ratio. After one hour, duplicate 50 mL aliquots of each culture were centrifuged at 3,000 RPM for 5 minutes. Nitrate reductase activity was determined using the method optimized for *H. akashiwo* in Stewart and Coyne (2011). The cell pellet was immediately flash frozen in liquid nitrogen, and sonicated in 200 mM KPi extraction buffer (pH 7.9) to extract the cellular contents. KNO<sub>3</sub> was added in excess to the extract and the reaction was quenched with 1M zinc acetate after 15

minutes. N-methylphenozium methyl sulfate, HCl, sulfanilamide, and N-(1-Naphthyl)-ethylenediamine hydrochloride were added to the standards and the samples and the absorbance was measured at 543 nm. The amount of nitrite present was determined using linear regression of nitrite standards absorbance. Activity was then normalized to protein content using the BCA protein assay kit (Pierce, Rockford, IL).

### **3.3.2 Evaluation of growth and productivity on flue gas and dairy wastewater**

The dairy waste water used in this study was collected from Kilby Dairy Farm in Colora, MD on October 6<sup>th</sup>, 2014. The wastewater was anaerobically digested at the farm and stored in an underground storage tank. Anaerobic digestion is the microbially-mediated biochemical degradation of complex organic materials into simple organic molecules and dissolved nutrients (Lansing et al. 2008). Upon collection, the waste water was autoclaved, filtered through 20  $\mu\text{M}$ , 5  $\mu\text{M}$ , and 1  $\mu\text{M}$  filters, and stored at 10°C. One liter of the wastewater was filtered through a 0.7  $\mu\text{m}$  filter and stored at 4°C. Nutrient concentrations were measured using the Seal nutrient auto-analyzer 3 (Mequon, WI). The nitrate, ammonium, and phosphate concentrations in the dairy wastewater were 12.95 mM, 72.2 mM, and 0.99 mM, respectively. The waste water was then diluted to 0.579 mM total nitrogen.

Growth media were prepared with 20 ppt artificial seawater buffered with 20 mM HEPES (pH=7.4). Phosphate was added to the dairy waste growth medium to bring the total phosphate concentration to 0.0362 mM. Iron, trace metals, and vitamins were in f/2 concentrations in the growth media for the ammonium treatments (Guillard 1975), but were not added to the dairy waste growth medium. *H. akashiwo* cultures were acclimated to each treatment in 800 mL glass columns with 150  $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$  illumination on a 12-hour light and dark cycle under the following conditions: (1)

9% CO<sub>2</sub>, 0.579 mM ammonium, and 0.0362 mM phosphate (control cultures) (designated NH<sub>4</sub>CO<sub>2</sub>), (2) industrial flue gas containing 55 ppm NO and 9% CO<sub>2</sub> with 0.579 mM ammonium and 0.0362 mM phosphate (designated NH<sub>4</sub>FG) , (3) dairy wastewater diluted to 0.579 mM total nitrogen supplemented with 9% CO<sub>2</sub> (designated ADCO<sub>2</sub>), and (4) dairy wastewater diluted to 0.579 mM total nitrogen supplemented with industrial flue gas (designated ADFG). Cultures were grown semi-continuously and were considered acclimated to these conditions when growth rates were constant (Table 4). Growth rates was calculated as:

$$\mu = \ln(N_2/N_1) / (t_2 - t_1)$$

where N is the dry weight (g/L) and t is the time in days.

Experimental cultures (n=4) were inoculated at 250,000 cells/mL and maintained semi-continuously. Growth was monitored every other day by dry weights and each culture was diluted back to the starting cell concentration of 250,000 cells/mL. Each culture was sampled every other day for oven dry weight and chlorophyll a concentration as described in Chapter 2.

Once the cultures reached a consistent growth rate each culture was sampled for carbohydrate analysis, fatty acid methyl ester analysis, and particulate carbon and nitrogen elemental analysis as described in Chapter 2. Nitrate reductase activity was measured as described in the previous section.

Table 5 Gas constituents, nitrogen, and phosphate concentrations in each treatment group where AD and FG refer to anaerobically-digested dairy wastewater and flue gas, respectively.

<b>Treatment</b>	<b>Gas</b>	<b>Total Nitrogen (mM)</b>	<b>Ammonium (mM)</b>	<b>Nitrate (mM)</b>	<b>Phosphate (mM)</b>
<b>NH<sub>4</sub><sup>+</sup> + CO<sub>2</sub></b>	9% CO <sub>2</sub>	0.579	0.579	0	0.0362
<b>AD + CO<sub>2</sub></b>	9% CO <sub>2</sub>	0.579	0.491	0.0883	0.0362
<b>NH<sub>4</sub><sup>+</sup> + FG</b>	9% CO <sub>2</sub> + 55 ppm Nitric oxide	0.579	0.579	0	0.0362
<b>AD + FG</b>	9% CO <sub>2</sub> + 55 ppm Nitric oxide	0.579	0.491	0.0883	0.0362

### **3.3.3 Evaluation of bioremediation potential for flue gas**

To evaluate bioremediation potential, each culture was sampled for dissolved inorganic carbon every other day as described in Chapter 2. The concentration of aqueous nitric oxide was measured every other day using a nitric oxide sensor (ISO-NOP, World Precision Instruments, Sarasota, FL), that can detect nitric oxide from 0.3 nM to 100  $\mu$ M, as described in Stewart and Coyne (2011). Nitric oxide concentrations were determined by linear regression using a sensor calibrated with  $\text{KNO}_2$ . Samples were also taken in triplicate from cell-free columns containing ammonium and flue gas (n=1), or dairy wastewater and flue gas (n=1) to evaluate the bioremediation potential of cultures with algae.

### **3.3.4 Statistical Analysis**

The data for both experiments were checked for normality and equal variances using the Shapiro-Wilk and Levene tests, respectively. For the nitrate reductase activity experiment, an ANOVA was performed to determine significant differences between treatments using  $p < 0.05$  as the cut-off for statistical significance. Once a statistical difference was detected by the one-way ANOVA, a Tukey's HSD post hoc test was performed. For the flue gas and dairy wastewater experiment, a two-way ANOVA was performed on full factorial models for each variable to determine if there was an interaction effect present between the gas constituents and nitrogen source. If there was a significant interaction, interaction plots were analyzed to determine where the interaction occurred. T-tests were used to compare the  $\text{NH}_4\text{CO}_2$  and  $\text{NH}_4\text{FG}$  treatments. All statistical tests were performed using JMP Pro 12.1.0 (SAS, Cary, NC).

## **3.4 Results**

### **3.4.1 Nitrate reductase activity under varying nitrate to ammonium ratios**

There was a significant difference in nitrate reductase activity with nitrate to ammonium ratios ( $p=0.0018$ ). The nitrate reductase activity in the 1:0 nitrate to ammonium treatment ( $0.0177 \pm 0.003$ ) was significantly higher than in the 1:10 ( $0.0109 \pm 0.002$ ) and the 1:40 treatment ( $0.008 \pm 0.0004$ ). Nitrate reductase activity was also significantly higher for the 1:1 treatment ( $0.0134 \pm 0.001$ ) as compared to the 1:40 treatment. There was no significant difference in nitrate reductase activity between the 1:0 and 1:1 treatment.

### **3.4.2 Bioremediation of flue gas and dairy waste**

#### **3.4.2.1 Growth**

Comparisons of all treatments were conducted by building a full factorial model, and then evaluating the model for interactions. If the p-value for the interaction term was less than 0.05, there was an interaction effect. Algal growth was highest for the  $\text{NH}_4\text{CO}_2$  cultures. Growth was lower for the  $\text{NH}_4\text{FG}$  cultures compared to the  $\text{NH}_4\text{CO}_2$  cultures, and was the lowest for both the  $\text{ADFG}$  and  $\text{ADCO}_2$  cultures ( $p<0.0001$ , Table 5). There was an interaction effect for the dry weights. Dry weights for the cultures supplemented with ammonium were the highest, while cultures supplemented with  $\text{ADCO}_2$  had the lowest dry weight ( $p<0.001$ , Table 5).

#### **3.4.2.2 Nitrate reductase activity**

There was an interaction effect between the gas constituent and nitrogen source for nitrate reductase activity ( $p=0.0121$ ). Nitrate reductase activity was highest for

ADFG cultures, and lowest for ADCO<sub>2</sub> cultures (Figure 10). Nitrate reductase activity was not significantly different in the NH<sub>4</sub>CO<sub>2</sub> and NH<sub>4</sub>FG treatments.

#### **3.4.2.3 Bioremediation potential of flue gas**

The bioremediation potential of *H. akashiwo* was evaluated by analyzing the dissolved inorganic carbon content and nitric oxide content in the NH<sub>4</sub>FG and ADFG cultures compared to identical columns with no added algae. There was no interaction between the nitrogen source and the presence of algae for dissolved inorganic carbon ( $p=0.215$ ), and no significant differences between the treatments (Table 7). The data for the nitric oxide concentration could not be normalized due to the low values of nitric oxide for the ammonium cultures with algae (Table 7). A comparison of the dairy waste cultures using a t-test showed no difference in nitric oxide concentration.

#### **3.4.2.4 Biochemical profile and biodiesel potential**

There was a significant interaction effect among the gas constituent and the nitrogen source for the percentage of nitrogen within the total biomass ( $p=0.0016$ , Table 8, Figure 12). The cultures supplemented with CO<sub>2</sub> had the highest percentage of nitrogen, where the ADCO<sub>2</sub> cultures had the most. The flue gas culture supplemented with ammonium had higher nitrogen than the dairy waste culture ( $p=0.0016$ , Table 8, Figure 12). Data for the percentage of carbon within the total biomass was not normal and could not be transformed, therefore it was not statistically compared.

There was a significant interaction effect for the monomeric sugar ( $p=0.0173$ ) and protein ( $p=0.0016$ ) content of *H. akashiwo*. Monomeric sugars were highest in the ADFG cultures and lowest in the ADCO<sub>2</sub> cultures (Figure 13). In contrast, the protein

content was highest in the ADCO<sub>2</sub> cultures, and lowest in the ADFG cultures (Figure 13). Lipid content was only compared for the NH<sub>4</sub>CO<sub>2</sub>, NH<sub>4</sub>FG, and ADFG cultures as there was not enough biomass to measure the lipid content accurately for the ADCO<sub>2</sub> treatment. There was no significant difference in the lipid content between treatments (Figure 13).

The main fatty acid constituents in all treatments were C14:0, C15, C16:1, and C20:5n3 plus C22:0 (Figure 15). There was no significant difference in the C14:0 content between treatments. C14:1 was significantly higher in the NH<sub>4</sub>CO<sub>2</sub> and NH<sub>4</sub>FG cultures than in the ADFG culture (p=0.001). C16:0, C17:0, and C18:1n9 significantly decreased for cultures supplemented with flue gas, compared to the NH<sub>4</sub>CO<sub>2</sub> cultures. C18:0 and C18:2n6 were significantly higher in the ADFG cultures, compared to the NH<sub>4</sub>CO<sub>2</sub> culture. There was no difference in the amount of C18:0 and C18:2n6 in the flue gas cultures, but there was a significant increase from the NH<sub>4</sub>CO<sub>2</sub> culture to the NH<sub>4</sub>FG culture. C20:5n3 and C22:0 have the same retention time, so they must be combined in the analysis. The amount of C20:5n3 plus C22:0 was significantly higher for the ADFG cultures compared to both the NH<sub>4</sub>CO<sub>2</sub> and NH<sub>4</sub>FG cultures. It should also be noted that C13:0 was present in the cultures supplemented with flue gas, but not present in the NH<sub>4</sub>CO<sub>2</sub> cultures. These could not be compared statistically due to non-normal distributions.

Saponification values for the ADFG cultures were significantly lower than both the NH<sub>4</sub>CO<sub>2</sub> and NH<sub>4</sub>FG cultures (Table 9). There was no significant difference in the iodine values (Table 9). Cetane numbers could not be compared between the 3 treatments due to non-normal data that could not be normalized by transformation.

### 3.5 Discussion

Nitrate reductase (NR) activity was detected in *H. akashiwo* even when ammonium was 40 times higher than nitrate in the growth media. Interestingly, ammonium has been shown to completely inhibit transcription of NR in other algae such as *Cylindrotheca fusiformis* and *Dunaliella tertiolecta* (Poulsen and Kroger 2005, Song and Ward 2004). Previous studies have found that the *NR1* transcript in *H. akashiwo* was still expressed even when cultures were acclimated to ammonium (Coyne 2010). *NR1* was also detected in nitrate starved cultures in this study, indicating that nitrate reductase may be constitutively expressed in this alga. Here, NR activity was still detected when ammonium was 10 and 40 times higher than nitrate in the growth media but there was a significant reduction in activity as compared to cultures supplemented with just nitrate. The total nitrogen concentration in the media remained the same in all treatments, so it is logical that the NR activity would decrease corresponding to the concentration of nitrate in the media, that is NR activity should be lower in cultures with less nitrate. NR activity in the 1:10 and 1:40 treatment was reduced by 1.6 and 2.2-fold, respectively, as compared to the control (1:0). This indicates that nitrate reductase activity is not strictly tied to the amount of nitrate in the medium and may not be influenced by the presence of ammonium.

The ammonium in the media may be repressing nitrate reductase transcription and/or activity. *NR1* transcript levels of *H. akashiwo* analyzed in Coyne 2010 after the addition of nitrate to ammonium-acclimated cultures increased. This indicated that *NR1* repression from ammonium can be lessened by the addition of nitrate (Coyne 2010). A similar result was demonstrated in this study where the presence of ammonium was shown to decrease, but not completely inhibit all NR activity. The presence of NR activity, even when ammonium is in higher concentrations than

nitrate, indicates that bioremediation of the ammonium in dairy wastewater and the nitric oxide in flue gas may occur simultaneously. If so, this offers a unique solution to pollution problems caused by dairy wastewater and flue gas, as both could now be simultaneously remediated using microalgae. The end product of this process would be algal biomass that could be sold commercially for biofuels.

*H. akashiwo* was supplemented with ammonium and carbon dioxide (designated as  $\text{NH}_4\text{CO}_2$ ), dairy wastewater and carbon dioxide (designated as  $\text{ADCO}_2$ ), ammonium and flue gas (designated as  $\text{NH}_4\text{FG}$ ), and dairy wastewater and flue gas (designated as  $\text{ADFG}$ ). Growth rates were lowest for both dairy waste treatments, indicating that growth medium made with dairy wastewater does not support algal growth as well as the modified f/2 medium used in the control treatment. This may be due to lower levels of trace metals or vitamins, as nitrogen and phosphate concentrations were the same for all of the treatments. Micronutrient concentrations in the dairy wastewater were not measured here. Trace metals and vitamins are common co-factors for many enzymes and therefore are necessary for optimal algal growth (Falkowski and Raven 2013). Alternatively, there may have been something in the dairy waste, such as bacteria or toxins, that inhibited algal growth.

Interestingly, there was also a decrease in growth rate from the  $\text{NH}_4\text{CO}_2$  cultures to the  $\text{NH}_4\text{FG}$  cultures. This indicates that the nitric oxide present may have an inhibitory effect on growth as compared to growth on  $\text{CO}_2$ . Inhibition of growth from high concentrations of NO has been observed in other algal species (Liu et al. 2014). Previous studies have demonstrated an increase in growth rate of *H. akashiwo* when it was cultured on flue gas compared to air (Stewart et al. 2015). Growth rates for the  $\text{NH}_4\text{FG}$  cultures in this study were similar to previously published growth rates

for *H. akashiwo* supplemented with flue gas (Stewart et al. 2015). It is possible that the CO<sub>2</sub> present in the flue gas has a more positive effect on growth than the nitric oxide does.

Nitrate reductase activity was the highest in the ADFG treatment and the lowest in the ADCO<sub>2</sub> treatment. This difference is likely due to the presence of nitric oxide in the flue gas. Interestingly, NR activity was higher in the ADFG cultures compared to the NH<sub>4</sub>FG cultures. About 15% of the total nitrogen in the dairy wastewater was nitrate (Table 4) so it may be that nitrate reductase was upregulated in response to the nitrate present in the growth medium rather than the introduction of nitric oxide with the flue gas. Nitrogen content was higher in cultures supplemented with CO<sub>2</sub>, with the highest in the ADCO<sub>2</sub> cultures indicating that although the NR activity was the lowest, these cultures were able to take up and assimilate more nitrogen than the cultures supplemented with flue gas.

Comparisons of the concentration of dissolved inorganic carbon in medium with and without added algae showed no significant difference indicating that *H. akashiwo* was not assimilating a significant amount of CO<sub>2</sub>. Previous studies of *H. akashiwo* comparing growth and productivity on air and flue gas demonstrated a significant increase in storage carbohydrates when the alga was given flue gas compared to air, suggesting that *H. akashiwo* was able to assimilate the excess CO<sub>2</sub> present in the flue gas (Stewart et al. 2015). In my experiment, the algae are most likely assimilating excess CO<sub>2</sub> as well, but the uptake was undetectable due to the high levels of CO<sub>2</sub>. The CO<sub>2</sub> was also most likely being supplied to the column faster than the algae could assimilate it. Future studies should examine the ability of *H. akashiwo*

to remediate flue gas that is recycled and bubbled into the algal growth media multiple times.

Similarly, no difference in nitric oxide was detected, indicating that *H. akashiwo* is not significantly reducing the amount of nitric oxide present in the flue gas. However, NR activity was detected in cultures without nitrate and supplemented with flue gas, indicating that the algae may be assimilating a small, undetectable amount of nitric oxide. Results of this experiment indicated that *H. akashiwo* can withstand high levels of CO<sub>2</sub> and nitric oxide making it suitable for the partial bioremediation of the carbon dioxide present in flue gas. Recycling flue gas to minimize fresh CO<sub>2</sub> demand is a method employed in micro-algal culturing for biofuel production (Davis et al. 2011). It may be possible for *H. akashiwo* to remove a significant amount of CO<sub>2</sub> and nitric oxide from flue gas after multiple remediation cycles.

The ADFG cultures had the highest monomeric sugar content at 17% of its total biomass, and the ADCO<sub>2</sub> cultures had the lowest content of monomeric sugar with about 3% of its total biomass. Conversely, the ADCO<sub>2</sub> cultures had the highest protein content while the ADFG cultures had the lowest. Here the largest cellular constituent was protein at 46-60% of the algal biomass, where cultures supplemented with CO<sub>2</sub> had a higher protein content than the flue gas cultures. This indicates that the nitric oxide present in the flue gas may be inhibiting protein production by altering gene expression. Nitric oxide can act as a signaling molecule in many plants and plays an important role in gene induction (Grun et al. 2005). This may also account for the lower growth rates observed in the NH<sub>4</sub>FG cultures compared to the NH<sub>4</sub>CO<sub>2</sub> cultures.

Monomeric sugars only comprised 3-17% of the biomass and lipids only comprised 2-5% of the biomass. These findings align closely with the biochemical make up reported in Chapter 2, where the algal biomass of *H. akashiwo* cultured on nitrate and ammonium was about 60% protein and 10% lipids. However, this does not correspond to the high lipid content (23%) achieved at the 16:1 nitrate to phosphate ratio in Chapter 2. The sum of these cellular constituents for all treatments equal 60-70% of the total algal biomass, indicating that there are cellular constituents that are unaccounted for. This may be due to limitations of the analytical techniques employed as all are sensitive to low biomass concentrations (Van Wychen and Laurens 2013, Laurens 2013).

The low percentage of lipids in *H. akashiwo* for this experiment suggests that this species is not suitable for biofuel production. Other studies have demonstrated that *H. akashiwo* can accumulate about 20-30% of its total biomass as lipids (Chapter 2, Fuentes-Grunewald et al. 2012, Fuentes-Grunewald et al. 2013, Bianco 2013). In contrast, *H. akashiwo* did accumulate a considerable amount of proteins (45-60%) in all treatments. Protein-rich algae have been targeted as a source of animal feed (Duong et al. 2015, Lum et al. 2013, Lodge-Ivey et al. 2014). Microalgae used for the treatment of swine manure wastewater can produce high-value animal feeds, accumulating 46% of its total biomass as protein and 4% of its total fatty acid content as omega-3 fatty acids (Zhou et al. 2012). Here, *H. akashiwo* accumulated up to 60% of its total biomass as protein and up to 20% of its total fatty acid content as the omega-3 fatty acid, EPA, indicating that *H. akashiwo* shows promise as a feed stock for animal fuels, especially when compared to similarly cultured algae. Unfortunately, *H. akashiwo* is known to be a harmful alga (Chang et al. 1990, Honjo et al. 1993),

although the toxicity of the Delaware Inland Bays strain used here is unclear. The toxicity of this strain of *H. akashiwo* would need to be fully evaluated before using it as an animal-feed. Alternatively, whole algae hydrothermal liquefaction could be used to produce biofuel from *H. akashiwo* (Bidby et al. 2013).

The predominant fatty acids profiled in all treatments were C14:0, C15:0, C16:0, C16:1, and C20:5n3 plus C22:0. This supports the findings of a previous study that demonstrated C16:0, C16:1, and C20:5n3 were the predominant fatty acids in *H. akashiwo* cultured on flue gas (Stewart et al. 2015). These specific fatty acids are involved in the formation of sulfoquinovosyl diacylglycerol (SQDG), a thylakoid sulfolipid (Keusgen et al. 1997, Benning 1998). SQDG is used to support an increase in the photosynthetic requirements when *H. akashiwo* is cultured with high concentrations of CO<sub>2</sub>. C14:0 was likely present in high concentrations as it is a precursor to C16:0 and most other long chain fatty acids (Cagliari et al. 2011). Interestingly, C13 content increased in the presence of flue gas from 0.35% of the total fatty acid profile to 5.7% and 9.1% in the NH<sub>4</sub>FG and ADFG cultures, respectively. C13:0 is such an uncommon fatty acid in algae that it is often used as an internal recovery standard (NREL, 2013), so the function of C13:0 in algal cells is unknown. The change in C13:0 could also be due to changes in the microbial community associated with *H. akashiwo* under nitric oxide exposure as the presence of odd chain fatty acids in algal cultures are often due to bacteria (Petkov et al. 2007). However, the presence of C13:0 in just the cultures supplemented with flue gas indicates that there may be a biological reason for the presence of odd chain fatty acids. C13:0 may be produced by NO-initiated lipid peroxidation where 18C fatty acids are cleaved at the C-13 position (Guschina et al. 2006, Hogg et al. 1999). The C18 fatty acids C18:0,

C18:1n9, and C18:2n6 are highest in the cultures supplemented with flue gas and may be acting as protection from harmful free radicals (Hogg et al. 1999).

### **3.6 Conclusion**

*H. akashiwo* can sustain growth on diluted dairy wastewater and industrial flue gas. In this study, *H. akashiwo* was unable to remediate the CO<sub>2</sub> in the flue gas. Future studies should examine the bioremediation potential of this alga where the flue gas is constantly recycled. Low total lipid contents indicate that *H. akashiwo* supplemented with dairy wastewater and/or flue gas may not be suitable for biofuel production. However, the lipid content of *H. akashiwo* supplemented with dairy waste and flue gas reached almost 20% indicating that adjustments in these culturing conditions, such as higher light levels or different nutrient concentrations, may induce more lipid accumulation. Additionally, high protein content supports the use of this alga for animal feed if it can be proven to be non-toxic.

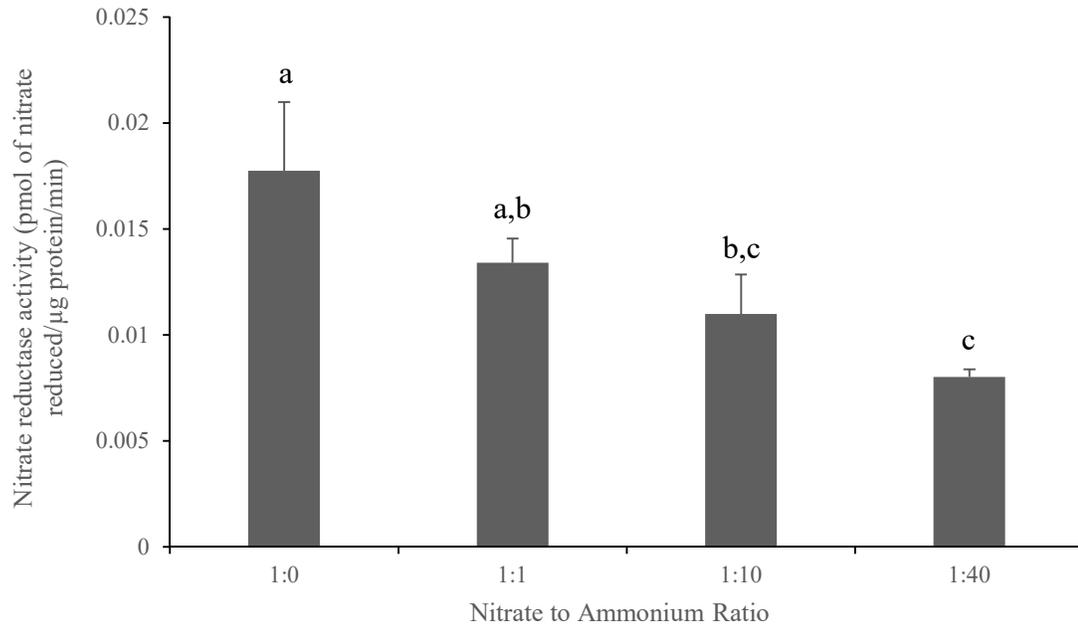


Figure 9: The nitrate reductase activity (pmoles of nitrate reduced/ $\mu\text{g}$  protein/min) in *H. akashiwo* following a three-week acclimation to culture conditions (total nitrogen= $200 \mu\text{M}$ ). Data are expressed as means  $\pm$  standard deviation ( $n=3$ ). Different letters denote significant differences between groups using a one-way ANOVA and Tukey-Kramer HSD ( $p<0.05$ ).

Table 6 The growth rate ( $d^{-1}$ ), cell density (cells/mL), dry weight (g/L), and chlorophyll *a* content ( $\mu\text{g/L}$ ) of *H. akashiwo* cultured semi-continuously on ammonium and carbon dioxide, dairy waste and carbon dioxide, ammonium and flue gas, and dairy waste and flue gas. Data are expressed as means  $\pm$  standard deviations (n=4) and an asterisk denotes significant interactions. Values are the average over days 2-14.

	<b>NH<sub>4</sub>CO<sub>2</sub></b>	<b>ADCO<sub>2</sub></b>	<b>NH<sub>4</sub>FG</b>	<b>ADFG</b>
Growth rate ( $d^{-1}$ ) *	0.59 $\pm$ 0.07	0.12 $\pm$ 0.07	0.34 $\pm$ 0.01	0.17 $\pm$ 0.04
Dry weight (g/L) *	0.09 $\pm$ 0.008	0.04 $\pm$ 0.009	0.09 $\pm$ 0.002	0.07 $\pm$ 0.008
Chl <i>a</i> ( $\mu\text{g/L}$ )	1804.5 $\pm$ 83.9	263.5 $\pm$ 60.2	1462.3 $\pm$ 205.7	499.8 $\pm$ 241.0

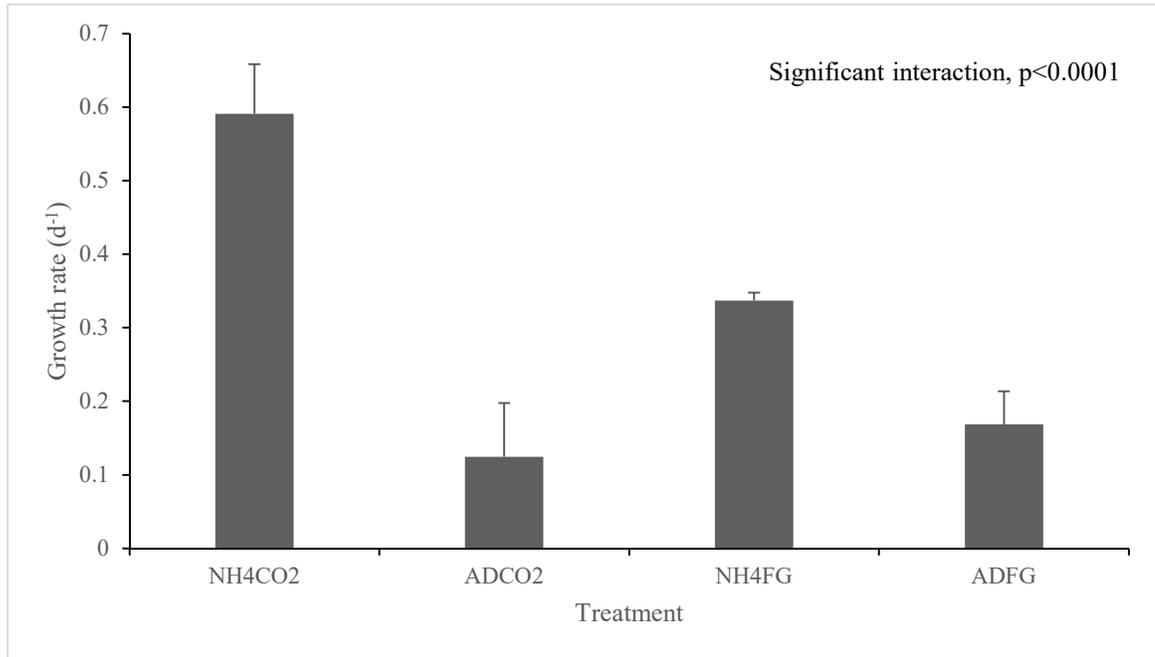


Figure 10: The growth rate ( $d^{-1}$ ) of *H. akashiwo* cultured semi-continuously on ammonium and carbon dioxide, dairy waste and carbon dioxide, ammonium and flue gas, and dairy waste and flue gas. Data are expressed as means  $\pm$  standard deviations ( $n=4$ ). Values are an average over days 2-14.

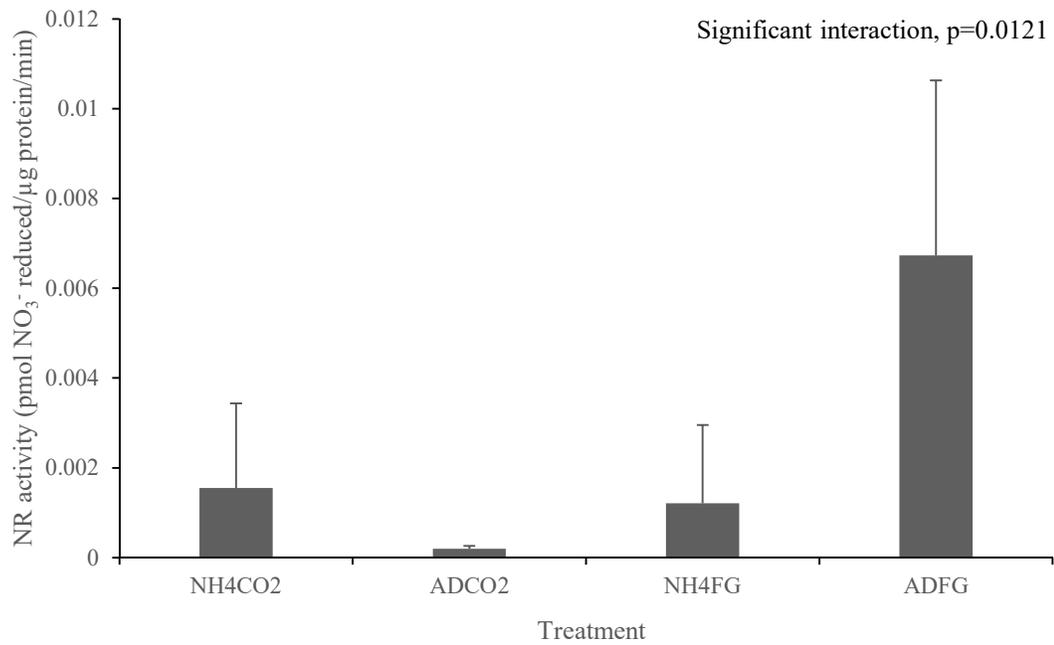


Figure 11: Nitrate reductase activity (pmole nitrate reduced/ $\mu$ g protein/min) for *H. akashiwo* cultures grown on ammonium and carbon dioxide, dairy waste and carbon dioxide, ammonium and flue gas, and dairy waste and flue gas. Data are expressed as means  $\pm$  standard deviations (n=4). Samples were taken once cultures reached a constant growth rate.

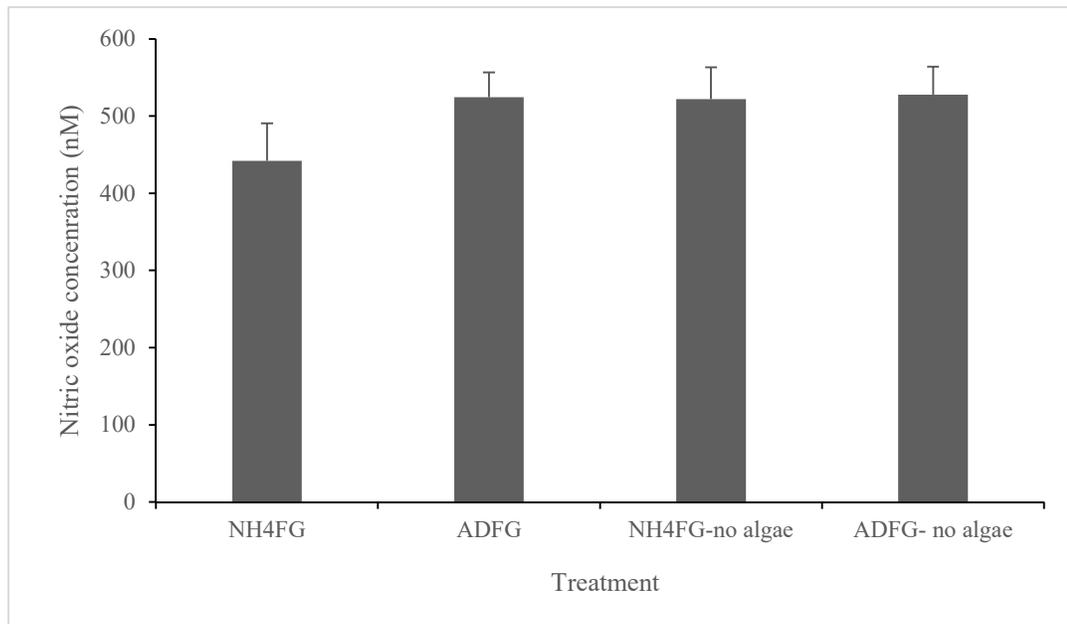


Figure 12: Aqueous nitric oxide concentration (nM) for *H. akashiwo* cultures grown on ammonium and carbon dioxide, dairy waste and carbon dioxide, ammonium and flue gas, and dairy waste and flue gas. Data are expressed as means  $\pm$  standard deviations (n=3). Values are averages over days 2-14. Samples without algae are technical replicates.

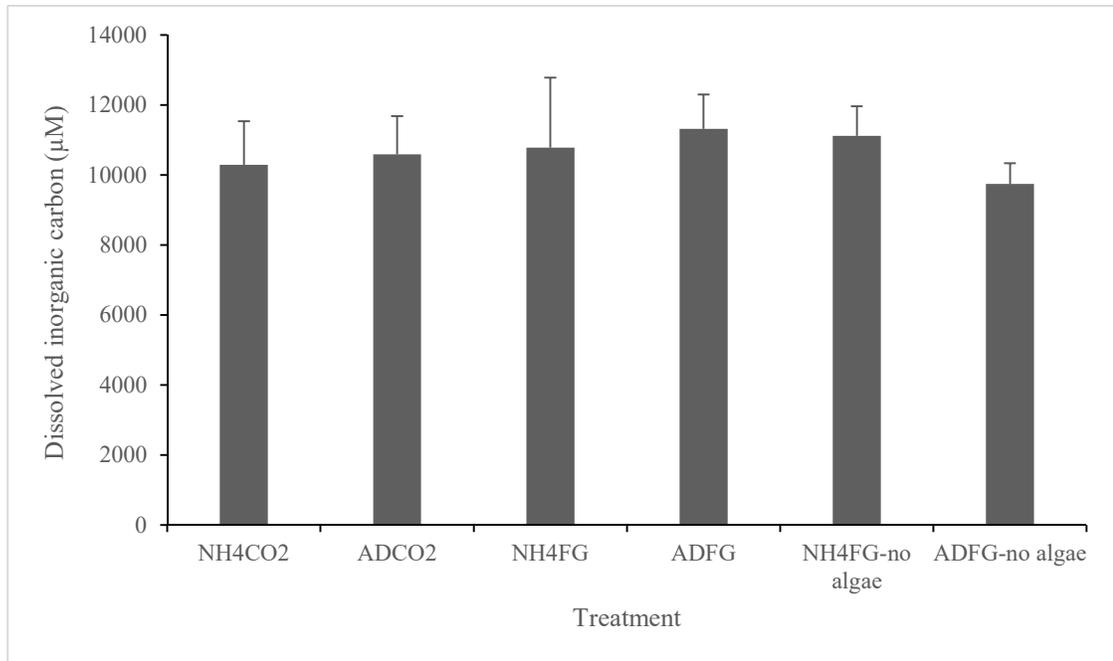


Figure 13: Dissolved inorganic carbon (DIC) concentration ( $\mu\text{M}$ ) for *H. akashiwo* cultures supplemented with ammonium and carbon dioxide, dairy waste and carbon dioxide, ammonium and flue gas, and dairy waste and flue gas. Data are expressed as means  $\pm$  standard deviations ( $n=3$ ). Values are averages over the final two sampling days for each treatment. Samples without algae are technical replicates. There were no significant interaction effects or differences.

Table 7 The percentage of carbon (% C) and nitrogen (% N) relative to the total biomass in *H. akashiwo* cultures supplemented with ammonium and carbon dioxide, dairy waste and carbon dioxide, ammonium and flue gas, and dairy waste and flue gas. Data are expressed as means  $\pm$  standard deviations (n=4). Samples were taken once cultures reached a constant growth rate. Asterisks denote significant interaction effect.

	<b>NH<sub>4</sub>CO<sub>2</sub></b>	<b>ADCO<sub>2</sub></b>	<b>NH<sub>4</sub>FG</b>	<b>ADFG</b>
% C	65.10 $\pm$ 7.49	46.93 $\pm$ 1.43	59.33 $\pm$ 12.55	47.38 $\pm$ 3.94
% N *	11.34 $\pm$ 0.59	12.59 $\pm$ 0.63	10.28 $\pm$ 0.57	9.73 $\pm$ 1.03

Table 8 The saponification value (SV), iodine value (IV), and cetane number (CN) for the fatty acid profile of *H. akashiwo* cultures supplemented with ammonium and carbon dioxide, dairy waste and carbon dioxide, ammonium and flue gas, and dairy waste and flue gas. Data are expressed as means  $\pm$  standard deviations (n=4). Samples were taken once cultures reached a constant growth rate. Letters denote significant differences.

	<b>NH<sub>4</sub>CO<sub>2</sub></b>	<b>ADCO<sub>2</sub></b>	<b>NH<sub>4</sub>FG</b>	<b>ADFG</b>
SV	190.75 $\pm$ 4.31 (a)	No data	170.94 $\pm$ 9.41 (a)	143.18 $\pm$ 17.41 (b)
IV	32.91 $\pm$ 0.86 (a)	No data	37.37 $\pm$ 8.43 (a)	31.55 $\pm$ 9.11 (a)
CN	67.52 $\pm$ 0.56	No data	69.88 $\pm$ 1.70	77.68 $\pm$ 2.59

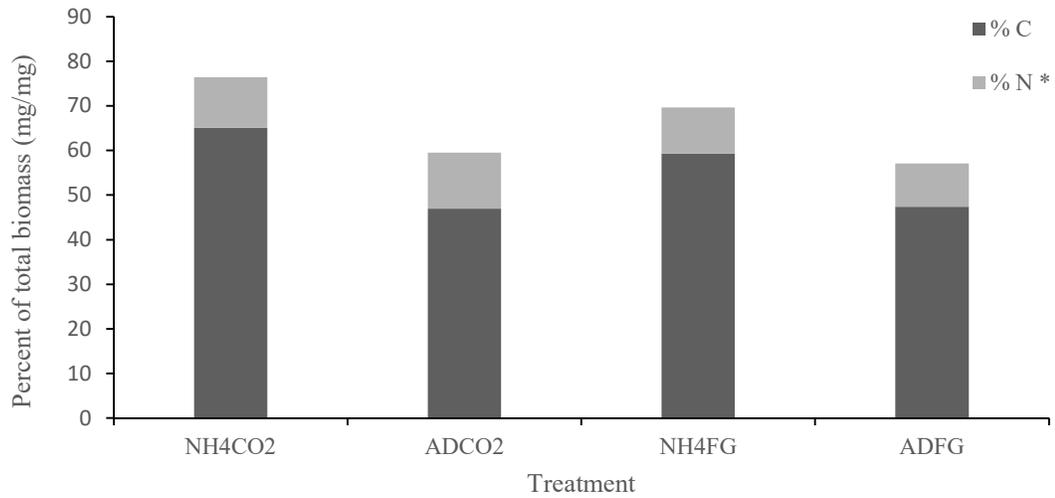


Figure 14: The percentage of carbon (% C) and nitrogen (% N) relative to the total biomass in *H. akashiwo* cultures supplemented with ammonium and carbon dioxide, dairy waste and carbon dioxide, ammonium and flue gas, and dairy waste and flue gas. Data are expressed as means (n=4). Samples were taken once cultures reached a constant growth rate. Asterisks indicates a significant interaction effect.

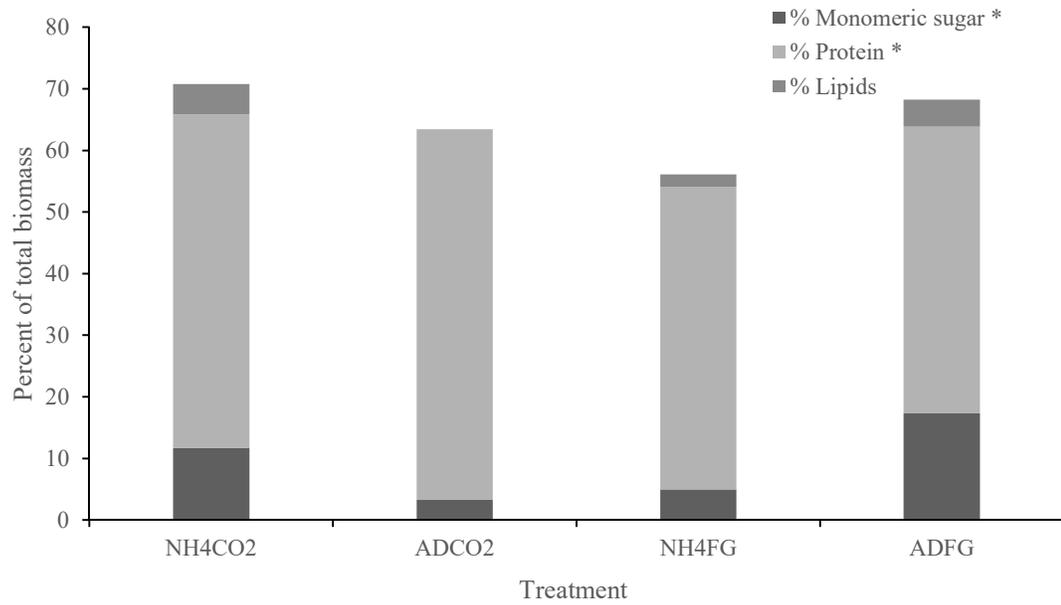


Figure 15: The percentage of monomeric sugars, protein, and lipids relative to the total biomass in *H. akashiwo* cultures supplemented with ammonium and carbon dioxide, dairy waste and carbon dioxide, ammonium and flue gas, and dairy waste and flue gas. Data are expressed as means (n=4). Asterisks indicate a significant interaction effect.

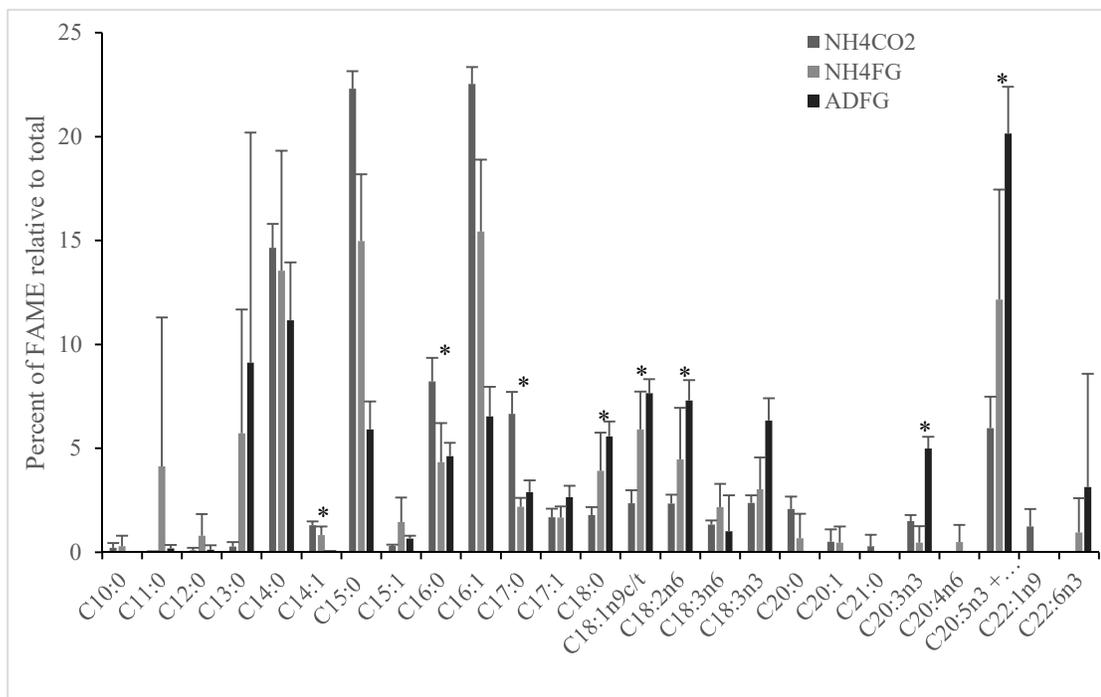


Figure 16: The percentage of fatty acid methyl esters (FAME) relative to the total amount of FAMES in *H. akashiwo* cultures supplemented with ammonium and carbon dioxide, and ammonium and flue gas. Data are expressed as means  $\pm$  standard deviations (n=4). Samples were taken once cultures reached a constant growth rate. Asterisks denote FAMES that are significantly different.

## REFERENCES

- An, J., Sim, S., Lee, J. S., & Kim, B. W. (2003). Hydrocarbon production from secondarily treated piggery wastewater by the green alga *Botryococcus braunii*. *Journal of Applied Phycology*, 15(2-3), 185-191.
- Benning, C. (1998). Biosynthesis and function of the sulfolipid sulfoquinovosyl diacylglycerol. *Annual Review of Plant Biology*, 49(1), 53-75.
- Bianco, C. (2013). The effects of flue gas, light intensity and nitrogen source on the carbon partitioning and biodiesel source potential of *Heterosigma akashiwo* and *Chattonella subsalsa*.
- Biddy, M., Davis, R., Jones, S., & Zhu, Y. (2013). Whole algae hydrothermal liquefaction technology pathway. National Renewable Energy Laboratory Golden.
- Bitton, G. (1994). Wastewater microbiology. New York: Wiley-Liss.
- Cagliari, A., Margis, R., dos Santos Maraschin, F., Turchetto-Zolet, A. C., Loss, G., & Margis-Pinheiro, M. (2011). Biosynthesis of triacylglycerols (TAGs) in plants and algae. *International Journal of Plant Biology*, 2(1), 10.
- Chang, F. H., Anderson, C., & Boustead, N. C. (1990). First record of a *Heterosigma* (raphidophyceae) bloom with associated mortality of cage-reared salmon in Big Glory bay, New Zealand. *New Zealand Journal of Marine and Freshwater Research*, 24(4), 461-469.
- Coyne, K. J. (2010). Nitrate reductase (NR1) sequence and expression in the harmful alga *Heterosigma akashiwo* (raphidophyceae). *Journal of Phycology*, 46(1), 135-142.
- Davis, R., Aden, A., & Pienkos, P. T. (2011). Techno-economic analysis of autotrophic microalgae for fuel production. *Applied Energy*, 88(10), 3524-3531.
- Duong, V. T., Ahmed, F., Thomas-Hall, S. R., Quigley, S., Nowak, E., & Schenk, P. M. (2015). High protein-and high lipid-producing microalgae from northern Australia as potential feedstock for animal feed and biodiesel. *Frontiers in Bioengineering and Biotechnology*, 3, 53.
- EPA. (2014). Overview of Greenhouse Gases. Available at: <https://www3.epa.gov/climatechange/ghgemissions/gases.html>

- EPA. (2015). Clean Power Plan for Existing Power Plants. Available at:  
<https://www.epa.gov/cleanpowerplan/clean-power-plan-existing-power-plants>
- Falkowski, P. G., & Raven, J. A. (2013). Aquatic photosynthesis. *Princeton University Press*.
- Fuentes-Grünewald, C., Garcés, E., Alacid, E., Rossi, S., & Camp, J. (2013). Biomass and lipid production of dinoflagellates and raphidophytes in indoor and outdoor photobioreactors. *Marine Biotechnology*, 15(1), 37-47.
- Fuentes-Grünewald, C., Garcés, E., Alacid, E., Sampedro, N., Rossi, S., & Camp, J. (2012). Improvement of lipid production in the marine strains *Alexandrium minutum* and *Heterosigma akashiwo* by utilizing abiotic parameters. *Journal of Industrial Microbiology & Biotechnology*, 39(1), 207-216.
- Glibert, P. M., Wilkerson, F. P., Dugdale, R. C., Raven, J. A., Dupont, C. L., Leavitt, P. R., . . . Kana, T. M. (2016). Pluses and minuses of ammonium and nitrate uptake and assimilation by phytoplankton and implications for productivity and community composition, with emphasis on nitrogen-enriched conditions. *Limnology and Oceanography*, 61(1), 165-197.
- Grün, S., Lindermayr, C., Sell, S., & Durner, J. (2006). Nitric oxide and gene regulation in plants. *Journal of Experimental Botany*, 57(3), 507-516.
- Guillard, R. R. (1975). Culture of phytoplankton for feeding marine invertebrates. *Culture of marine invertebrate animals* (pp. 29-60) Springer.
- Guschina, I. A., & Harwood, J. L. (2006). Lipids and lipid metabolism in eukaryotic algae. *Progress in Lipid Research*, 45(2), 160-186.
- Honjo, T. (1993). Overview on bloom dynamics and physiological ecology of *Heterosigma akashiwo*. *Toxic Phytoplankton Blooms in the Sea*, 33-41.
- Huang, G., Chen, F., Kuang, Y., He, H., & Qin, A. (2016). Current techniques of growing algae using flue gas from exhaust gas industry: A review. *Applied Biochemistry and Biotechnology*, 178(6), 1220-1238. doi:10.1007/s12010-015-1940-4
- Jha, P., Ali, A., & Raghuram, N. (2007). Nitrate-induction of nitrate reductase and its inhibition by nitrite and ammonium ions in *Spirulina platensis*. *Physiology and Molecular Biology of Plants*, 13(2), 163.

- Kao, C., Chen, T., Chang, Y., Chiu, T., Lin, H., Chen, C., . . . Lin, C. (2014). Utilization of carbon dioxide in industrial flue gases for the cultivation of microalga *Chlorella* sp. *Bioresource Technology*, 166, 485-493.
- Keusgen M, Curtis JM, Thibault P, Walter JA, Windust A, Ayer SW,. (1997). Sulfoquinovosyl diacylglycerols from the alga *Heterosigma carterae*. *Lipids*, 32(10), 1101-12.
- Lansing, S., Botero, R. B., & Martin, J. F. (2008). Waste treatment and biogas quality in small-scale agricultural digesters. *Bioresource Technology*, 99(13), 5881-5890.
- Laurens, L. M. (2013). Summative mass analysis of algal Biomass—Integration of analytical procedures. Contract, 303, 275-3000.
- Liu, C., Kieber, D. J., Yang, G., Xue, C., Wang, L., & Liu, H. (2014). Evidence for the mutual effects of dimethylsulfoniopropionate and nitric oxide during the growth of marine microalgae. *Nitric Oxide*, 42, 54-61.
- Lodge-Ivey, S., Tracey, L., & Salazar, A. (2014). Ruminant nutrition symposium: The utility of lipid extracted algae as a protein source in forage or starch-based ruminant diets. *Journal of Animal Science*, 92(4), 1331-1342.
- Lum, K. K., Kim, J., & Lei, X. G. (2013). Dual potential of microalgae as a sustainable biofuel feedstock and animal feed. *Journal of Animal Science and Biotechnology*, 4(1), 1.
- Mulbry, W., Kondrad, S., & Buyer, J. (2008). Treatment of dairy and swine manure effluents using freshwater algae: Fatty acid content and composition of algal biomass at different manure loading rates. *Journal of Applied Phycology*, 20(6), 1079-1085.
- National Research Council (U.S.), Committee on the Sustainable Development of Algal Biofuels., National Research Council (U.S.), Board on Agriculture and Natural Resources., National Research Council (U.S.), Board on Energy and Environmental Systems., National Academies Press (U.S.),. (2012). Sustainable development of algal biofuels in the united states. Washington, D.C.: National Academies Press.
- Petkov, G., & Garcia, G. (2007). Which are fatty acids of the green alga *Chlorella*? *Biochemical Systematics and Ecology*, 35(5), 281-285.
- Poulsen, N., & Kröger, N. (2005). A new molecular tool for transgenic diatoms. *Febs Journal*, 272(13), 3413-3423.

- Razzak, S. A., Hossain, M. M., Lucky, R. A., Bassi, A. S., & de Lasa, H. (2013). Integrated CO<sub>2</sub> capture, wastewater treatment and biofuel production by microalgae culturing—a review. *Renewable and Sustainable Energy Reviews*, 27, 622-653.
- Song, B., & Ward, B. B. (2004). Molecular characterization of the assimilatory nitrate reductase gene and its expression in the marine green alga *Dunaliella tertiolecta* (chlorophyceae). *Journal of Phycology*, 40(4), 721-731.
- Stewart, J. J., Bianco, C. M., Miller, K. R., & Coyne, K. J. (2015). The marine microalga, *Heterosigma akashiwo*, converts industrial waste gases into valuable biomass. *Frontiers in Energy Research*, 3, 12.
- Stewart, J. J., & Coyne, K. J. (2011). Analysis of raphidophyte assimilatory nitrate reductase reveals unique domain architecture incorporating a 2/2 hemoglobin. *Plant Molecular Biology*, 77(6), 565-575.
- Van Wychen, S., & Laurens, L. M. (2013). Determination of total carbohydrates in algal biomass. *Contract*, 303, 275-3000.
- Van Wychen, S., & Laurens, L. (2013). Determination of total lipids as fatty acid methyl esters (FAME) by in situ transesterification. *Contract*, 303, 375-300.
- Vergara, Juan J., Berges, John A., Falkowski, Paul G., (1998). Diel periodicity of nitrate reductase activity and protein levels in the marine diatom *Thalassiosira weissflogii* (bacillariophyceae). *JPY Journal of Phycology*, 34(6), 952-961.
- Wang, L., Li, Y., Chen, P., Min, M., Chen, Y., Zhu, J., & Ruan, R. R. (2010). Anaerobic digested dairy manure as a nutrient supplement for cultivation of oil-rich green microalgae *Chlorella* sp. *Bioresource Technology*, 101(8), 2623-2628.
- Wilson, M., Groppo, J., Placido, A., Graham, S., Morton III, S., Santillan-Jimenez, E., . . . Andrews, R. (2014). CO<sub>2</sub> recycling using microalgae for the production of fuels. *Applied Petrochemical Research*, 4(1), 41-53.
- Woertz, I., Feffer, A., Lundquist, T., & Nelson, Y. (2009). Algae grown on dairy and municipal wastewater for simultaneous nutrient removal and lipid production for biofuel feedstock. *Journal of Environmental Engineering*, 135(11), 1115-1122.

- Zhang, Y., Fu, F., Whereat, E., Coyne, K. J., & Hutchins, D. A. (2006). Bottom-up controls on a mixed-species HAB assemblage: A comparison of sympatric *Chattonella subsalsa* and *Heterosigma akashiwo* (Raphidophyceae) isolates from the Delaware Inland Bays, USA. *Harmful Algae*, 5(3), 310-320.
- Zhou, W., Hu, B., Li, Y., Min, M., Mohr, M., Du, Z., . . . Ruan, R. (2012). Mass cultivation of microalgae on animal wastewater: A sequential two-stage cultivation process for energy crop and omega-3-rich animal feed production. *Applied Biochemistry and Biotechnology*, 168(2), 348-363.