

THE EFFECTS OF FLUE GAS, LIGHT INTENSITY AND NITROGEN
SOURCE ON THE CARBON PARTITIONING AND
BIODIESEL SOURCE POTENTIAL OF *HETEROSIGMA AKASHIWO*
AND *CHATTONELLA SUBSALSA*

by

Colleen M. Bianco

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ABSTRACT

This work aims to evaluate *Heterosigma akashiwo* and *Chattonella subsalsa* as biodiesel feedstocks when grown on CO₂ and NO rich model flue gas. The growth rates of *H. akashiwo* and *C. subsalsa* were higher when the cultures were bubbled with flue gas compared to when bubbled with air. The ability of *H. akashiwo* and *C. subsalsa* to survive high concentrations of NO may be due to a hybrid nitrate reductase, (NR2-2/2HbN), that may detoxify and convert nitric oxide to nitrate. *H. akashiwo* was able to grow to a higher cell density when bubbled with flue gas compared to when bubbled with air under nitrate deficient conditions, indicating that *H. akashiwo* may be able to use NO as an inorganic nitrogen source. When grown on flue gas, both *H. akashiwo* and *C. subsalsa* accumulated significantly more carbohydrates than when grown on air. This indicates that the high CO₂ concentration in the flue gas was assimilated as carbohydrates, a direct product of photosynthesis. When *H. akashiwo* was grown at a high light intensity, the growth rate was not significantly different from that at a low light intensity, which suggests that light limitation during mass cultivation would not be an issue during biodiesel production. This work also explored the transcript abundance of key fatty acid synthesis genes to further understand fatty acid synthesis in microalgae. β -ketoacyl-ACP synthase transcript abundance varied with almost every treatment and may be a key regulator when *H. akashiwo* and *C. subsalsa* are under environmental stress. Overall, this work demonstrates that both *H. akashiwo* and *C. subsalsa* can grow on model flue gas and be producers of high quality biodiesel.

Chapter 1

INTRODUCTION

Microalgae have been identified as a potential biofuel source due to their high productivity, high lipid yields and high growth rates (reviewed by Williams et al. 2010). As a biodiesel feedstock, microalgae are advantageous over terrestrial, first-generation biofuels such as crop plants. In addition to having high growth rates, microalgae do not require as much land or fresh water as terrestrial plants. Theoretical calculations based on photosynthetic efficiency and growth potential of microalgae predict an annual oil production of over 30,000 liters or 200 barrels of oil per hectare of land. This oil production estimate is 100-times higher than the current oil production of soybeans, which the United States is currently using for biodiesel (reviewed by Hu et al. 2008).

1.1 Carbon Partitioning

The composition of the algal biomass with respect to carbohydrates, proteins and lipids will determine the overall biodiesel potential of a microalga. Carbon is assimilated into microalgae through photosynthesis and partitioned into carbohydrates, proteins and lipids (Figure 1.1). Williams et al. (2010) calculated the average proportions of lipid (24.2%), protein (48.3%), and carbohydrate (27.5%) during active growth for a dataset with multiple alga species, but these proportions can vary greatly depending on species. Generally, protein comprises 20-60% of algal content. Proteins are embedded in lipid membranes, where they serve structural and metabolic functions. Furthermore, proteins are catalysts for cell metabolism and are the major determinants of cellular growth rates (reviewed by Williams et al. 2010). Carbohydrates also have both structural and metabolic functions, as well as being energy reserves. They usually

comprise 10-50% of algal cell content (reviewed by Williams et al. 2010). In microalgae, carbohydrates are the early products of photosynthesis and serve as the starting point for the synthesis of other molecules. Similar to carbohydrates, lipids serve as energy storage compounds. However, the oxidation of lipids releases more than twice the energy released from the oxidation of carbohydrates (Nelson et al. 2008). Lipids also moderate cellular activity and are the main structural component of cell membranes (reviewed by Williams et al. 2010). Lipids are composed of a fatty acid molecule bonded to a head group, such as glycerol (Halim et al. 2012). Most lipids can be classified into two categories depending on the polarity of the head group. A neutral lipid is formed when the carboxylate end of the fatty acid molecule is bonded to an uncharged head group while a polar lipid is formed when the carboxylate end of the fatty acid molecule is bonded to a charged head group (Halim et al. 2012).

The synthesis of starch, a major storage carbohydrate, shares common precursors with lipid synthesis. However, the interaction between starch metabolism and lipid metabolism is poorly understood. Li et al. (2011) showed that starch synthesis was up-regulated in *Pseudochlorococcum* sp. under high light and nitrogen-limited conditions, suggesting that starch is used for primary carbon and energy storage. When nitrogen was depleted, starch content decreased and neutral lipid content increased, indicating that carbon partitioning was switched to neutral lipids for secondary carbon and energy storage. The transient accumulation of starch followed by a gradual accumulation of neutral lipids also suggests that starch and neutral lipids can be inter-converted. This was demonstrated by Roessler (1988) who showed that carbon previously assimilated into carbohydrates can be converted into lipids in the diatom *Cyclotella cryptica*, under silicon limited conditions. Similar to Roessler (1988), Lee et al. (2012) demonstrated that the immediate N deprivation response of *Haematococcus pluvialis* was to accumulate carbohydrates. However, on the third day of N starvation, fatty acid productivity remained constant while carbohydrate synthesis ceased. Overall, this observation suggested that the immediate stress response is to mass-produce carbohydrates, which can then be converted to fatty acids.

Yang et al. (2013) analyzed transcriptional changes in the diatom *Thalassiosira pseudonana* under N deprivation and suggested that catabolism of carbohydrates may lead to TAG accumulation. In terms of carbon fixation, most plants rely on photosynthesis and glycolysis to produce pyruvate. Yang et al. (2013) observed that glycolysis was up-regulated while photosynthesis was inhibited under N deprivation. This may indicate that under N deprivation, *T. pseudonana* may rely on glycolysis to form pyruvate, which is the source of acetyl-CoA for fatty acid biosynthesis.

1.2 Lipid Synthesis and Triacylglycerol

Microalgae produce several types of lipids including phospholipids, glycolipids, mono-, di- and triglycerides. While free fatty acids comprise 1-2% of lipids in microalgae, most fatty acids are bound to a glycerol molecule to form a neutral acylglycerol (Nasimento et al. 2013). Triacylglycerol (TAG) is a form of acylglycerol that contains three fatty acid chains and provides energy for immediate use in algal cells (Halim et al. 2012). TAGs are the only acylglycerol that can easily be converted into biodiesel. Generally, TAGs have a lower degree of unsaturation than polar lipids, so they produce fatty acid methyl esters with higher oxidative stability (Halim et al. 2012).

In microalgae, *de novo* synthesis of fatty acids begins in the chloroplast (Figure 1.2; Li et al. 2011). The commitment step in fatty acid synthesis is the conversion of acetyl-CoA to malonyl-CoA. This 2-step reaction is catalyzed by a single enzyme complex, acetyl-CoA carboxylase (ACC). Fatty acids are then transferred from the chloroplast to the endoplasmic reticulum membrane where they are synthesized into polar membrane lipids and neutral storage lipids. Lipid membranes, which are composed of a bilayer of strongly polar molecules such as phospholipids and glycolipids, surround the algal organelles. Neutral storage lipids, specifically TAGs, are important energy reserves. After being synthesized, TAGs are deposited in the cytoplasm as densely packed lipid bodies. Many microalgae accumulate significant amounts of TAGs, making up to 20-50% of total dry weight (Wahlen et al. 2011).

Manipulation of culture conditions and physiological status can increase lipid content and alter fatty acid composition of microalgae. For example, microalgae harvested during the stationary phase have a lower polar lipid content than obtained in the logarithmic growth phase (Halim et al. 2012). Under unfavorable environmental conditions, such as high light irradiance and nitrogen limitation, many microalgae alter their lipid biosynthetic pathways to accumulate neutral lipids (Rismani-Yazdi et al. 2012; Li et al. 2011, reviewed by Hu et al. 2008). Light intensity has also been shown to alter the fatty acid composition of many algae. High levels of polyunsaturated fatty acids, which are typically incorporated into membrane lipids, are produced under low light intensities. Saturated and mono-saturated fatty acids, which make up neutral lipids, are predominantly synthesized under high light intensities (reviewed by Hu et al. 2008). High light intensities may also affect growth rates. Li et al. (2011), for example, observed that moderate photoinhibition of photosynthesis induced by high light intensity was needed for maximum formation and accumulation of neutral lipids, but further increased light intensities inhibited growth. Furthermore, the synthesis of neutral lipids may be a stress response by serving as an electron sink when the cell is under photo-oxidative stress (reviewed by Hu et al. 2008; Li et al. 2011).

Yang et al. (2013) hypothesized that the inhibition of genes involved in photosynthesis is a genetic response to N deprivation, which implies that under N deprivation, light is not required to produce neutral lipids. This approach to the production of bio-fuels would be an important finding for industrial scale biodiesel production because the availability of light would not be a limiting factor in algal productivity. Furthermore, if photosynthesis were inhibited, the cell would rely on the elevated citric acid cycle for carbon fixation. Although nitrogen limitation has been shown to induce increases in neutral lipid concentration, it has been shown to have little effect on fatty acid composition (Liang et al. 2013; Fuentes-Grünewald et al. 2012).

As mentioned, under nutrient stress, algae favor the synthesis of neutral lipids over polar lipids. The primary effect of N deprivation is the reduced synthesis of nitrogen containing cellular components, such as nucleic acids, amino acids, proteins, and

chlorophyll (Yang et al. 2013; reviewed by Williams et al. 2010). Yang et al. (2013) analyzed transcriptional changes in the diatom, *Thalassiosira pseudonana*, under N deprivation. It was determined that N deprivation caused an increase in amino acid catabolism to ultimately increase acetyl-CoA and succinyl-CoA. Transcriptional analysis also revealed that gluconeogenesis was inhibited and the carbon was redirected towards neutral lipid accumulation. The transcript abundance of pyruvate dehydrogenase also increased under N deprivation, suggesting that pyruvate was largely converted to acetyl-CoA. Acetyl-CoA is the precursor to fatty acid synthesis so an increase in acetyl-CoA concentration may lead to an increase in fatty acid production.

1.3 Biodiesel Quality

The properties of the biodiesel depend heavily on the fatty acid composition the microalgae feedstock. The fatty acid composition of biodiesel will be identical to the fatty acid composition of the raw material (Ciubota-Rosie et al. 2013). Chain length and degree of unsaturation of the fatty acids have the most significant effect on the biodiesel quality (Nascimento et al. 2013).

Cetane number (CN) is the primary indicator of diesel quality and measures the ignition delay when the diesel is injected into the combustion chamber. A high CN correlates to a short ignition time. Diesel with a low CN causes diesel knocking and may not completely combust, causing high levels of particulate exhaust emissions (Ciubota-Rosie et al. 2013). CN increases with increasing length of fatty acid chains and decreases with increasing degree of unsaturation. The American Society for Testing and Material set a minimum CN of 47 for diesel while Europe has a standard CN of 51 (Nascimento et al. 2013). However, saturated fatty acids have higher melting points compared to unsaturated fatty acids. When diesel contains a high amount of saturated fatty acids, crystallization can occur during normal engine operating temperatures and clog fuel lines. For this reason, biodiesel feedstocks should not contain high amounts of saturated fatty acids.

Iodine value (IV) indicates the ability of biodiesel to react with oxygen at ambient temperature. IV is related to the degree of unsaturation of the FAMES: the higher the degree of unsaturation, the higher the IV. A high IV indicates a high tendency of the biodiesel to oxidize. Diesel with a high IV polymerizes in the engine to form deposits on injector nozzles and piston rings when the engine is heated (Ciubota-Rosie et al. 2013).

Similar to IV, oxidation stability is also an important parameter. Diesel with high levels of polyunsaturated FAMES has low oxidation stability because the methylene groups adjacent to the double bonds are susceptible to oxidative attack (Ciubota-Rosie et al. 2013). Specifically, linolenic acid (C18:3(n-3)) has very low oxidation stability because it contains two bis-allylic positions at the C-11 and C-14. Primary oxidative products, such as hydroperoxides, can polymerize to form deposits within the engine. Further oxidation can yield ketones, aldehydes and short-chain carboxylic acid, which cause corrosion within the engine (Ciubota-Rosie et al. 2013).

IV and CN can be measured directly or estimated using the empirical formulas found in Appendix A.

1.4 Metabolic Engineering

Metabolic engineering is believed to be an effective solution to the high production cost of microalgal biodiesel and may be key to obtaining a favorable fatty acid composition. Although the lipid metabolic pathway of higher plants has been characterized extensively, the process in microalgae has yet to be thoroughly investigated (Misra et al. 2013). Several enzymes in lipid biosynthetic pathways may be good candidates for metabolic engineering. Generally, the activity of acetyl CoA carboxylase (ACC) is considered to be highly regulated and to be a major determinant of the overall rate of fatty acid synthesis (Baud et al. 2003). ACC catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, the first committed step in fatty acid synthesis (Figure 1.2). Fatty acid synthesis from malonyl-CoA occurs through a series of reactions catalyzed by multiple enzymes. The malonyl group is transferred from malonyl-CoA to the thiol

group of acyl carrier protein (ACP) by malonyl-CoA-ACP transferase (MCT) to form malonyl-ACP. Throughout fatty acid synthesis, ACP carries the acyl groups of the fatty acid intermediates through a thioester linkage. The formation of malonyl-ACP adds a two-carbon acetyl unit to the growing fatty acyl chain. This step is followed by six or seven cycles of condensation, reduction, dehydration and another reduction to produce C16:0 or C18:0, respectively. These reactions are catalyzed by β -ketoacyl-ACP synthase (KAS), β -ketoacyl-ACP reductase, β -hydroxyacyl-ACP dehydrase and enoyl-ACP reductase, respectively. In this study, the mRNA abundance of KAS was investigated. KAS is considered a key regulator of fatty acid chain length because it is a condensing enzyme responsible for the two carbon elongations of substrates from C4:0 to C14:0. The role of KAS in fatty acid synthesis ceases after C16:0 or C18:0 is produced.

The end products of fatty acid synthesis are C16:0 and C18:0, which are converted into long chain fatty acids and polyunsaturated fatty acids (PUFA) through a series of desaturation and elongation steps. Both *Chattonella subsalsa* and *Heterosigma akashiwo* produce high levels of the PUFA, eicosapentaenoic fatty acid (C20:5, EPA), a precursor to many lipid regulators and complex lipid molecules (Wen et al. 2003). Two enzymes, Δ 6 elongase (FAE) and Δ 5 desaturase (DES), are responsible for the production of EPA. Yang et al. (2013) determined that the transcript abundance of genes involved in fatty acid elongation decreased under N deprivation in *T. pseudonana*. Specifically, Δ 6 elongase (FAE) decreased by 16% which was consistent with a 26% decrease in C20:5. In the same experiment, the transcript abundance of Δ 5 desaturase (DES) increased 32-fold, which was consistent with a 1.6-fold increase in mono-unsaturated fatty acid concentration.

Acyl-CoA binding proteins (ACBP) are involved in transport of saturated fatty acids from plastid to endoplasmic reticulum. Xiao et al. (2010) observed that ACBP transcript abundance was upregulated in *Arabidopsis thaliana* during senescence. Moreover, transgenic *A. thaliana* overexpressing ACBP had accelerated leaf senescence. ACBP transcript abundance may be indicative of senescence in *H. akashiwo* and *C.*

subsalsa. Because ACBP is involved in the transport of saturated fatty acids, which increase biodiesel quality, ACBP transcript abundance may be a proxy for biodiesel quality.

1.5 *Chattonella subsalsa* and *Heterosigma akashiwo* as Model Species

Heterosigma akashiwo and *Chattonella subsalsa* are unicellular flagellated algae (class Raphidophyceae) that are known to form high-density blooms. Both raphidophytes tolerate large fluctuations in temperature and salinity. Handy et al. (2005) report that both *H. akashiwo* and *C. subsalsa* cross large physical and chemical gradients as they vertically migrate in the Delaware Inland bays. Both strains actively grow between a salinity of 5-30 psu and temperatures between 10-30⁰C (Zhang et al. 2006). Optimal growth of *C. subsalsa* occurs between 20-30⁰C and 15-25 psu. Similarly, optimal growth *H. akashiwo* occurs between 16-30⁰C and 10-30 psu (Zhang et al. 2006).

H. akashiwo has been identified as a promising biofuel feedstock candidate due to its high productivity, high lipid yields and fast growth rate (Fuentes-Grünewald et al. 2009). *H. akashiwo* has been shown to produce 73% of its dry weight in lipids under nitrogen limiting conditions at 25⁰C (Fuentes-Grünewald et al. 2012). Changes in fatty acid profiles with culture age and environmental conditions are not well understood for raphidophytes. However, it is known that raphidophytes increase their lipid quantity with culture age (Fuentes-Grünewald et al. 2012). Fuentes-Grünewald et al. (2012) determined that the highest TAG concentrations in *H. akashiwo* occurred during the stationary growth phase under nitrogen-deficient conditions

Growth of *H. akashiwo* can vary significantly among strains. Fuentes-Grünewald et al. (2012) investigated the growth of *H. akashiwo* isolated from the Mediterranean Sea and achieved a maximum growth rate (μ) of $0.51 \pm 0.03 \text{ day}^{-1}$ at 25⁰C and 330 μM nitrate in the medium. Furthermore, Lopes et al. (2012) determined the maximum growth rate of a Paran Bay (Brazil) isolate to be 1.33 day^{-1} at 21⁰C and 880 μM nitrate in the medium. Zhang et al. (2006) determined the maximum growth rates

of the Delaware Inland Bays' strains of *H. akashiwo* and *C. subsalsa* to be 0.99 ± 0.1 day⁻¹ and 0.87 ± 0.1 day⁻¹, respectively.

Lopes et al. (2012) extensively characterized carbohydrate content in a Brazilian strain of *H. akashiwo*. It was determined that carbohydrate content increased to and peaked (119 pg carbohydrate/cell) at day 14 of growth, which was when this strain reached early stationary growth. Fuentes-Grünewald et al. (2012) also worked with a Mediterranean Sea isolate of *H. akashiwo*. When this strain was gently aerated with air, it produced 7.5% of its dry weight in lipids when the medium contained 880 μ M nitrate. Lipid content increased to 73% of its dry weight when the medium contained only 330 μ M nitrate. The interaction among lipids, carbohydrates and proteins has not been studied in the Delaware Inland Bay isolate of *H. akashiwo* or *C. subsalsa*.

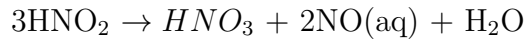
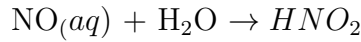
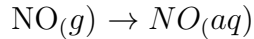
1.6 Flue Gas

Microalgal biodiesel can potentially decrease fossil fuel dependence and also reduce pollutants, such as carbon dioxide (CO₂) and nitrogen oxides (NO_x) present in flue gas. Microalgae have long been considered for the removal of CO₂ from the flue gas of fossil fuel power stations (Brown 1996; reviewed by Van den Hende et al. 2012). Electrical power plants release 2.2×10^9 tons of CO₂ per year into the atmosphere, which is more than 1/3 of U.S emissions (reviewed by Van den Hende et al. 2012). It has been shown that microalgae can consume high quantities of CO₂ and may have the potential to diminish the release of CO₂ into the atmosphere (Brown 1996; Van den Hende et al. 2012; Yoo et al. 2010). Specifically, Brown (1996) determined that some species of microalgae could not only survive high concentrations of CO₂ but also have a 90% CO₂ trapping efficiency.

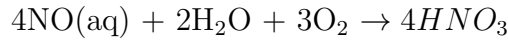
In addition to being 15-20% CO₂, flue gas also contains 100-300 ppm NO_x (95% NO and 5% NO₂). NO_x are precursors to ground level ozone and contribute to fine particulate pollution and smog (Hogue 2011). In 2011, the EPA ruled that beginning in 2012, power plants in twenty-three states along the east coast and in the Midwest must reduce the emission of NO_x 54% from 2005 levels by 2014. This new rule is expected

to increase the operational costs of power plants by \$ 800 million per year and \$ 1.6 billion annually for thirty years for investments in pollution control equipment (Hogue 2011). Remediation of the flue gas from power plants by microalgae may help reduce the emission of NO_x .

Current research has focused on the removal of CO_2 from flue gas but has ignored NO_x (reviewed by Van Den Hende et al. 2012). NO in flue gas can also potentially be a nitrate source for the microalgae (reviewed by Farrelly et al. 2013). NO dissolves in water to form nitrous acid HNO_2 and nitric acid HNO_3 .



When oxygen is present in the culture medium, NO can react to directly form HNO_3



When the pH of the culture medium is above 4, HNO_2 and HNO_3 are present as NO_2^- and NO_3^- , respectively (reviewed by Van Den Hende et al. 2012). NO_3^- is the preferred inorganic nitrogen source of *H. akashwio* (Zhang et al. 2006).

Brown (1996) demonstrated that the green alga, *Monoraphidium minutum*, could tolerate 150 ppm nitric oxide. NO_2^- concentration in the culture medium bubbled with flue gas was twice the NO_2^- concentration in the controls, which were not bubbled with flue gas. Furthermore, the flue gas cultures used less NO_3^- than the control cultures but cell growth was unaffected. These observations lead Brown (1996) to suggest that the NO dissolved in the medium and was available as a nitrogen source for the algae. Nagase et al. (2001) suggested that NO can be directly assimilated by *Dunaliella tertiolecta* by permeating directly into the cells through diffusion. In this case, NO was preferentially utilized as a N source over NO_3^- .

Negoro et al. (1991) evaluated the growth of microalgae to determine if CO_2 in flue gas could be used as a carbon source. In contrast to studies described above, however, the NO inhibited the growth of nine of the ten algae investigated. The green

alga, *Nannochloris* sp. was able to grow at a maximum concentration of 300 ppm NO and assimilate a small amount of NO. *Nannochloris* sp. was investigated again by Yoshihara et al. (1996). The addition of NO inhibited the growth of *Nannochloris* sp. during the lag phase, but when NO was added during the exponential phase, growth rate was not inhibited and *Nannochloris* sp. could eliminate 50% of the NO bubbled into the medium.

Stewart and Coyne (2011) demonstrated that nanomolar concentrations of NO had no effect on the growth rate of *H. akashiwo*. This may be because *H. akashiwo* contains a hybrid nitrate reductase (NR2-2/2HbN) that may detoxify and convert nitric oxide to nitrate (Stewart and Coyne, 2011). Nitrate reductase catalyzes the rate-limiting step in nitrate assimilation; the reduction of nitrate to nitrite. Stewart and Coyne (2011) report the presence of NR2-2/2HbN in both *H. akashiwo* and *C. subsalsa*. This hybrid nitrate reductase consists of a 2/2 hemoglobin inserted into the hinge 2 region of prototypical nitrate reductase. When NO was added to cultures of *H. akashiwo*, it was shown that NR2-2/2HbN transcript abundance was higher in cultures exposed to NO, indicating a link between the removal of NO from culture medium by *H. akashiwo* and the increase in expression of this enzyme (Stewart and Coyne 2011). Lastly, Stewart and Coyne (2011) demonstrated that *H. akashiwo* removed NO from the growth medium. The effect of NO addition on *C. subsalsa*, however, has never been examined. The presence of NR2-2/2HbN in these algae may reduce the high production cost of microalgal biodiesel. Nitrogen accounts for 45% of energy input (reviewed by Chisti, 2008); therefore harnessing NO from flue gas as a free nitrogen source will reduce the cost of biodiesel production from microalgae.

1.7 Objectives

This work aims to evaluate *H. akashiwo* and *C. subsalsa* as biodiesel feedstocks. Specific objectives were to:

1. Evaluate the long-term impacts of model flue gas on carbon metabolism of *H.*

akashiwo and *C. subsalsa* and the ability of *H. akashiwo* to use NO present in flue gas as a nitrogen source

2. Determine the environmental conditions (light intensity and nitrogen stress) that produce the highest quantity of lipids and the highest quality of fatty acids in *H. akashiwo*

3. Explore the transcript abundance of key fatty acid synthesis genes to further understand fatty acid synthesis in microalgae and target candidate genes for metabolic engineering

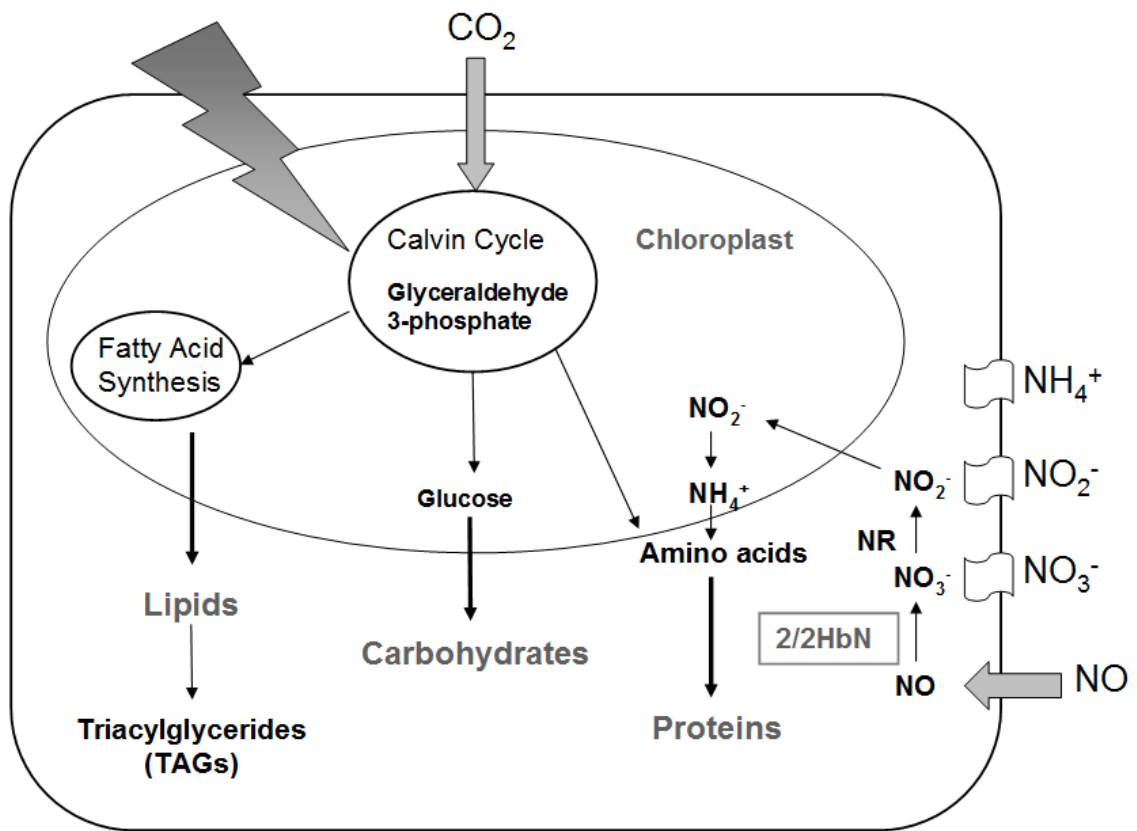


Figure 1.1: Carbon and nitrogen assimilation in microalgae: Carbon is assimilated into microalgae through photosynthesis and partitioned into carbohydrates, proteins and lipids. Depending on its form, nitrogen is diffused or transported across the cell wall and incorporated largely into amino acids.

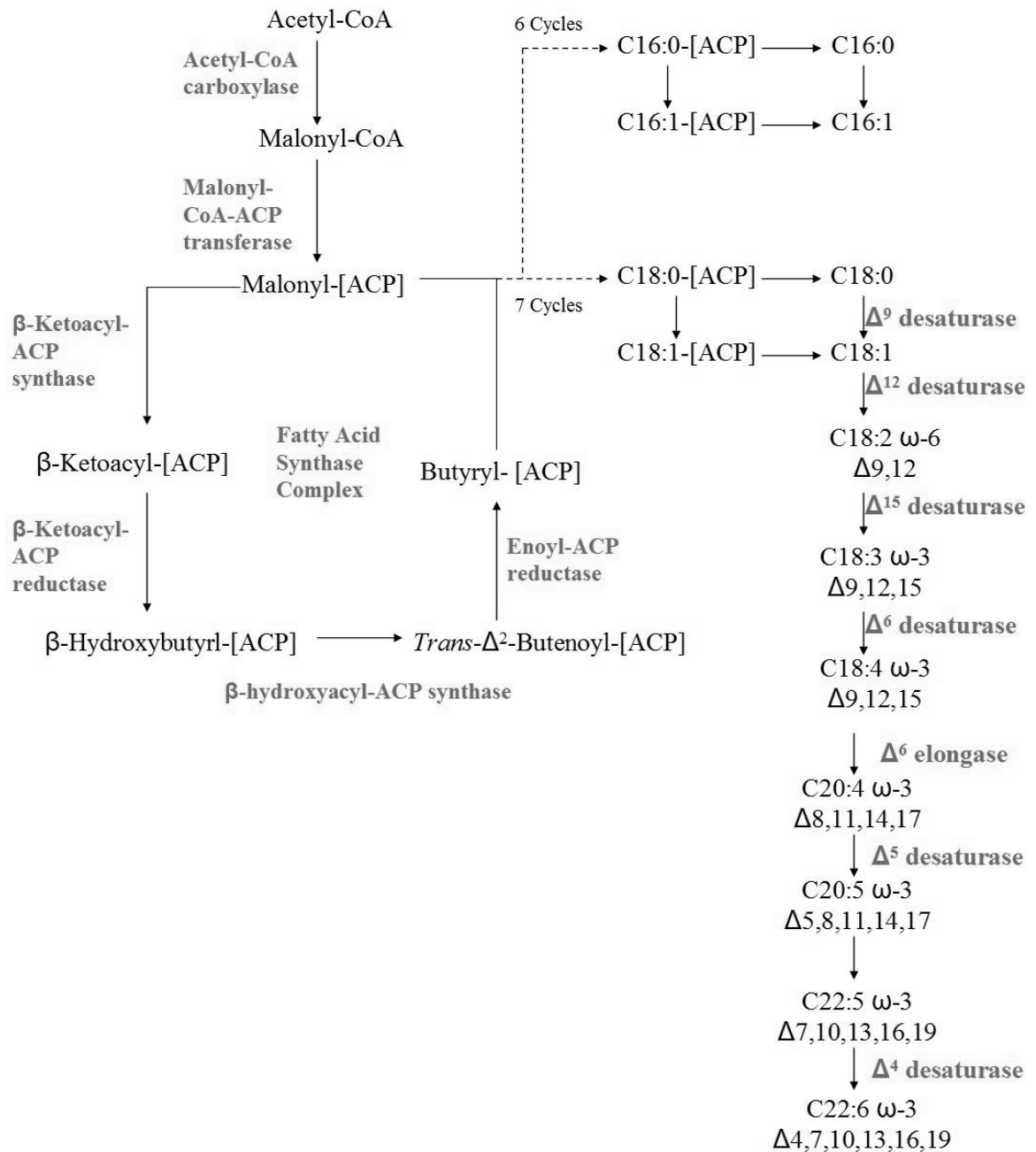


Figure 1.2: The pathways of fatty acid biosynthesis. ACC catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, the first committed step in fatty acid synthesis. Fatty acid synthesis from malonyl-CoA occurs through a series of reactions catalyzed by multiple enzymes (gray) to form C16:0 and C18:0, which are then converted into long chain fatty acids and polyunsaturated fatty acids (PUFA) through a series of desaturation and elongation steps. Modified from: Rismani-Yazdi et al. (2012) and Wen et al. (2003).

1.8 References

Brown, L.M. 1996, "Uptake of carbon dioxide from flue gas by microalgae", *Energy Conversion and Management*, vol. 37, no. 6-8, pp. 1363-1367.

Chisti, Y. 2008, "Biodiesel from microalgae beats bioethanol", *Trends in biotechnology*, vol. 26, no. 3, pp. 126-131.

Ciubota-Rosie, C., Ramon Ruiz, J., Jesus Ramos, M. Perez, A. 2013, "Biodiesel from *Camelina sativa*: A comprehensive characterisation", *Fuel*, vol. 105, pp. 572-577.

Farrelly, D.J., Everard, C.D., Fagan, C.C. McDonnell, K.P. 2013, "Carbon sequestration and the role of biological carbon mitigation: A review.", *Renewable Sustainable Energy Reviews*, vol. 21, pp. 712-727.

Fuentes-Grünewald, C., Garces, E., Alacid, E., Rossi, S. Camp, J. 2013, "Biomass and Lipid Production of Dinoflagellates and Raphidophytes in Indoor and Outdoor Photobioreactors", *Marine Biotechnology*, vol. 15, no. 1, pp. 37-47.

Fuentes-Grünewald, C., Garces, 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*, vol. 39, no. 1, pp. 207-216.

Halim, R., Danquah, M.K. Webley, P.A. 2012, "Extraction of oil from microalgae for biodiesel production: A review", *Biotechnology Advances*, vol. 30, no. 3, pp. 709-732.

Handy, S., Coyne, K., Portune, K., Demir, E., Doblin, M., Hare, C., Cary, S. Hutchins, D. 2005, "Evaluating vertical migration behavior of harmful raphidophytes in the

Delaware Inland Bays utilizing quantitative real-time PCR RID C-6195-2008", *Aquatic Microbial Ecology*, vol. 40, no. 2, pp. 121-132.

Hogue, C. 2011, *Clearing Skies*.

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", *Plant Journal*, vol. 54, no. 4, pp. 621-639.

Lei, A., Chen, H., Shen, G., Hu, Z., Chen, L. Wang, J. 2012, "Expression of fatty acid synthesis genes and fatty acid accumulation in *Haematococcus pluvialis* under different stressors", *Biotechnology for Biofuels*, vol. 5, pp. 18.

Li, Y., Fei, X. Deng, X. 2012, "Novel molecular insights into nitrogen starvation-induced triacylglycerols accumulation revealed by differential gene expression analysis in green algae *Micractinium pusillum*", *Biomass Bioenergy*, vol. 42, pp. 199-211.

Li, Y., Han, D., Sommerfeld, M. Hu, Q. 2011, "Photosynthetic carbon partitioning and lipid production in the oleaginous microalga *Pseudochlorococcum* sp (Chlorophyceae) under nitrogen-limited conditions RID D-2553-2010", *Bioresource technology*, vol. 102, no. 1, pp. 123-129.

Liang, C., Cao, S., Zhang, X., Zhu, B., Su, Z., Xu, D., Guang, X. Ye, N. 2013, "De Novo Sequencing and Global Transcriptome Analysis of *Nannochloropsis* sp (Eustigmatophyceae) Following Nitrogen Starvation", *Bioenergy Research*, vol. 6, no. 2, pp. 494-505.

Lopes, D.C., Baron Maurer, J.B., Stevan-Hancke, F.R., de Oliveira Proenca, L.A. Zawadzki-Baggio, S.F. 2012, "Chemical analysis of exopolysaccharide fractions and

lipid compounds of the microalga *Heterosigma akashiwo* grown in vitro”, *Botanica Marina*, vol. 55, no. 6, pp. 565-580.

Misra, N., Patra, M.C., Panda, P.K., Sukla, L.B. Mishra, B.K. 2013, ”Homology modeling and docking studies of FabH (beta-ketoacyl-ACP synthase III) enzyme involved in type II fatty acid biosynthesis of *Chlorella variabilis*: a potential algal feedstock for biofuel production.”, *Journal of Biomolecular Structure Dynamics*, vol. 31, no. 3, pp. 241-257.

Nascimento, I.A., Izabel Marques, S.S., Dominguez Cabanelas, I.T., Pereira, S.A., Druzian, J.I., de Souza, C.O., Vich, D.V., de Carvalho, G.C. Nascimento, M.A. 2013, ”Screening Microalgae Strains for Biodiesel Production: Lipid Productivity and Estimation of Fuel Quality Based on Fatty Acids Profiles as Selective Criteria”, *Bioenergy Research*, vol. 6, no. 1, pp. 1-13.

Negoro, M., Shioji, N., Miyamoto, K. Miura, Y. 1991, ”Growth of Microalgae in High CO₂ Gas and Effects of SO_x and NO_x”, *Applied Biochemistry and Biotechnology*, vol. 28-9, pp. 877-886.

Nelson, D.L. Cox, M.M. 2008, *Lehninger Principles of Biochemistry*, 5th edn, W.H Freeman, New York.

Recht, L., Zarka, A. Boussiba, S. 2012, ”Patterns of carbohydrate and fatty acid changes under nitrogen starvation in the microalgae *Haematococcus pluvialis* and *Nannochloropsis* sp.”, *Applied Microbiology and Biotechnology*, vol. 94, no. 6, pp. 1495-1503.

Rismani-Yazdi, H., Haznedaroglu, B.Z., Hsin, C. Peccia, J. 2012, ”Transcriptomic analysis of the oleaginous microalga *Neochloris oleoabundans* reveals metabolic insights

into triacylglyceride accumulation”, *Biotechnology for Biofuels*, vol. 5, pp. 74.

Roessler, P. 1988, ”Changes in the Activities of various Lipid and Carbohydrate Biosynthetic-Enzymes in the Diatom *Cyclotella-Cryptica* in Response to Silicon Deficiency”, *Archives of Biochemistry and Biophysics*, vol. 267, no. 2, pp. 521-528.

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*, vol. 77, no. 6, pp. 565-575.

Van den Hende, S., Vervaeren, H. Boon, N. 2012, ”Flue gas compounds and microalgae: (Bio-)chemical interactions leading to biotechnological opportunities”, *Biotechnology Advances*, vol. 30, no. 6, pp. 1405-1424.

Wahlen, B.D., Willis, R.M. Seefeldt, L.C. 2011, ”Biodiesel production by simultaneous extraction and conversion of total lipids from microalgae, cyanobacteria, and wild mixed-cultures”, *Bioresource technology*, vol. 102, no. 3, pp. 2724-2730.

Wen, Z.Y. Chen, F. 2003, ”Heterotrophic production of eicosapentaenoic acid by microalgae”, *Biotechnology Advances*, vol. 21, no. 4, pp. 273-294.

Williams, P.J.I.B. Laurens, L.M.L. 2010, ”Microalgae as biodiesel biomass feedstocks: Review analysis of the biochemistry, energetics economics”, *Energy Environmental Science*, vol. 3, no. 5, pp. 554-590.

Yang, Z., Niu, Y., Ma, Y., Xue, J., Zhang, M., Yang, W., Liu, J., Lu, S., Guan, Y. Li, H. 2013, ”Molecular and cellular mechanisms of neutral lipid accumulation in diatom following nitrogen deprivation.”, *Biotechnology for biofuels*, vol. 6, no. 1, pp. 67-67.

Yoshihara, K.I., Nagase, H., Eguchi, K., Hirata, K. Miyamoto, K. 1996, "Biological elimination of nitric oxide and carbon dioxide from flue gas by marine microalga NOA-113 cultivated in a long tubular photobioreactor", Journal of Fermentation and Bioengineering, vol. 82, no. 4, pp. 351-354.

Zhang, Y., Fu, F., Whereat, E., Coyne, K. Hutchins, D. 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, vol. 5, no. 3, pp. 310-320.

Chapter 2

IMPACTS OF MODEL FLUE GAS AND CULTURE AGE ON FATTY ACID COMPOSITION AND BIODIESEL SOURCE POTENTIAL OF *HETEROSIGMA AKASHIWO* AND *CHATTONELLA SUBSALSA*

2.1 Abstract

The aim of this study was to evaluate the biodiesel potential of *Heterosigma akashiwo* and *Chattonella subsalsa* (class: Raphidophyceae) when bubbled with model flue gas and at different culture ages. Culture age had a significant effect on the biodiesel quality of both *H. akashiwo* and *C. subsalsa*. While the flue gas treatment increased the growth rates of both species, control cultures grown on air and in stationary phase had ideal fatty acid profiles for biodiesel production due to the low content of polyunsaturated fatty acids. Carbohydrate content was higher than lipid and protein content in all treatments of *H. akashiwo*, which suggests that this species may be a candidate for bioethanol production. Lipids were the most abundant biomolecule in all treatments of *C. subsalsa*. Lastly, the flue gas treatment resulted in an increase in carbohydrate content in both species. Biodiesel from both cultures, when bubbled with air and in stationary phase, were calculated to have a cetane number of 48-52, indicating the potential biodiesel is of high quality.

2.2 Introduction

Compared to land-crop feedstocks, microalgae have a higher photosynthetic efficiency, a faster growth rate and can accumulate larger quantity of lipids (Nascimento et al. 2013). Conservatively, microalgae are estimated to produce 20,000 liters of oil $\text{ha}^{-1} \text{year}^{-1}$, which is three times the oil production of palm (Nascimento et al. 2013). Despite this impressive estimate, biodiesel from microalgae is expensive and not economically feasible compared to traditional fossil fuels. To make microalgal biodiesel

more economical, resilient and productive algal species should be chosen and cultivation conditions should be optimized. Previous studies have identified *Heterosigma akashiwo* (Raphidophyceae) as a promising biodiesel feedstock candidate due to its high productivity, high lipid yields and fast growth rate (Fuentes-Grünewald et al. 2012). Furthermore, Fuentes-Grünewald et al. (2013) determined that *H. akashiwo* could be scaled up from the laboratory to a large-scale biodiesel production. However, *Chattonella subsalsa* (Raphidophyceae) has not been investigated.

While proper species selection is important, it is also beneficial to reduce the cost of cultivation. Carbon makes up 35-65% of algal dry mass and is a key nutrient resource. Large-scale cultivation of microalgae cannot depend on the diffusion of CO₂ from the atmosphere due to the low CO₂ concentration in the air and slow diffusion rates (reviewed by Van den Hende et al., 2012). Van den Hende et al., (2012) estimated that the addition of CO₂ accounts for 41% of raw material costs. Furthermore, it is estimated that 45% of energy input during the production of biodiesel from microalgae comes from the addition of nitrogen (reviewed by Chisti, 2008).

Flue gas is a possible nutrient source that has yet to be fully utilized for microalgal biodiesel (Van den Hende et al. 2012). Electrical power plants release 2.2 x 10⁹ tons of CO₂ per year into the atmosphere, which is more than 1/3 of U.S emissions (reviewed by Van den Hende et al. 2012). It has been shown that microalgae can consume high quantities of CO₂ and may have the potential to diminish the release of CO₂ into the atmosphere (Brown 1996; Van den Hende et al. 2012; Yoo et al. 2010). Specifically, Brown (1996) determined that some species of microalgae could not only survive high concentrations of CO₂ but also have a 90% CO₂ trapping efficiency.

Because microalgae can consume high quantities of CO₂, they have been considered for the removal of CO₂ from the flue gas of fossil fuel power stations, but flue gas also contains 100-300 ppm NO_x (>95% as NO), which has largely been ignored (reviewed by Van den Hende, 2012). Flue gas can also potentially be a nitrogen source for microalgae. NO reacts with oxygen and water to form NO₂⁻ and NO₃⁻, under basic conditions, which are N sources and can be assimilated by algae. Nagase et al.

(2001) suggested that NO can also be directly assimilated by *Dunaliella tertiolecta* by permeating directly into the cells through diffusion. In *D. tertiolecta*, NO was preferentially utilized as an N source over NO_3^- . Both *C. subsalsa* and *H. akashiwo* contain a hybrid nitrate reductase (NR2-2/2HbN) that may detoxify and convert NO to nitrate (Stewart and Coyne, 2011). This enzyme may allow *H. akashiwo* and *C. subsalsa* to harness NO from flue gas as a free nitrogen source and thus reduce the cost of biodiesel production from microalgae.

The chemical composition of the biodiesel derived from microalgae will be very similar to the microalgal fatty acid profile, thus it is important to thoroughly investigate the effect of culture age on fatty acid composition. In a recent study, Fuentes-Grünewald et al. (2012) investigated changes in the fatty acid profiles of *H. akashiwo* with culture age. Saturated fatty acid content, especially palmitic acid (C16:0), increased as *H. akashiwo* reached the stationary phase. Due to their lack of double bonds, saturated fatty acids are a good source of biodiesel because they are stable against oxidation in the engine block. Low oxidative stability of biodiesel is caused by polyunsaturated fatty acids (PUFA) (Ciubota-Rosie et al., 2013). This low stability is due to the methylene groups adjacent to the double bonds (bis-allylic positions), which are susceptible to oxidative attack by free radicals during the combustion process (Ciubota-Rosie et al., 2013). Fuentes-Grünewald et al. (2012) determined that *H. akashiwo* greatly reduced its PUFA content in the stationary phase. This decrease in PUFA content and the associated increase in saturated fatty acids indicate that *H. akashiwo* will produce the best fatty acid profile for biodiesel production during stationary phase. However, the effects of flue gas (increased CO_2 and N in the form of NO) on carbon partitioning and fatty acid profiles of *H. akashiwo* or other raphidophyte species is unknown.

The goal of this study was to compare and assess carbon partitioning and the biodiesel potential of *H. akashiwo* and *C. subsalsa* when bubbled with model flue gas compared to air. Fatty acid composition was evaluated by gas chromatography and used to calculate biodiesel quality. To better understand lipid metabolism in

microalgae, the expression patterns of genes involved in fatty acid synthesis were also investigated. Results show that when bubbled with air, *C. subsalsa* is an ideal biodiesel feedstock and *H. akashiwo* is a potential bioethanol feedstock.

2.3 Materials and Methods

2.3.1 Experimental Design

Heterosigma akashiwo (Delaware Inland Bay isolate, CCMP 2393) and *Chattonella subsalsa* (Delaware Inland Bay isolate, CCMP 2393) are deposited in the National Center for Marine Algae and Microbiota (formerly the Center for the Culture of Marine Phytoplankton). Cultures were grown in 20 psu f/2 medium buffered with 20 mM HEPES (pH 7.4) at 25°C on a 12:12 hour light: dark cycle with an irradiance of 100 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$. Batch cultures of *H. akashiwo* (seeded at 180,000 cells/mL) and *C. subsalsa* (seeded at 50,000 cells/mL) were bubbled on air for five weeks (transferred every 7 days) then separated into control and treatment cultures. The control cultures continued to be bubbled (2 mL minute⁻¹) with air, while the treatment cultures were bubbled (2 mL minute⁻¹) with model flue gas (nitrogen gas containing 12% carbon dioxide and 150 ppm nitric oxide). After the treatment cultures began to grow on flue gas, control and treatment cultures were separated into replicates (N=4). Cell density was measured for all cultures over two growth cycles in order to determine when each culture would reach stationary phase. Prior to the third growth cycle, replicates for control and treatment cultures were recombined and separated into replicates (N=4). To determine when the cultures entered each growth phase, cell density and *in vivo* chl *a* were measured every day. Samples were collected for analysis of total lipid content, lipid composition, carbon partitioning, and gene expression during exponential and stationary phases. Sampling and analytical methods are described in detail in Appendix A.

2.3.2 Statistical Analysis

The experiment was performed in four replicates for the control and treatment. Data represent means \pm standard deviations. A one-way ANOVA was performed to determine the significant differences between all treatments. A Tukey's HSD post hoc test was performed when a one-way ANOVA detected a significant difference. Kolmogorov-Smirnov and Levenes tests were used to determine if the data were normally distributed and had equal variances, respectively. The transcript abundance data were not normally distributed and treatments did not have equal variances so a square root transformation was performed. After this transformation, all one-way ANOVA assumptions were met. A paired t-test was used to determine differences between the exponential and stationary phases of the same culture. A p-value of 0.05 was used as the standard for statistical significance. Statistical analyses were conducted with IBM SPSS Statistics (International Business Machines Corporation, USA)

2.4 Results

2.4.1 Growth, Particulate Nitrogen and Particulate Carbon

During exponential phase, *H. akashiwo* grew 2.3 times faster when bubbled with flue gas compared to when bubbled with air ($p < 0.01$; Table 2.1). Similarly, the growth rate of *C. subsalsa* was higher when bubbled with flue gas compared to the control cultures grown on air, however, this difference was not significant (Table 2.2). Furthermore, in both species, the cultures bubbled with flue gas were in the exponential growth phase for a longer period of time compared to the cultures bubbled with air (Figure 2.1). *H. akashiwo* was in exponential phase for 10 days when bubbled with flue gas and 5 days when bubbled with air. *C. subsalsa* was in exponential phase for 11 days when bubbled with flue gas and 6 days when bubbled with air. It should be noted that samples in exponential phase were collected at the same time for each treatment so the *H. akashiwo* cultures bubbled with air may have already been in the stationary phase when the exponential phase sampling occurred (Figure 2.1).

The C/N ratio of *H. akashiwo* grown on flue gas was not significantly different from cultures grown on air during the exponential phase. However, during the stationary phase, the cultures grown on flue gas had a significantly higher C/N ratio than the cultures grown on air ($p < 0.001$). In *C. subsalsa*, the C/N ratio of the stationary culture bubbled on flue gas was significantly higher than all other treatments (Table 2.2). This increase in C/N may be due to the significant increase in carbon from the exponential to the stationary phase in the cultures bubbled with flue gas.

2.4.2 Carbon Partitioning

When bubbled with air, there were no significant changes in carbohydrate, lipid or protein content with culture age in *H. akashiwo* (Figure 2.2). Protein and lipid content were higher than carbohydrate content in both growth phases. However, when bubbled with model flue gas, carbohydrate and protein content of *H. akashiwo* were higher than lipid content in both growth phases. *H. akashiwo* accumulated significantly more carbohydrates in the stationary phase compared to the exponential phase (Tukey's HSD, $p < 0.01$) so that carbohydrates were the most abundant biomolecule in the flue gas treatment. When comparing between gas treatments, protein content in the flue gas treatment was significantly higher than in cultures bubbled with air during exponential growth and cultures grown on flue gas had significantly more carbohydrates in both phases compared to the cultures grown on air (one-way ANOVA, $p < 0.001$).

Gas treatment had a significant effect of the carbon partitioning of *C. subsalsa* (Figure 2.3). When bubbled with air, lipid content of *C. subsalsa* was higher than carbohydrate and protein content in both growth phases. The cultures bubbled with flue gas and in stationary phase had significantly more carbohydrates than the other cultures (one-way ANOVA, $p < 0.001$) while the cultures bubbled with air had significantly more lipids than the cultures bubbled with flue gas (one-way ANOVA, $p < 0.001$). Protein content was significantly higher in the cultures grown on flue gas compared to the cultures grown on air (one-way ANOVA, $p > 0.05$).

2.4.3 Fatty Acid Profile

The major fatty acids in both air and flue gas treatments of *H. akashiwo* were myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), linolenic (C18:3), stearidonic (C18:4) and eicosapentaenoic (C20:5, EPA) (Figure 2.4). Under both gas treatments, the percent of saturated fatty acids were significantly higher and polyunsaturated fatty acids (PUFA) significantly lower during stationary phase compared to exponential phase (Table 2.3). This is due to the accumulation of significantly more C14:0 (one-way ANOVA, $p < 0.01$) and C15:0 (one-way ANOVA, $p < 0.05$) in stationary phase of both gas treatments (Figure 2.4). In the air treatment, there was also a significant decrease in C18:3, C18:4 and C20:5, along with a significant increase in C20:1 content as a percent of total fatty acids during stationary phase, resulting in a significant increase in the proportion of monounsaturated fatty acids (MUFAs). The largest change in fatty acid content in the cultures grown on air was C20:5, which decreased significantly from $19 \pm 1\%$ in the exponential phase to $0.6 \pm 0.2\%$ in the stationary phase (paired t-test, $p < 0.01$).

Fatty acid profiles were also significantly different between gas treatments for *H. akashiwo* cultures. The flue gas cultures had a significantly higher C18:3 content than cultures grown on air (one-way ANOVA, $p < 0.001$). However, the air treatment resulted in a significantly higher gadoleic acid (C20:1) content compared to cultures grown on flue gas (one-way ANOVA, $p < 0.001$). The cultures bubbled with flue gas contained trace amounts of C20:4 and C18:2, which were not detected in the cultures grown on air. In contrast to the cultures bubbled with air, the C20:5 content for cultures bubbled with flue gas did not vary between the exponential and stationary phase. In addition, the cultures grown on flue gas did not exhibit large variation in fatty acid content between growth phases that were observed for cultures bubbled with air. The only significant changes for the cultures grown on flue gas were in C14:0, C14:1 and C15:0 content, which were significantly higher in the stationary phase compared to the exponential phase.

The major fatty acids in both gas treatments of *C. subsalsa* were C14:0, C16:0,

C16:1, C18:3, C18:4 and C20:5 (Figure 2.5). Overall, PUFA content as a percent of total fatty acids was significantly higher in the flue gas treatment compared to the air treatment (one-way ANOVA, $p < 0.01$; Table 2.4) while MUFA content was significantly higher in the air treatment (one-way ANOVA, $p < 0.01$). Under both gas treatments, saturated fatty acid content as a percent of total fatty acid content was higher during the exponential phase compared to the stationary phase (Table 2.4). C14:0 content was significantly higher in the exponential phase than in the stationary phase for both treatments (one-way ANOVA, $p < 0.001$). C15:0 (one-way ANOVA, $p < 0.001$) and C16:0 (one-way ANOVA, $p < 0.001$), as percents of total fatty acid content were significantly higher in the stationary phase than in the exponential phase.

The flue gas also had a significant effect on fatty acid profiles in *C. subsalsa* when compared to the air treatments. The flue gas treatment resulted in a significantly higher C15:0 (one-way ANOVA, $p < 0.001$), C18:3 (one-way ANOVA, $p < 0.01$) and C20:5 (one-way ANOVA, $p < 0.05$) content than the cultures grown with air. However, C18:0 content was significantly higher in the air treatment compared to the flue gas treatment (one-way ANOVA, $p < 0.01$). While the fatty acid content of the air treatment contained C14:1, this MUFA was not detected in the cultures grown on flue gas. The cultures bubbled on flue gas also contained C18:2 and C22:1, which were not detected in the cultures bubbled on air.

In both *H. akashiwo* and *C. subsalsa*, cultures bubbled with flue gas contained C18:2, which was not detected in the cultures bubbled with air. *H. akashiwo* contained 20-carbon fatty acids (C20:1, C20:3 and C20:4), which were not detected in *C. subsalsa*. Interestingly, *C. subsalsa* contained C22:1, the product of the elongation of C20:1, which was not detected in *H. akashiwo*.

2.4.4 Transcript Abundance

Relative messenger RNA levels of six key genes involved in fatty acid synthesis were investigated in *H. akashiwo* (Figure 2.6 and 2.7). There were no significant differences in acyl carrier protein (ACP) or $\Delta 5$ desaturase (DES) transcript abundance

among the treatments. The cultures bubbled on air had significantly more acyl CoA-binding protein (ACBP) transcript abundance during the stationary phase compared to all other treatments (one-way ANOVA, $p < 0.01$). In the cultures bubbled with flue gas, the transcript abundance of acetyl-CoA carboxylase (ACC) and beta-ketoacyl-ACP synthase (KAS) were significantly higher in stationary phase compared to exponential phase and to cultures bubbled with air (one-way ANOVA, $p < 0.05$ and $p < 0.01$, respectively). $\Delta 6$ fatty acid elongase (FAE) transcript abundance was higher in the cultures bubbled with air compared to the cultures bubbled with flue gas (one-way ANOVA, $p < 0.01$).

Relative messenger RNA levels of five key genes involved in fatty acid synthesis also were investigated in *C. subsalsa* (Figure 2.8 and 2.9). There were no significant differences in ACC or FAE transcript abundance among the treatments. Similar to *H. akashiwo*, transcript abundance of ACBP was significantly higher during the stationary phase of the cultures bubbled with air compared to all other treatments (one-way ANOVA, $p < 0.01$). Transcript abundance of malonyl-CoA-ACP-transferase (MCT) was significantly higher during the exponential phase of the cultures bubbled with flue gas compared to all other treatments (one-way ANOVA, $p < 0.05$). In contrast to *H. akashiwo*, DES transcript abundance in *C. subsalsa* was significantly higher during the stationary phase of the cultures bubbled with air compared to all other treatments (one-way ANOVA, $p < 0.05$).

2.4.5 Biodiesel Quality

Culture age had a significant effect on biodiesel quality of both *H. akashiwo* and *C. subsalsa* (Tables 2.5 and 2.6). For both species, cetane number (CN) was significantly higher during stationary phase compared to exponential phase (one-way ANOVA, Ha $p < 0.01$; Cs $p < 0.05$). However, there was no significant difference of the CN between gas treatments. Biodiesel from the *C. subsalsa* air culture during stationary phase had the lowest iodine number (111 ± 3) and the highest CN (48 ± 1) than the other treatments (Table 2.4). Similarly, biodiesel from the *H. akashiwo*

air culture during stationary phase had the lowest iodine number (92 ± 12) and the highest CN (52 ± 3) than the other treatments (Table 2.3), indicating that it is the best biodiesel candidate of both species and all treatments.

2.5 Discussion

Biodiesel from microalgae is not currently economically feasible, in part due to the high cost of cultivation. Using flue gas as a nutrient source may be an effective way to reduce these costs. While flue gas has been shown to inhibit the growth of most algae (Negoro et al. 1991), both *H. akashiwo* and *C. subsalsa* had a significantly higher growth rate and achieved greater cell density when bubbled with flue gas compared to when bubbled with air. This flue gas-induced increase in growth rate and biomass is highly desirable in terms of biodiesel.

The composition of the algal biomass with respect to carbohydrates, proteins and lipids will determine the overall biodiesel potential of a *H. akashiwo* and *C. subsalsa*. When bubbled with air, there were no differences in lipid, carbohydrate or protein concentration between the exponential and the stationary phase for *H. akashiwo*. In contrast to these results, Fuentes-Grünewald et al. (2009) observed a 43% decrease in lipid concentration from the exponential phase to the stationary phase in a Mediterranean Sea isolate of *H. akashiwo*, highlighting the value of investigating alternate strains of this species for biomass production.

H. akashiwo had significantly higher protein content during both growth phases when bubbled with flue gas compared to exponential growth phase cultures bubbled with air. Protein content of exponential phase cultures in the flue gas treatment was also significantly higher than protein content of cultures grown on air. Proteins contain more N than both lipids and carbohydrates (reviewed by Williams et al. 2010). Thus, this increase in protein may have been due to uptake of NO present in the flue gas.

When bubbled with flue gas, *H. akashiwo* contained significantly more carbohydrates than when bubbled with air. Under flue gas treatment, carbohydrate content also increased in stationary phase compared to exponential phase. This suggests that

starch is used for primary carbon and energy storage. These results are consistent with those of Lopes et al. (2012), which determined that carbohydrate content increased in the stationary phase in *H. akashiwo*. Carbohydrate synthesis has important implications for bioethanol production. Although the structure of *H. akashiwo* carbohydrates were not identified in this study, Lopes et al (2012) determined that the major carbohydrates in *H. akashiwo* were rhamnose, fucose, mannose, galactose and glucose. For bioethanol production, the storage carbohydrates are hydrolyzed into simple sugars (usually glucose), which are then converted into ethanol by microbial fermentation. In spite of extensive research demonstrating that *Dunaliella* has potential to be a renewable biomass for production of bioethanol, bioethanol from microalgae is not currently mass-produced (Doan et al. 2012).

Similar to *H. akashiwo*, *C. subsalsa* also accumulated carbohydrates in the stationary phase. Carbohydrates were the most abundant biomolecule for the *C.subsalsa* cultures bubbled with flue gas while lipids were the most abundant biomolecule for the cultures bubbled with air. Both *H. akashiwo* and *C. subsalsa* shifted their resources to production of carbohydrates when bubbled with flue gas. This is probably due to the high amount of CO₂ (12%) in the flue gas, which is converted into simple carbohydrates through photosynthesis. For *C. subsalsa*, this increase in carbohydrate content may be explained by the increase in particulate carbon in the stationary phase.

In microalgae, the fatty acids in triglycerides are easily converted into biodiesel. Thus, the fatty acid composition has an important influence on biodiesel quality. Generally, saturated and monounsaturated fatty acid concentrations increase as microalgae age (reviewed by Hu et al. 2008). The fatty acid profiling reported in this study suggests that both *H. akashiwo* and *C. subsalsa* are ideal candidates for biodiesel production. C16:0 was the principle fatty acid in both species and under all treatments and is an ideal fatty acid for biodiesel due to its chain length and degree of unsaturation (Nascimento et al. 2013). In *H. akashiwo*, C14:0 and C15:0 significantly increased during stationary phase compared to the exponential phase, which contributed to the increase in saturated fatty acid content during the stationary phase. These findings are

similar to those of Fuentes-Grünewald et al. (2012), who reported that *H. akashiwo* increased its saturated fatty acid content and decreased its PUFA content upon reaching the stationary phase. In the air cultures, this decrease in PUFA content is due to the significant decrease in C20:5 during stationary phase.

Metabolic engineering is believed to be an effective solution to the high production cost of microalgal biodiesel and may be a way to obtain a favorable fatty acid composition (Lei et al. 2012). Both *H. akashiwo* and *C. subsalsa* contain high amounts of the omega-3 fatty acid, eicosapentaenoic acid (C20:5, EPA). The biosynthesis of C20:5 was specifically investigated in this work because C20:5 significantly decreases biodiesel quality due to its high degree of unsaturation. The enzymes, $\Delta 6$ fatty acid elongase (FAE) and $\Delta 5$ desaturase (DES), aid in the production of C20:5. However, neither FAE nor DES is specific for EPA production. DES converts C20:4 into C20:5 but also desaturates C20:3 to form C20:4. FAE elongates C18:4 to form C20:4 and C18:3 to form C20:3. It was expected that the decrease in C20:5 in the *H. akashiwo* cultures bubbled with air would correlate to a decrease in mRNA abundance of these genes but there was no difference between the growth phases. However, C20:5 may have been elongated to longer chain fatty acids, which were not detected in this study. Furthermore, C20:4 (a product of FAE) was not detected in the cultures bubbled with air, which indicates that FAE may have worked on a substrate other than C18:4. DES may have immediately converted C20:4 to C20:5. Another possibility is that C20:5 is produced through the ω -6 pathway via $\Delta 17$ desaturase. Further investigation into the gene abundance of $\Delta 17$ desaturase is needed to fully understand C20:5 synthesis in *H. akashiwo*.

Acyl-CoA binding proteins (ACBP) are involved in transport of saturated fatty acids from plastid to endoplasmic reticulum. Previous studies have shown that ACBP is upregulated during biological aging (senescence) in plants (Xiao et al. 2010). In this study, ACBP transcript abundance was higher in *H. akashiwo* during stationary phase compared to exponential phase. This observation indicates that ACBP transcript abundance may be indicative of senescence in *H. akashiwo*. Furthermore, ACBP

transcript abundance was also positively correlated to increase in saturated and monounsaturated fatty acid content at stationary phase. Because these fatty acid types increase biodiesel quality, ACBP transcript abundance may be a proxy for biodiesel quality.

3-ketoacyl ACP reductase (KAS) elongates substrates between C4:0 and C14:0 by the addition of 2 carbon atoms. KAS transcript abundance was upregulated during the stationary phase in *H. akashiwo* cultures bubbled with flue gas. However, this high KAS transcript abundance was not correlated to a high saturated fatty acid content as expected. Moreover, there were no differences in monounsaturated or polyunsaturated fatty acid content between the stationary phase in *H. akashiwo* cultures bubbled with flue gas and all other treatments. This uncorrelated KAS transcript abundance may indicate that the saturated fatty acids produced by KAS were elongated to long chain fatty acids (> C:22), which were not measured in this study.

Malonyl CoA-ACP transferase (MCT) transcript abundance was significantly higher in the exponential phase of the *C. subsalsa* culture bubbled with flue gas compared to all other treatments. MCT catalyzes the initiation of fatty acid elongation by transferring the malonyl moiety from malonyl-CoA on to ACP. Because MCT initiates the two-carbon addition, the resulting change in fatty acid profile would probably be in the short-chain fatty acids, which were not measured in this study. DES transcript abundance was significantly higher in the stationary phase of the culture bubbled with air, compared to all other treatments. Inconsistently, C20:5 content was lowest in this treatment compared to the others. However, C20:4, which is a product of FAE and a substrate of DES, was not detected in the cultures bubbled with air. This may indicate that DES acted on another substrate, as noted above.

Cetane number (CN) is the primary indicator of diesel quality and measures the ignition delay when the diesel is injected into the combustion chamber. Iodine value (IV) indicates the tendency of the biodiesel to oxidize. Both of these parameters are negatively affected by a high degree of unsaturation, while CN is positively related to fatty acid chain length. Thus, the high proportions of the long chain saturated fatty

acid C16:0 in both *H. akashiwo* and *C. subsalsa* contributes to a high CN. In both *H. akashiwo* and *C. subsalsa*, CN was significantly higher during stationary phase compared to exponential phase but there was no significant difference of the CN between gas treatments. For both species, biodiesel from the air culture during stationary phase had a lowest iodine value and a highest CN than the other treatments.

In both species, the cultures bubbled with air and in stationary phase were the only treatments with IV below the European maximum of 120 (Nascimento et al. 2013). The air cultures of *H. akashiwo* in stationary phase had significantly less C20:5 than all other treatments, which contributed to its good CN and IV. Furthermore, saturated fatty acids as a percent of total fatty acid concentration were significantly higher and PUFA as a percent of total fatty acid concentration was significantly lower in both air cultures during stationary phase compared to all other treatments, which contributed to the high quality of the biodiesel.

2.6 Conclusion

Figure 2.12 summarizes the effects of excess CO₂ and NO in flue gas on *H. akashiwo* and *C. subsalsa*. When bubbled with flue gas, both species had higher carbohydrate and protein content compared to when bubbled with air. This indicates that the excess CO₂ was partitioned into the carbohydrates while the excess NO increased protein content. When bubbled with air, *C. subsalsa*, had a higher lipid content than when bubbled with flue gas. However, on a per cell basis, *C. subsalsa* had a higher lipid content than *H. akashiwo*, which indicates that *C. subsalsa* may be a better biodiesel feedstock. In the cultures bubbled with flue gas, carbohydrate content increased in stationary phase. There was no change in lipid, protein or carbohydrate content with growth phase in the culture bubbled with air.

Overall, culture age was shown to have a significant effect on the biodiesel quality of both *H. akashiwo* and *C. subsalsa*. The stationary phase cultures of both species grown on air had ideal fatty acid profiles for biodiesel production due to the

high amount of saturated fatty acids and low amount of PUFA. However, the flue gas treatment increased the growth rates of both species.

Table 2.1: Growth rate and particulate carbon and nitrogen of *H. akashiwo* expressed as means \pm standard deviation, n= 4. C/N = moles carbon/ moles nitrogen. Different letters denote significant differences.

	Exponential		Stationary	
	Control	Flue Gas	Control	Flue Gas
Growth Rate (day ⁻¹)	0.16 \pm 0.03 (a)	0.36 \pm 0.05 (b)		
Carbon (pg/ cell)	710 \pm 43 (a)	898 \pm 160 (a)	670 \pm 130 (a)	603 \pm 130 (a)
Nitrogen (pg/ cell)	110 \pm 16 (a)	110 \pm 26 (a)	164 \pm 34 (b)	92 \pm 18 (a)
C/N	7.8 \pm 1.1	9.5 \pm 0.9	4.8 \pm 0.1	7.8 \pm 0.5

Table 2.2: Growth rate and C/N of *C. subsalsa* expressed as means \pm standard deviation, n= 4. C/N = moles carbon/ moles nitrogen. Different letters denote significant differences.

	Exponential		Stationary	
	Control	Flue Gas	Control	Flue Gas
Growth Rate (day^{-1})	0.15 ± 0.05	0.23 ± 0.04		
Carbon (pg/ cell)	3860 ± 297 (a)	2047 ± 283 (b)	6372 ± 353 (c)	3133 ± 246 (d)
Nitrogen (pg/ cell)	1009 ± 101 (a)	509 ± 86 (b)	1561 ± 85 (c)	553 ± 41 (b)
C/N	4.5 ± 0.1 (a)	4.7 ± 0.2 (a)	4.8 ± 0.1 (a)	6.6 ± 0.6 (b)

Table 2.3: Biodiesel quality of oil derived from *H. akashiwo* under gas treatment at different culture ages (expressed as means \pm standard deviation, n= 4). Different letters denote significant differences between groups using a one-way ANOVA (p<0.05).

	Control		Flue Gas	
	Exponential	Stationary	Exponential	Stationary
Iodine Value	187 \pm 12 (a)	92 \pm 17 (b)	165 \pm 16 (c)	133 \pm 44 (b c)
Saponification Number	201 \pm 4 (a)	201 \pm 6 (a)	201 \pm 1 (a)	204 \pm 3 (a)
Cetane Number	31 \pm 9 (a)	52 \pm 3 (b)	36 \pm 1 (a)	43 \pm 9 (a)
Cetane Number without 20:5(n3)	54 \pm 1 (a)	59 \pm 4 (a)	50 \pm 2 (b)	56 \pm 3 (a)

Table 2.4: Biodiesel quality of oil derived from *C. subsalsa* under gas treatment at different culture ages (expressed as means \pm standard deviation, n= 4)

	Control		Flue Gas	
	Exponential	Stationary	Exponential	Stationary
Iodine Value	154 \pm 18	111 \pm 3	145 \pm 12	126 \pm 6
Saponification Number	240 \pm 32	201 \pm 1	198 \pm 1	202 \pm 1
Cetane Number	34 \pm 7	48 \pm 1	41 \pm 3	44 \pm 2
Cetane Number without 20:5(n3)	48 \pm 6	57 \pm 1	56 \pm 1	62 \pm 2

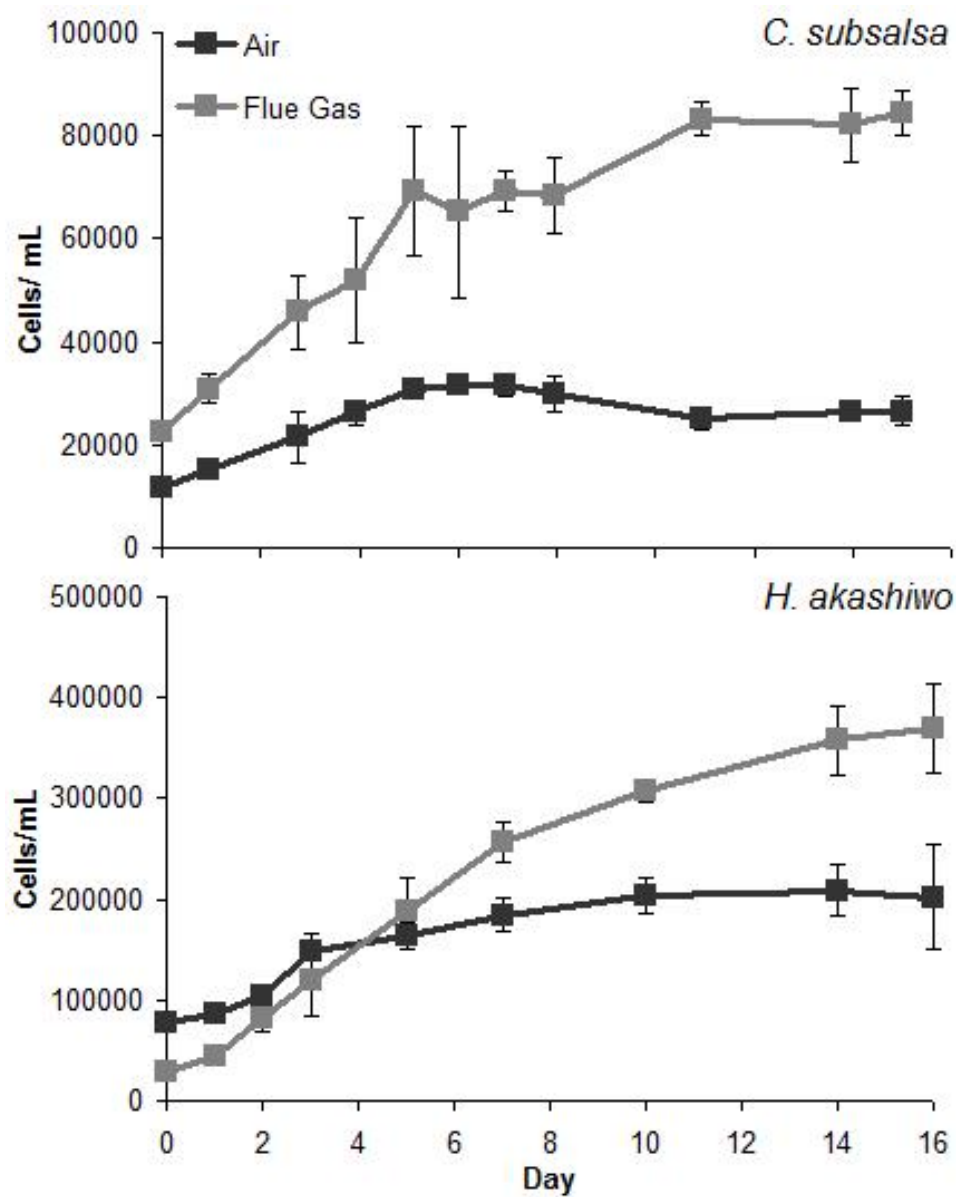


Figure 2.1: Growth curves of *C. subsalsa* and *H. akashiwo*. Data are expressed as means and error bars represent one standard deviation (n=4). Samples were taken on days indicated with black squares.

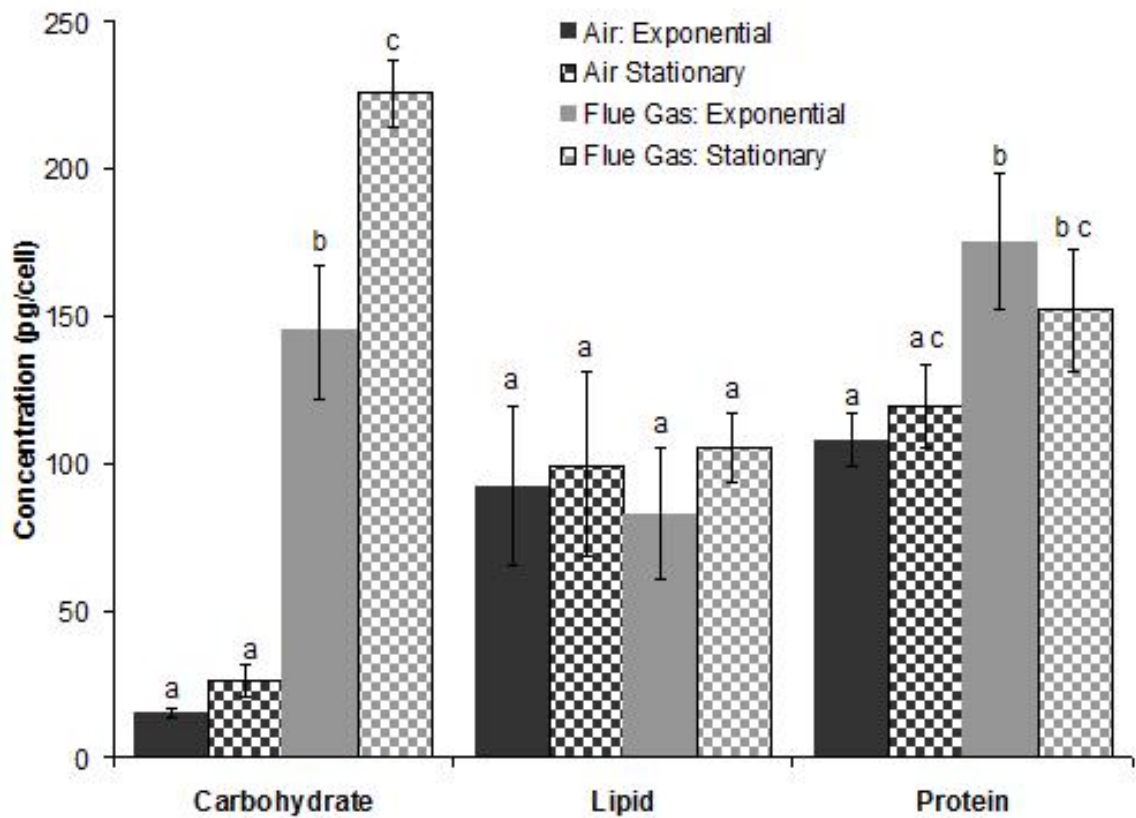


Figure 2.2: Effect of gas treatment and growth phase on carbohydrate, lipid and protein content of *H. akashiwo*. Data are expressed as means and error bars represent one standard deviation (n=4). Different letters denote significant differences between groups using a one way ANOVA ($p < 0.05$).

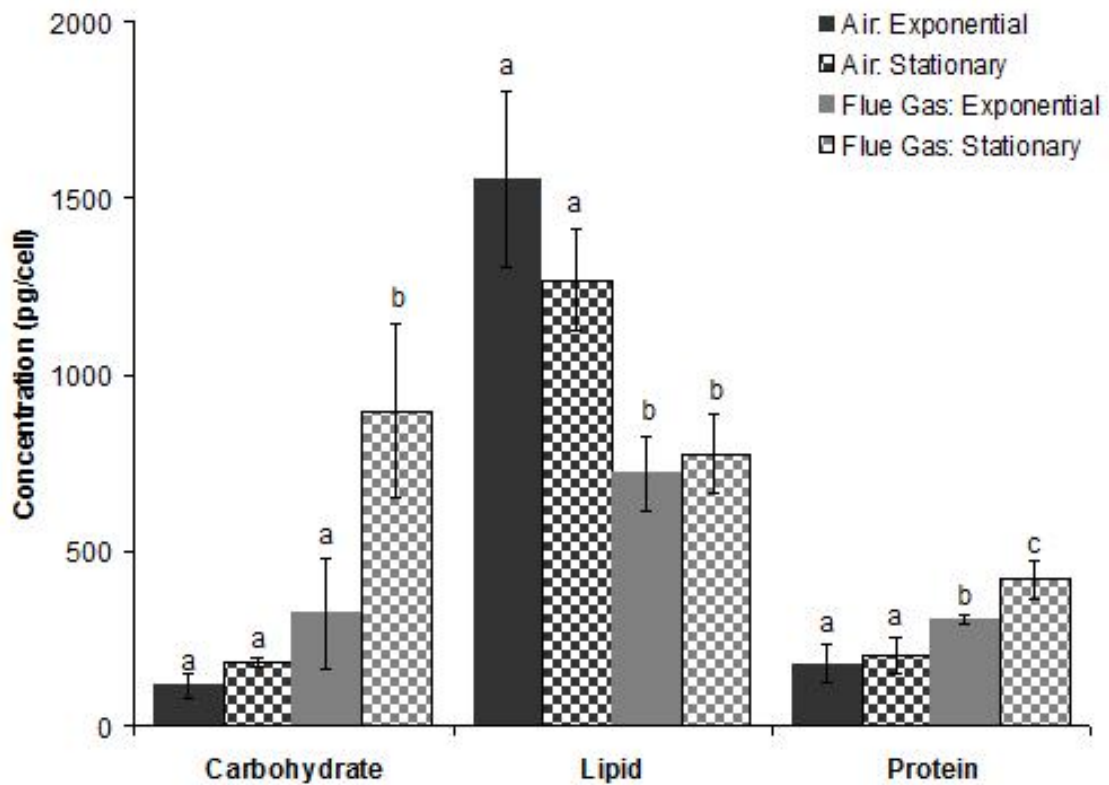


Figure 2.3: Effect of gas treatment and growth phase on carbohydrate, lipid and protein content of *C. subsalsa*. Data are expressed as means and error bars represent one standard deviation (n=4). Different letters denote significant differences between groups using a one way ANOVA ($p < 0.05$).

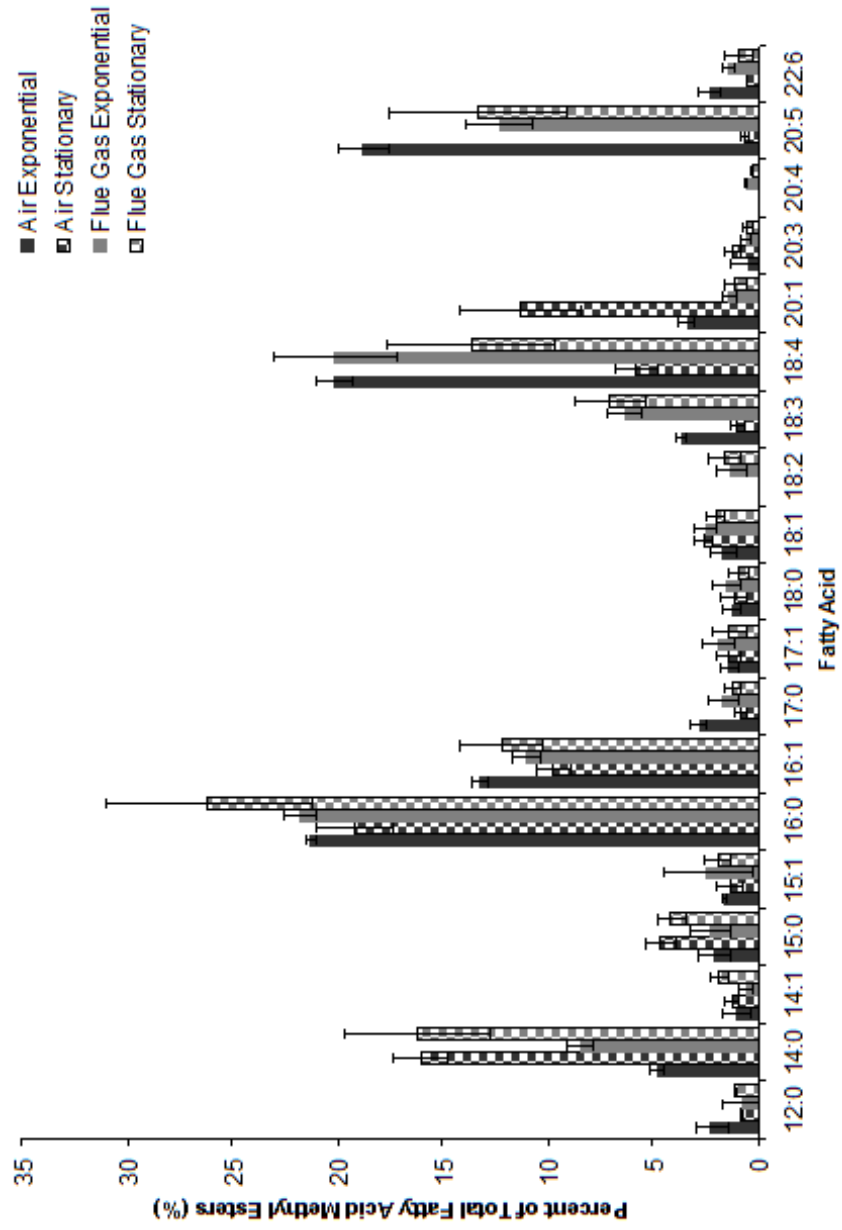


Figure 2.4: Fatty acid profile of *H. akashiwo* grown on flue gas or on air at different growth phases. Data are expressed as means and error bars represent one standard deviation (n=4).

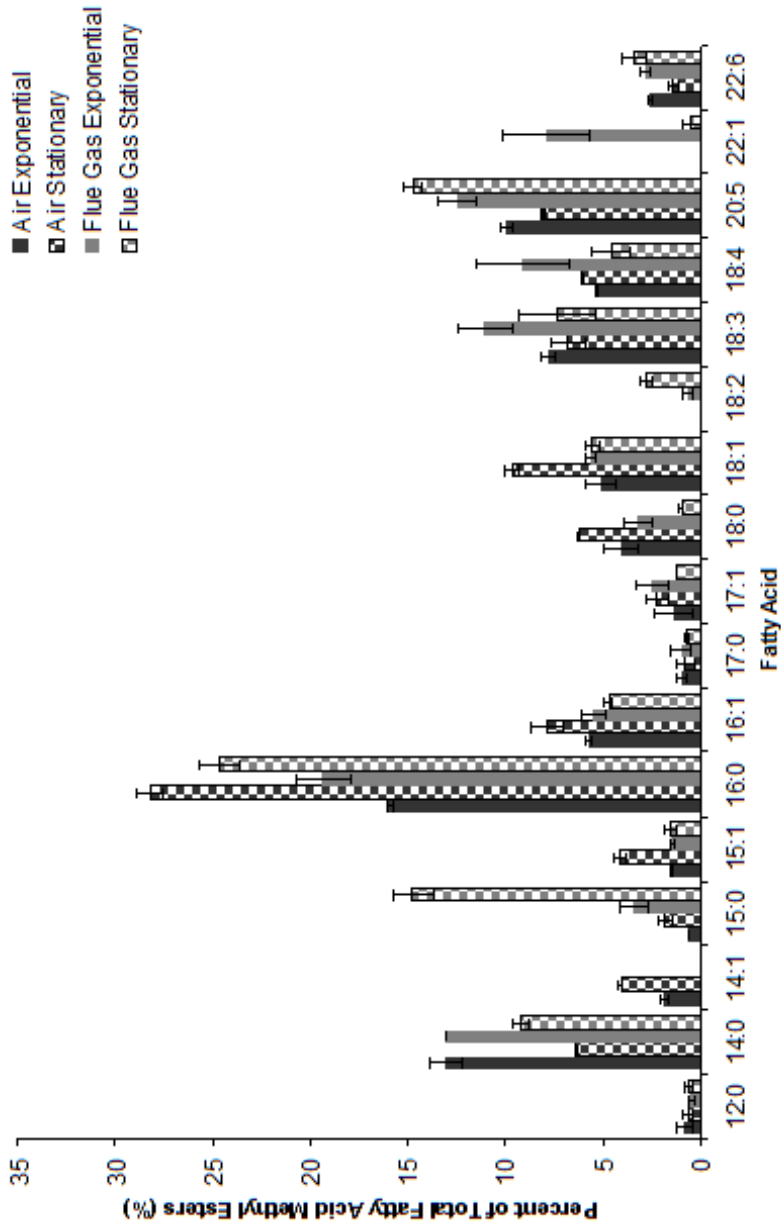


Figure 2.5: Fatty acid profile of *C. subsalsa* grown on flue gas or on air at different growth phases. Data are expressed as means and error bars represent one standard deviation (n=4).

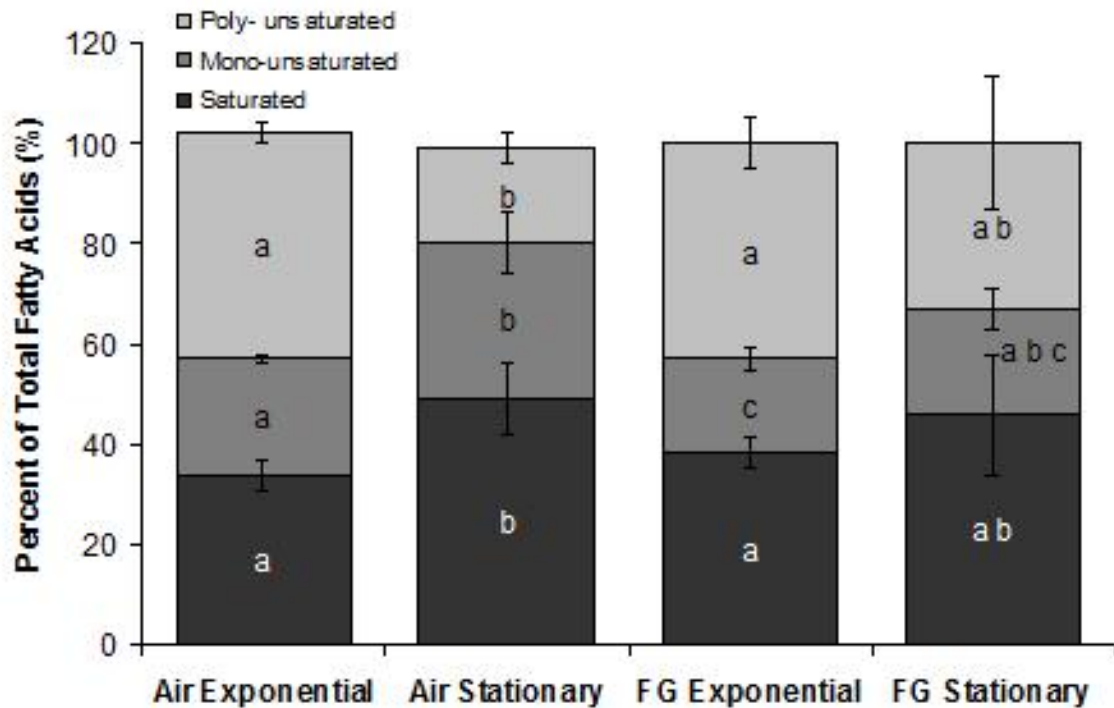


Figure 2.6: Fatty acid type as a percent of total fatty acid content in *H. akashiwo* under gas treatment at different culture ages (expressed as means \pm standard deviation, n=4). Different letters denote significant differences between groups using a one-way ANOVA ($p < 0.05$).

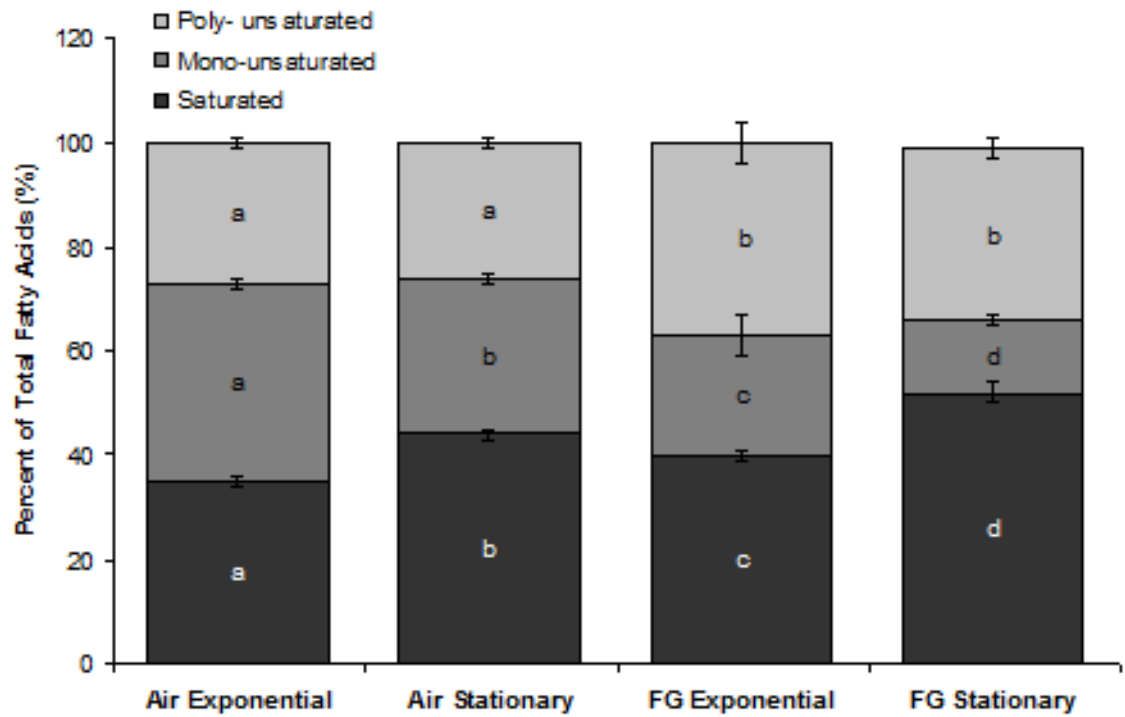


Figure 2.7: Fatty acid type as a percent of total fatty acid content in *C. subsalsa* under gas treatment at different culture ages (expressed as means \pm standard deviation, n=4). Different letters denote significant differences between groups using a one-way ANOVA ($p < 0.05$).

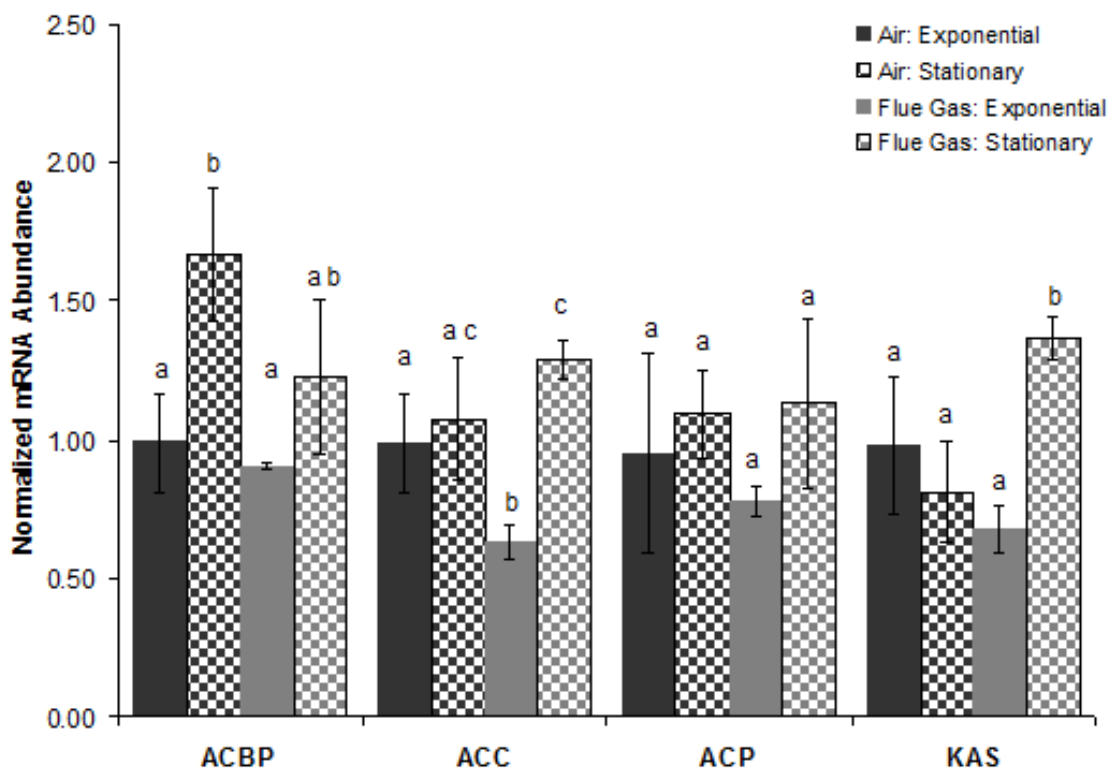


Figure 2.8: Effects of gas treatment and growth phase on the mRNA abundance of four key genes involved in fatty acid synthesis in *H. akashiwo*. ACBP: acyl CoA-binding protein, ACC: acetyl-CoA carboxylase, ACP: acyl-carrier protein, KAS: beta-ketoacyl-ACP synthase. Gene expression is normalized to 18S rRNA transcript abundance and expressed relative to the average transcript abundance of the exponential phase culture in the air treatment. Data are expressed as means and error bars represent one standard deviation (n=4). Different letters denote significant differences among treatments using a one-way ANOVA followed by Tukey's HSD means comparison test ($p < 0.05$).

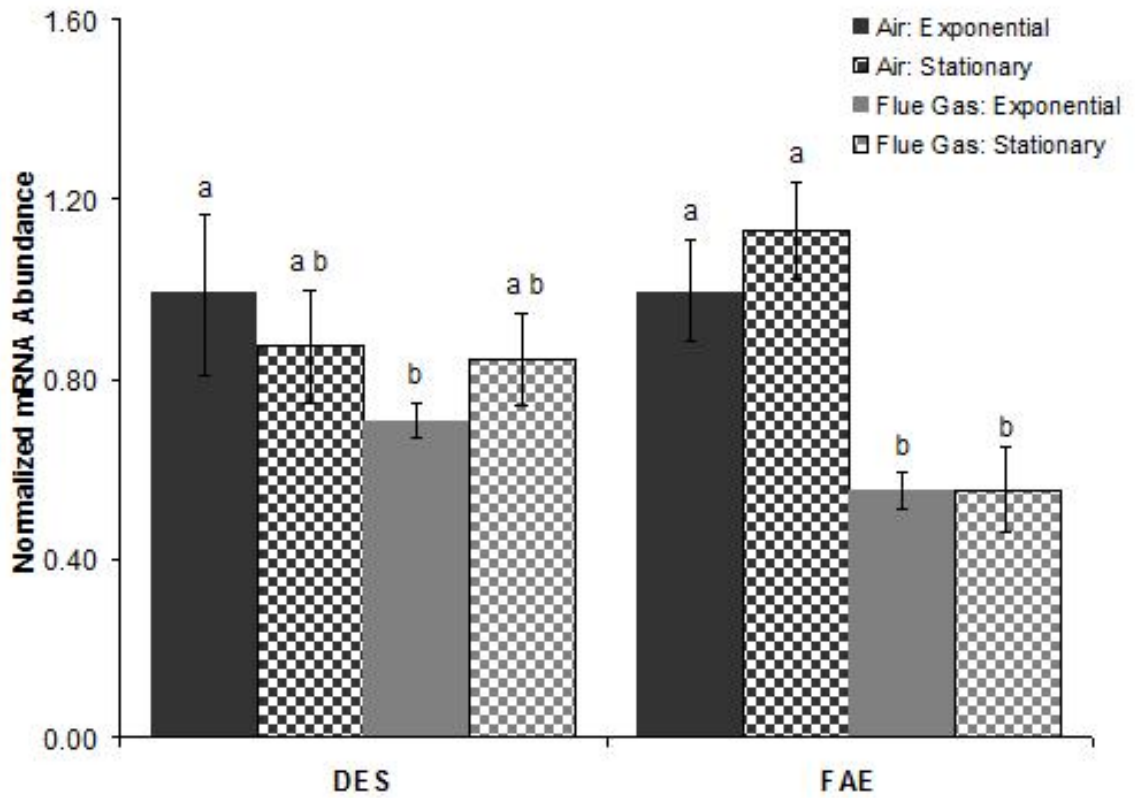


Figure 2.9: Effects of gas treatment and growth phase on $\Delta 5$ desaturase (DES) and $\Delta 6$ elongase (FAE) mRNA abundance in *H. akashiwo*. Gene expression is normalized to 18S rRNA transcript abundance and expressed relative to the average transcript abundance of the exponential phase culture in the air treatment. Data are expressed as means and error bars represent one standard deviation (n=4). Different letters denote significant differences among treatments using a one-way ANOVA followed by a Tukey's HSD means comparison test ($p < 0.05$).

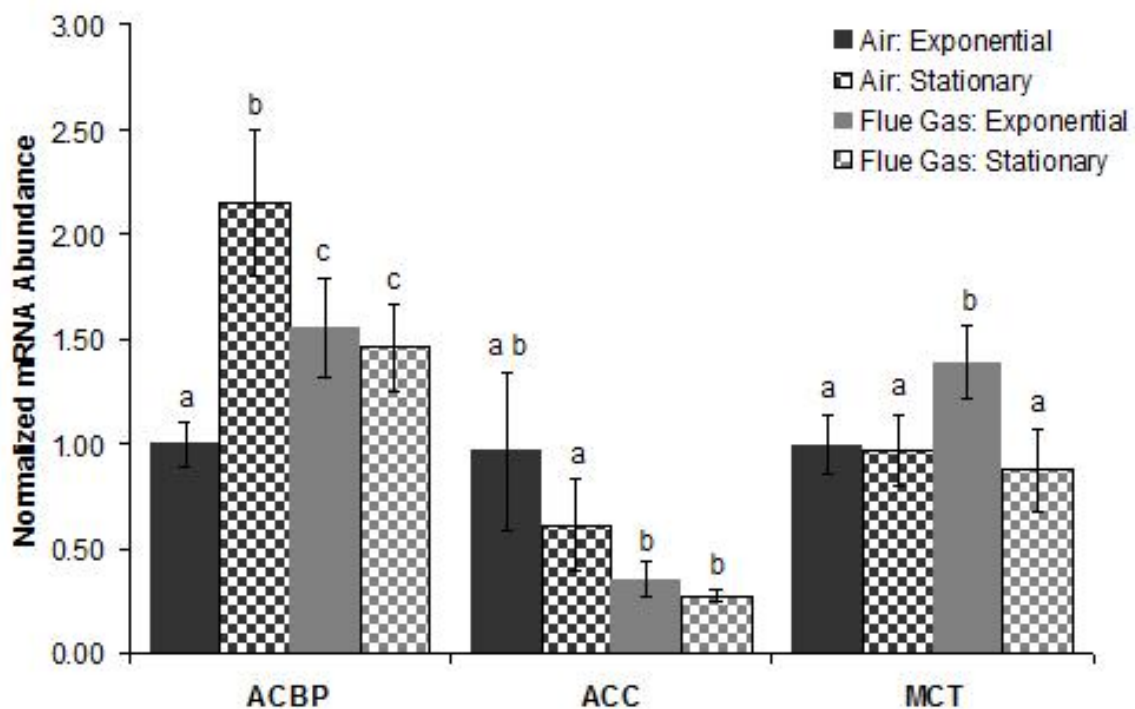


Figure 2.10: Effects of gas treatment and growth phase on the mRNA abundance of three key genes involved in fatty acid synthesis in *C. subsalsa*. ACBP: acyl CoA-binding protein, ACC: acetyl-CoA carboxylase, MCT: malonyl-CoA-ACP transferase. Gene expression is normalized to 18S rRNA transcript abundance and expressed relative to the average transcript abundance of the exponential phase culture in the air treatment. Data are expressed as means and error bars represent one standard deviation (n=4). Different letters denote significant differences among treatments using a one-way ANOVA followed by a Tukey's HSD means comparison test ($p < 0.05$).

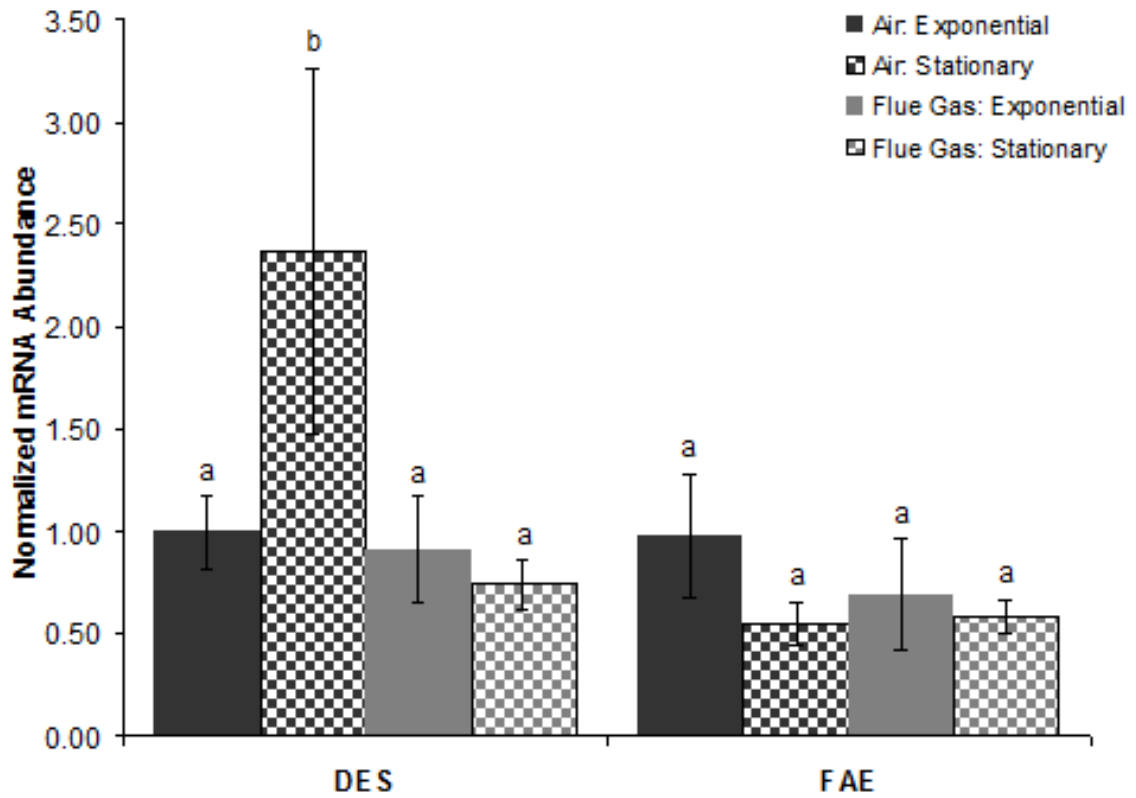


Figure 2.11: Effects of gas treatment and growth phase on $\Delta 5$ desaturase (DES) and $\Delta 6$ elongase (FAE) in *C.subsalsa*. Gene expression is normalized to 18S rRNA transcript abundance and expressed relative to the average transcript abundance of the exponential phase culture in the air treatment. Data are expressed as means and error bars represent one standard deviation (n=4). Different letters denote significant differences between groups using a one-way ANOVA followed by a Tukey's HSD means comparison test (p<0.05).

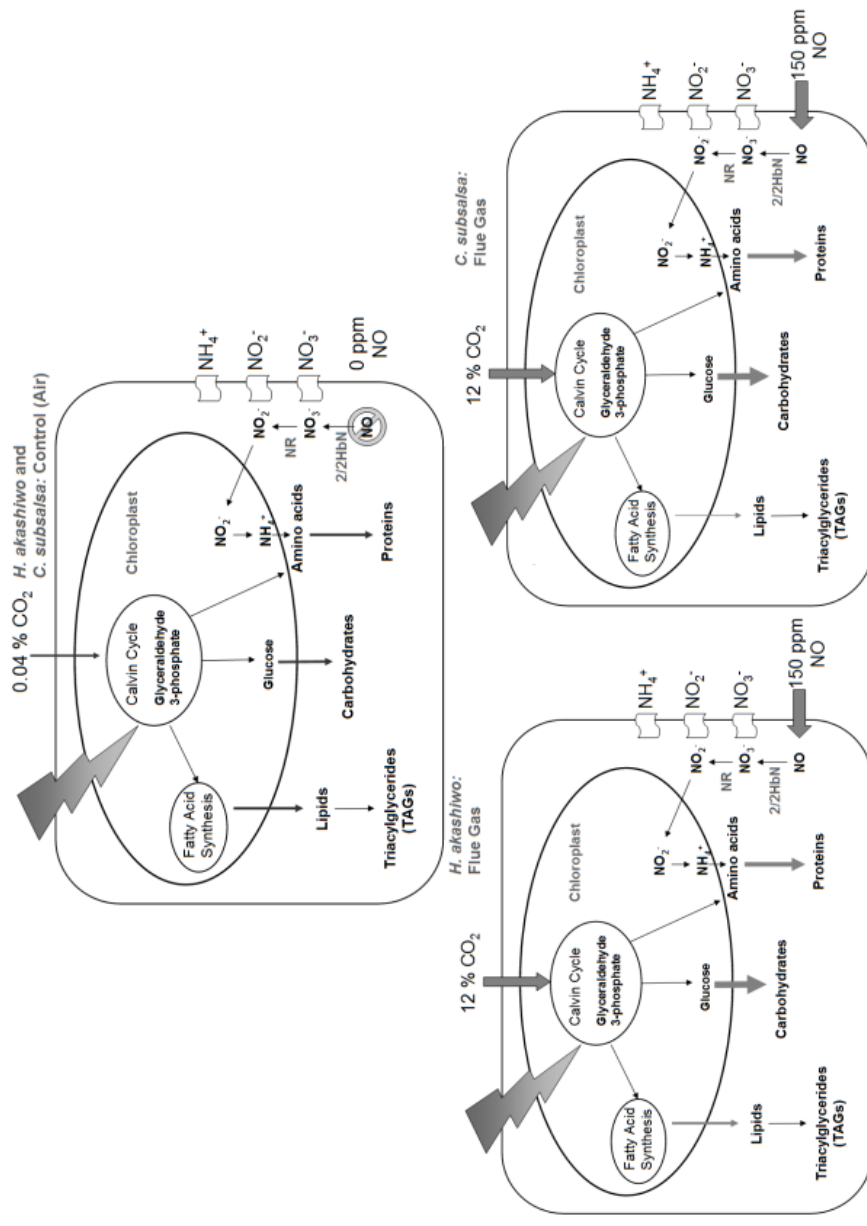


Figure 2.12: Summary of Chapter Two: *H. akashiwo* and *C. subsalsa* grown on flue gas or on air at different culture ages. Intensity of arrows indicates degree of increase due to flue gas treatment.

2.7 References

Brown, L.M. 1996, "Uptake of carbon dioxide from flue gas by microalgae", *Energy Conversion and Management*, vol. 37, no. 6-8, pp. 1363-1367.

Chisti, Y. 2008, "Biodiesel from microalgae beats bioethanol", *Trends in biotechnology*, vol. 26, no. 3, pp. 126-131.

Ciubota-Rosie, C., Ramon Ruiz, J., Jesus Ramos, M. Perez, A. 2013, "Biodiesel from *Camelina sativa*: A comprehensive characterisation", *Fuel*, vol. 105, pp. 572-577.

Fuentes-Grünewald, C., Garces, E., Alacid, E., Rossi, S., Camp, J. 2013, "Biomass and Lipid Production of Dinoflagellates and Raphidophytes in Indoor and Outdoor Photobioreactors", *Marine Biotechnology*, vol. 15, no. 1, pp. 37-47.

Fuentes-Grünewald, C., Garces, 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*, vol. 39, no. 1, pp. 207-216.

Halim, R., Danquah, M.K., Webley, P.A. 2012, "Extraction of oil from microalgae for biodiesel production: A review", *Biotechnology Advances*, vol. 30, no. 3, pp. 709-732.

Hogue, C. 2011, *Clearing Skies*.

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", *Plant Journal*, vol. 54, no. 4, pp. 621-639.

Lei, A., Chen, H., Shen, G., Hu, Z., Chen, L. Wang, J. 2012, "Expression of fatty acid synthesis genes and fatty acid accumulation in *Haematococcus pluvialis* under different stressors", *Biotechnology for Biofuels*, vol. 5, pp. 18.

Li, Y., Han, D., Sommerfeld, M. Hu, Q. 2011, "Photosynthetic carbon partitioning and lipid production in the oleaginous microalga *Pseudochlorococcum* sp (Chlorophyceae) under nitrogen-limited conditions RID D-2553-2010", *Bioresource technology*, vol. 102, no. 1, pp. 123-129.

Liang, C., Cao, S., Zhang, X., Zhu, B., Su, Z., Xu, D., Guang, X. Ye, N. 2013, "De Novo Sequencing and Global Transcriptome Analysis of *Nannochloropsis* sp (Eustigmatophyceae) Following Nitrogen Starvation", *Bioenergy Research*, vol. 6, no. 2, pp. 494-505.

Lopes, D.C., Baron Maurer, J.B., Stevan-Hancke, F.R., de Oliveira Proenca, L.A. Zawadzki-Baggio, S.F. 2012, "Chemical analysis of exopolysaccharide fractions and lipid compounds of the microalga *Heterosigma akashiwo* grown in vitro", *Botanica Marina*, vol. 55, no. 6, pp. 565-580.

Nascimento, I.A., Izabel Marques, S.S., Dominguez Cabanelas, I.T., Pereira, S.A., Druzian, J.I., de Souza, C.O., Vich, D.V., de Carvalho, G.C. Nascimento, M.A. 2013, "Screening Microalgae Strains for Biodiesel Production: Lipid Productivity and Estimation of Fuel Quality Based on Fatty Acids Profiles as Selective Criteria", *Bioenergy Research*, vol. 6, no. 1, pp. 1-13.

Negoro, M., Shioji, N., Miyamoto, K. Miura, Y. 1991, "Growth of Microalgae in High CO₂ Gas and Effects of Sox and Nox", *Applied Biochemistry and Biotechnology*, vol. 28-9, pp. 877-886.

Rawat, I., Kumar, R.R., Mutanda, T. Bux, F. 2013, "Biodiesel from microalgae: A critical evaluation from laboratory to large scale production", *Applied Energy*, vol. 103, pp. 444-467.

Rismani-Yazdi, H., Haznedaroglu, B.Z., Hsin, C. Peccia, J. 2012, "Transcriptomic analysis of the oleaginous microalga *Neochloris oleoabundans* reveals metabolic insights into triacylglyceride accumulation", *Biotechnology for Biofuels*, vol. 5, pp. 74.

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*, vol. 77, no. 6, pp. 565-575.

Van den Hende, S., Vervaeren, H. Boon, N. 2012, "Flue gas compounds and microalgae: (Bio-)chemical interactions leading to biotechnological opportunities", *Biotechnology Advances*, vol. 30, no. 6, pp. 1405-1424.

Wen, Z.Y. Chen, F. 2003, "Heterotrophic production of eicosapentaenoic acid by microalgae", *Biotechnology Advances*, vol. 21, no. 4, pp. 273-294.

Williams, P.J.L.B. Laurens, L.M.L. 2010, "Microalgae as biodiesel biomass feedstocks: Review analysis of the biochemistry, energetics economics", *Energy Environmental Science*, vol. 3, no. 5, pp. 554-590.

Yang, Z., Niu, Y., Ma, Y., Xue, J., Zhang, M., Yang, W., Liu, J., Lu, S., Guan, Y. Li, H. 2013, "Molecular and cellular mechanisms of neutral lipid accumulation in diatom following nitrogen deprivation.", *Biotechnology for biofuels*, vol. 6, no. 1, pp. 67-67.

Chapter 3

EFFECTS OF NITRATE CONCENTRATION ON CARBON PARTITIONING AND FATTY ACID CONTENT OF *HETEROSIGMA AKASHIWO* ON MODEL FLUE GAS

3.1 Abstract

In this study, the ability of *Heterosigma akashiwo* (class: Raphidophyceae) to use nitric oxide (NO) in model flue gas as a nitrogen source was evaluated. The effects of model flue gas and three levels of nitrate addition (0, 220 and 880 μM nitrate) on the fatty acid composition and carbon partitioning were also investigated during exponential growth and after reaching stationary phase. Consistent with results described in Chapter Two, *H. akashiwo* grew faster when bubbled with flue gas at each nitrate level compared to air. It is noteworthy that cultures grown with the flue gas and no added nitrate (1) achieved the same growth rate as the cultures grown on air with 220 and with 880 μM nitrate, and (2) reached higher cell density than no nitrate added cultures when grown on air. The high levels of CO_2 and NO in the flue gas may have caused this increase in growth rate. This suggests that NO supplied in the flue gas was used as a nitrogen source. In addition, the concentration of the nitrogen rich molecules, protein and chl *a* in exponential phase cultures grown with flue gas without added nitrate was not significantly different from cultures grown with 220 μM nitrate added. When bubbled with flue gas, *H. akashiwo* accumulated more carbohydrates than lipids in both exponential and stationary growth stages. This is probably due to the high amount of CO_2 (12%) in the flue gas, which is converted into simple carbohydrates through photosynthesis. In contrast, lipids were the most abundant biomolecule in cultures bubbled with air and in exponential phase. When considering biodiesel quality, the highest predicted cetane number (CN) was obtained from the stationary phase cultures

grown on air with addition of 220 μM and 880 μM nitrate despite the observation that the 0 μM nitrate addition culture produced the highest lipid concentration.

3.2 Introduction

Microalgae can produce high concentrations of neutral lipids called triacylglycerides that can be easily transesterified to produce biodiesel. Under environmental stress conditions, such as nutrient deprivation, several microalgae can alter their metabolic pathways to overproduce triacylglycerides (Rismani-Yazdi et al. 2012). This energy stored as TAG can then be used to maintain cellular metabolic requirements (reviewed by Hu et al. 2008; Fuentes-Grünewald et al. 2012). *Heterosigma akashiwo* has been shown to produce 73% of its dry weight as lipids under nitrogen-limiting conditions (Fuentes-Grünewald et al. 2012).

Previous work (Bianco Chapter Two) determined that *H. akashiwo* stores energy primarily as carbohydrates rather than lipids when bubbled with flue gas. The synthesis of starch, a major storage carbohydrate, shares common precursors with lipid synthesis but the interaction between starch metabolism and lipid metabolism is poorly understood. Li et al. (2011) showed that starch synthesis was up-regulated in *Pseudochlorococcum* sp. under high light and nitrogen-limited conditions, suggesting that starch is used for primary carbon and energy storage. When nitrogen was depleted, starch content decreased and neutral lipid content increased, indicating that carbon partitioning was switched to neutral lipids for secondary carbon and energy storage. The transient accumulation of starch followed by a gradual accumulation of neutral lipids also suggests that starch and neutral lipids can be inter-converted. This was demonstrated by Roessler (1988) who showed that carbon previously assimilated into carbohydrates can be converted into lipids in the diatom *Cyclotella cryptica*, under silicon limited conditions. However, in Chapter Two, it was determined that *H. akashiwo* continued to accumulate carbohydrates in the stationary phase while lipid content did not change between growth phases.

In another study, Yang et al. (2013), analyzed transcriptional changes in the diatom *Thalassiosira pseudonana* under N deprivation. Their results suggested that catabolism of carbohydrates may lead to TAG accumulation. In terms of carbon fixation, most plants rely on photosynthesis and glycolysis to produce pyruvate. Yang et al. (2013) observed that glycolysis was up-regulated while photosynthesis was inhibited under N deprivation. This indicated that under N deprivation, *T. pseudonana* may rely on glycolysis to form pyruvate, which may then be converted to acetyl-CoA for fatty acid biosynthesis. Although nitrogen limitation has been shown to induce increases in neutral lipid concentration, it has been shown to have little effect on fatty acid composition (Liang et al. 2013; Fuentes-Grünewald et al. 2012).

Nitrate deprivation may reduce the cost of biomass cultivation for biodiesel. It is estimated that 45% of energy input during the production of biodiesel from microalgae comes from the addition of nitrogen (reviewed by Chisti, 2008). Thus, an investigation of fatty acid profiles and carbon partitioning under nitrogen deprivation can be used to optimize biodiesel production and potentially decrease the production cost of biodiesel. In addition, NO_x present in flue gas can be a potential source of nitrate for microalgae (reviewed by Farrelly et al. 2013), but little is known about its effect on carbon partitioning and fatty acid profiles in microalgae.

NO reacts with oxygen in water to form NO_2^- and NO_3^- (reviewed by Van Den Hende et al. 2012). As detailed in previous chapters, several studies have demonstrated that NO from model flue gas can dissolve in the medium and be available as a nitrogen source for algae (Negoro et al. 1991; Brown 1996; Nagase et al. 2001). Both *C. subsalsa* and *H. akashiwo* may be exposed to high NO levels during algal blooms (Stewart and Coyne, 2011). Zhang et al. (2006) demonstrated that NO_3^- is the preferred inorganic N source of *H. akashiwo*. To use NO_3^- as a nitrogen source, it must be reduced to NH_4^+ using nitrate reductase and nitrite reductase. *C. subsalsa* and *H. akashiwo* contain a hybrid nitrate reductase (NR2-2/2HbN) that may couple dioxygenase activity of 2/2 hemoglobin to oxidize NO to NO_3^- , followed by the reduction of NO_3^- to NO_2^- (Stewart and Coyne 2011). In support of this hypothesis, Stewart and Coyne (2011)

conducted a short-term experiment to demonstrate that NO concentration decreased in cultures of *H. akashiwo*. Work described in Chapter Two also showed that *H. akashiwo* could be cultured for long periods of time in the presence of NO supplied by model flue gas. These previous experiments were conducted with high concentrations of nitrate, however, and it is not known if *H. akashiwo* is able to use NO as a sole nitrogen source.

In this study, the ability of *H. akashiwo* to use NO supplied in model flue gas as a nitrogen source was evaluated. The effects of model flue gas, nitrate addition and growth stage on the fatty acid composition and carbon partitioning were also investigated. To better understand the effects of N-source and concentrations on lipid metabolism in microalgae, the expression patterns of genes involved in fatty acid synthesis were also investigated.

3.3 Materials and Methods

3.3.1 Experimental Design

Heterosigma akashiwo (Delaware Inland Bay isolate, CCMP 2393) was grown in 20 psu f/2 medium (with varying nitrate concentrations) buffered with 20 mM HEPES (pH 7.4) at room temperature (25°C) on a 12:12 hour light: dark cycle with an irradiance of 100 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$. Batch cultures of *H. akashiwo* (seeded at 180,000 cells/mL) were bubbled for five weeks (transferred every 7 days). The control cultures were bubbled (2 mL minute⁻¹) with air, while the treatment cultures were bubbled (2 mL minute⁻¹) with model flue gas (nitrogen gas containing 12% carbon dioxide and 150 ppm nitric oxide). After five weeks, for both control and flue gas cultures, replicate cultures (N=4) were grown in culture medium with three nitrate concentrations; 0 μM , 220 μM , and 880 μM . For the 880 μM nitrate treatment, cultures were seeded at 30,000 cells/ mL while the 0 μM and 220 μM cultures were seeded at 150,000 cells/mL. Cell counts were measured daily for all cultures. For the 880 μM nitrate treatment, cell counts were measured with a Z series Coulter Counter (Beckman Coulter, USA). Cell counts for the 0 μM and 220 μM nitrate treatment were measured with a Neubauer hemocytometer. Samples were collected for analysis of

lipid composition, carbon partitioning, chlorophyll *a* concentration and gene expression during the exponential and stationary phases. Sampling and analytical methods are described in Appendix A.

3.3.2 Statistical Analysis

The experiment was performed in four replicates for each nitrate concentration and gas treatment. Data represents means \pm standard deviations. A two-way ANOVA was used to determine the statistical significance of the treatments. Specified factors were gas treatment (air or flue gas) and nitrate concentration (0 μM , 220 μM or 880 μM). A Tukey's HSD test was used to determine significant differences between mean values for each nitrate treatment. Kolmogorov-Smirnov and Levenes tests were used to determine if the data were normally distributed and had equal variances, respectively. The transcript abundance data were not normally distributed and treatments did not have equal variances so a square root transformation was performed. After this transformation, all one-way ANOVA assumptions were met. A paired t-test was used to determine differences between the exponential and stationary phases of the same culture. Statistical analyses were conducted with IBM SPSS Statistics (International Business Machines Corporation, USA). Two-way ANOVA tables are in Appendix B.

3.4 Results

3.4.1 Growth

Overall, *H. akashiwo* grew significantly faster when bubbled with flue gas compared to when bubbled with air under nitrate replete conditions (880 μM ; Table 3.1; one-way ANOVA, $p < 0.05$). Cultures bubbled with flue gas and no added nitrate achieved the same growth rate as the cultures grown on air with 220 and with 880 μM nitrate, while the cultures grown with air and no added nitrate had a significantly lower growth rate than all other treatments (one-way ANOVA; Tukeys HSD, $p < 0.01$ for all comparisons). The cultures bubbled with flue gas with no additional nitrate in the medium (0 μM) grew significantly faster than the complimentary culture bubbled

with air (one-way ANOVA; Tukeys HSD, $p < 0.001$). Within the 0 μM added treatment, the culture bubbled with flue gas reached a higher cell density compared to the culture bubbled with air (one-way ANOVA; Tukeys HSD, $p < 0.01$). For the 0 μM added treatment, the culture bubbled with flue gas reached $462,700 \pm 32,000$ cells/ mL when the stationary phase was sampled (on day 11). Meanwhile, the culture bubbled with air reached $282,700 \pm 21,000$ cells/ mL when the stationary phase was sampled (on day 11).

3.4.2 Carbon Partitioning

The concentrations of carbohydrate, lipid and protein in each treatment during the exponential phase are shown in Figure 3.2. For the carbohydrate analysis, a two-way ANOVA revealed significant interaction between gas and nitrate treatments during exponential growth for the cultures bubbled with flue gas ($p < 0.05$). Furthermore, the cultures bubbled with flue gas had significantly higher carbohydrate content than the cultures bubbled with air (two-way ANOVA, $p < 0.01$). While carbohydrate content increased with nitrate concentration within the flue gas treatment, carbohydrate content was highest in the 0 μM nitrate added within the air treatment. Overall, the combined effects of flue gas and a high nitrate concentration lead to the highest carbohydrate content. Lastly, carbohydrate content of the cultures with 0 μM nitrate added to the medium did not vary between the cultures bubbled with flue gas and the cultures bubbled with air.

The cultures bubbled with air had a significantly higher lipid content than the cultures bubbled with flue gas (two-way ANOVA, $p < 0.001$). Specifically, the cultures bubbled with air and 0 μM nitrate added to the algal medium had significantly more lipids than all other treatments (one-way ANOVA, $p < 0.001$, Tukey's HSD, $p < 0.01$ for all comparisons). A one-way ANOVA was used to determine that in the culture bubbled with flue gas; lipid content did not vary significantly among the nitrate concentrations ($p < 0.05$).

During the exponential phase, protein content increased with an increase in the amount of nitrate added to the culture (two-way ANOVA, $p < 0.001$). Although the average protein content was higher in cultures with no added nitrate in the flue gas treatment compared to the air treatment, the differences were not significant. In addition, protein content was not significantly different between the cultures bubbled with flue gas with no additional nitrate compared to those with $220 \mu\text{M}$ nitrate added to the medium, while protein content in cultures bubbled with air and $220 \mu\text{M}$ nitrate were significantly higher than those with no added nitrate.

Figure 3.3 shows the alteration of carbohydrate, lipid and protein content of the various treatments during stationary phase. For the carbohydrate analysis, a two-way ANOVA revealed that the cultures bubbled with flue gas had significantly higher carbohydrate content than that the cultures bubbled with air ($p < 0.01$). Furthermore, according to the two-way ANOVA, nitrate concentration did not have a significant effect on carbohydrate content. However, there was a significant interaction between the gas and nitrate treatments ($p < 0.05$). A one-way ANOVA determined that the culture bubbled with flue gas containing $220 \mu\text{M}$ and $880 \mu\text{M}$ nitrate had significantly more carbohydrates than all other treatments (Tukey's HSD, $p < 0.05$ for all comparisons) but did not differ from each other. As observed during the exponential phase, the combined effects of flue gas and a high nitrate concentration lead to the highest carbohydrate content.

A two-way ANOVA determined that gas and nitrate treatments had a significant interaction effect on algal lipid content. Through a one-way ANOVA, it was determined that this interaction effect occurred in the culture bubbled with air. The culture bubbled with air with $880 \mu\text{M}$ nitrate added to the medium had the highest lipid content of all the treatments (one-way ANOVA, $p < 0.01$, Tukey's HSD, $p < 0.05$ for all comparisons). Similar to what was observed during exponential phase; lipid content did not vary significantly among the nitrate concentrations within the culture bubbled with flue gas.

A two-way ANOVA determined that there were no significant differences in

protein content between the gas treatments during stationary phase ($p>0.05$). Air treatment and nitrate concentration had a significant interaction on protein content ($p<0.05$). Protein content was not significantly different between each of the nitrate treatments for the cultures bubbled on flue gas, while cultures bubbled with air had significantly higher protein with each increase in nitrate concentration (one-way ANOVA, Tukey's HSD $p>0.05$ for all comparisons within the flue gas treatment). When comparing between gas treatments, protein content in cultures grown on flue gas without added nitrate was similar to protein content of cultures grown on air with $220 \mu\text{M}$ nitrate but also not significantly different than cultures grown on air with no additional nitrate added. Protein content in cultures grown on air with $880 \mu\text{M}$ nitrate was significantly higher than all other treatments.

Figure 3.4 shows the effect of gas treatment, nitrate concentration and growth phase on chlorophyll *a* (chl *a*) content of *H. akashiwo*. During the exponential phase, protein content increased with nitrate concentration in the culture bubbled with air. However, this trend was not observed in the culture bubbled with flue gas. Chl *a* content of the culture without additional nitrate in the medium ($0 \mu\text{M}$) was not significantly different from the culture with $220 \mu\text{M}$ nitrate added to the medium (one-way ANOVA, Tukeys HSD, $p<0.01$). However, in the stationary phase, chl *a* concentration increased with an increase in the amount of nitrate added to the culture for both gas treatments (one-way ANOVA, $p<0.001$).

3.4.3 Fatty Acid Profile

For both the exponential and stationary phases, the major fatty acids in all treatments were myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), linolenic (C18:3), stearidonic (C18:4) and eicosapentaenoic (C20:5, EPA) (Figures 3.5-3.8). In cultures bubbled with flue gas, saturated fatty acid content as a percent of total fatty acid content was higher during the stationary phase compared to the exponential phase (paired t-test, $p<0.01$). Conversely, in the cultures bubbled with air, saturated fatty acid content as a percent of total fatty acid content remained the same in both phases

(paired t-test, $p > 0.01$). However, from the exponential phase to the stationary phase, the air cultures there was a sharp decrease in polyunsaturated fatty acids (PUFA) in cultures bubbled with air accompanied by an increase in monounsaturated fatty acids (MUFA). This can be attributed to a significant decrease in C20:5 content as a percent of total fatty acids. C20:5 content decreased significantly from $21 \pm 1\%$ in the exponential phase to $11.2 \pm 0.2\%$ in the stationary phase (paired t-test, $p < 0.01$).

During the exponential growth phase, C16:0 content as a percent of total fatty acids increased significantly with a decrease in nitrate concentration in both gas treatments. This was most apparent in the flue gas treatments, where changes were significant between each nitrate concentration. For the air treatment, this increase in C16:0 was significant only between the $880 \mu\text{M}$ nitrate treatment, compared to the $0 \mu\text{M}$ and $220 \mu\text{M}$ (one-way ANOVA, $p < 0.05$). At stationary phase, there was a significant difference in C16:0 content for the cultures bubbled with air, while the C16:0 content in the no nitrate addition culture in the flue gas treatment was significantly higher than the other two nitrate treatments. Conversely, C18:4 content increase significantly with an increase in nitrate concentration for cultures bubbled with flue gas (one-way ANOVA, $p < 0.01$). The C18:4 content in air treatments were not affected by nitrate concentration at each growth stage.

3.4.4 Transcript Abundance

Relative messenger RNA levels of five key genes involved in fatty acid synthesis were investigated (Figures 3.9-3.12). Acyl carrier protein (ACP), acyl CoA-binding protein (ACBP) and beta-ketoacyl-ACP synthase (KAS) aid in fatty acid chain growth while $\Delta 5$ desaturase (DES) and $\Delta 6$ elongase (FAE) desaturate and elongate fatty acids.

During exponential phase, gas treatment only had a significant effect on the transcript abundance of KAS (two-way ANOVA; statistics in Appendix B). KAS transcript abundance was significantly higher in the culture bubbled with flue gas compared to the culture bubbled with air (two-way ANOVA, $p < 0.001$).

Also during exponential phase, nitrate concentration only had a significant effect on the transcript abundance of ACBP and FAE (statistics in Appendix B). The culture with 880 μM nitrate added had significantly more ACBP transcript abundance compared to the other treatments (two-way ANOVA, $p < 0.001$ for all proteins). FAE transcript abundance was significantly higher in the culture with 0 μM nitrate added to the medium compared to the other treatments (two-way ANOVA, $p < 0.001$). Nitrate treatment did not have a significant effect on the transcript abundance of the other genes investigated (two-way ANOVA; statistics in Appendix B).

A one-way ANOVA was also used to determine significant differences among all treatments. During exponential phase, FAE, DES, ACP, ACBP and KAS transcript abundance was significantly different among treatments. Both cultures with 880 μM nitrate added to the medium (bubbled with air or with flue gas) had significantly less ACBP transcript abundance compared to the other treatments ($p < 0.01$). KAS transcript abundance was significantly higher in the cultures bubbled with flue gas with 880 μM and 220 μM nitrate added to the medium compared to all other treatments ($p < 0.01$). FAE transcript abundance did not vary with in the flue gas treatment but was significantly highest in the culture bubbled with air when 880 and 220 μM nitrate was added to the medium, compared to when there was no additional nitrate in the air culture medium ($p < 0.01$).

During stationary phase, transcript abundances of only four proteins were investigated: ACBP, KAS, DES and FAE. RNA was not recovered from the cultures bubbled on air with 0 μM nitrate added and reverse transcript reactions were unsuccessful in the cultures bubbled with flue gas with 0 μM nitrate added. Furthermore, transcript abundance of ACC and ACP were too low to be accurately measured. The transcript abundance of all investigated genes did not vary between the cultures bubbled with air and the cultures bubbled with flue gas (two-way ANOVA, data in Appendix B). However, nitrate concentration had a significant effect of the transcript abundance of all proteins investigated (two-way ANOVA, data in Appendix B) and an one-way ANOVA was used to further investigate this effect.

This one-way ANOVA determined that FAE, DES, ACBP and KAS transcript abundance was significantly different among treatments during stationary phase. Within the air treatment, FAE transcript abundance was significantly higher in the culture with 220 μM nitrate compared to the culture with 880 μM nitrate added to the medium. DES transcript abundance was significantly higher in the cultures bubbled with flue gas and 220 μM nitrate added to the medium compared to all other treatments ($p < 0.01$). Similar to observations in exponential phase, ACBP transcript abundance was significantly lower in the cultures with 880 μM nitrate added to the medium compared to the cultures with 220 μM nitrate added to the medium. KAS transcript abundance was significantly lower in the cultures bubbled with air with 880 μM nitrate added to the medium compared to all other treatments ($p < 0.01$).

3.4.5 Biodiesel Quality

Nitrate concentration did not have a significant effect on biodiesel quality of *H. akashiwo* (Table 3.2 and 3.3). Overall, the cultures bubbled with flue gas had a significantly higher CN compared to the cultures bubbled with air in the exponential phase (two way ANOVA, $p < 0.01$). However, in the stationary phase, the cultures grown on air had a higher CN compared to the cultures grown on flue gas (two way ANOVA, $p < 0.01$). The highest CN was achieved in the 220 μM (53 ± 3) and the 880 μM (53 ± 2) nitrate treatment in the cultures bubbled with air during the stationary phase, indicating that these conditions provide the best potential biodiesel. As previously mentioned, C20:5 significantly decreases biodiesel quality due to its high degree of unsaturation. All of the nitrate treatments contained a high percentage of C20:5. If C20:5 were removed prior to biodiesel production, the biodiesel quality of all treatments significantly increases to between 51-65, which is above the US standard of 47.

3.5 Discussion

Various methods have been implemented to enhance the lipid quantity and quality of microalgae (Fuentes-Grünwald et al. 2012). Environmental stresses, such

as high light or nutrient starvation, for example, have been reported to increase fatty acid accumulation (Lei et al. 2012). In this experiment, and as determined previously (Bianco Chapter 2), *H. akashiwo* grew faster when bubbled with flue gas than when bubbled with air. This flue gas-induced increase in growth rate is highly desirable in terms of biodiesel and may be due to high levels of CO₂ and NO in the flue gas. In addition, results of this work provide evidence that *H. akashiwo* may be capable of assimilating NO supplied in flue gas as a nitrogen source.

There was a positive growth rate for all treatments, even with no added N. This may indicate that there was nitrogen in the seawater used to prepare the medium. After this nitrogen was used up, the cultures grown on air ceased growth while the cultures bubbled with the flue gas continued to grow. Cultures grown with the flue gas and no added nitrate achieved the same growth rate as the cultures grown on air with 220 and 880 μM nitrate, while the cultures grown with air and no added nitrate had a significantly lower growth rate than all other treatments. Additionally, the cultures bubbled with flue gas and 0 μM nitrate reached a higher cell density compared to the culture bubbled with air and 0 μM nitrate. This observation supports the hypothesis that *H. akashiwo* can use NO as a nitrogen source.

While Zhang et al. (2006) demonstrated that NO₃⁻ is the preferred inorganic N source of *H. akashiwo*, in order to use NO₃⁻ as a nitrate source, it must be reduced to NH₄⁺, using nitrate reductase and nitrite reductase. The hybrid nitrate reductase (NR2-2/2HbN) present in *H. akashiwo* may couple the oxidation of NO to NO₃⁻ followed by the reduction of NO₃⁻ to NO₂⁻ (Stewart and Coyne 2011). This would mean that *H. akashiwo* is capable of directly assimilating the NO from the culture medium bubbled with flue gas. This was also shown to be possible in *Dunaliella tertiolecta*, which was able to directly assimilate NO entering the cells though diffusion (Nagase et al. 2001). In this case, NO was preferentially utilized as an N source over NO₃⁻.

Another possibility is that NO dissolved in the medium to form NO₂⁻ and NO₃⁻. This would increase the availability of NO₃⁻ for *H. akashiwo*. Previous studies (Negoro et al. 1991, Brown 1996, Nagase et al. 2001) have demonstrated that NO

from model flue gas can dissolve in the medium and be available as a nitrogen source for algae.

Protein content may also provide evidence of NO use by *H. akashiwo*. Proteins contain more nitrogen compared to lipids, carbohydrates and chlorophyll *a* (reviewed by Williams et al. 2012), thus it is expected that protein abundance will be most affected by nitrogen concentration. This assumption held true in both the exponential and the stationary phases of cultures grown on air because protein quantity per cell decreased with a decrease in added nitrate. However, there was no significant difference in protein content per cell for cultures grown with flue gas with no nitrate added compared to those with 220 μM nitrate added at exponential growth or when compared to both the 220 μM and 880 μM nitrate added cultures in stationary phase. The results suggest that the cultures with no nitrate added maintained protein content by assimilation of NO.

In contrast to protein content, chl *a* concentrations were also affected by nitrate limitation in both gas treatments. For each mole of carbon, chl *a* contains 0.07 moles nitrogen, while the nitrogen contained in proteins is 0.26 moles N per one mole carbon. In exponential and stationary phases of both the cultures grown on air and the cultures grown on flue gas, chl *a* quantity per cell decreased as nitrate decreased.

Lipid quantity was highest in the 0 μM and 880 μM nitrate cultures that were bubbled on air during both the exponential and the stationary phases, respectively. However, lipid content did not vary among the nitrate treatments within the flue gas treatment. Because lipid content generally increases under N-limitation (Rismani-Yazdi et al. 2012), this observation suggests that the cultures grown on air were more N-limited than the cultures grown on flue gas. As established in Chapter Two, cultures grown with flue gas used carbohydrates rather than lipids to store energy. This is probably due to the high amount of CO₂ in the flue gas, which is converted into simple carbohydrates through photosynthesis.

Chapter Two also investigated the carbon partitioning of *H. akashiwo* when 880 μM nitrate was added to the medium and cultures were grown on flue gas or

on air (Figure 2.2). While protein content was similar between the two experiments, carbohydrate and lipid content was higher for the nitrate addition experiment. This difference may be due to the time at which the cultures were sampled. Yang et al. (2013) reported that carbohydrates are rapidly produced during the light period of the day and consumed during the dark period of the day. Sampling for the Chapter Two experiment occurred in the late morning (10:30 am) while sampling for the Chapter Three experiment occurred in the late afternoon (3:00 pm). Thus, *H. akashiwo* sampled for Chapter Three (this experiment) was further into the day period compared to in Chapter Two and had more time to produce carbohydrates. Additionally, because lipids are an indirect product of photosynthesis, it is expected that lipid content would have a similar trend to carbohydrates.

Consistent with results of work reported in previous chapters of this thesis, C16:0 was the principle fatty acid under all treatments. This is highly desirable in terms of biodiesel quality due to its chain length and degree of unsaturation (Nascimento et al. 2013). Other studies have reported enhanced C16:0 content in microalgae under N-limitation. A recent study by Rismani-Yazdi et al. (2012), for example, demonstrated that under nitrogen limitation, *Neochloris oleoabundans* accumulated a higher content of C16:0 and C18:0 compared to cultures grown under nitrogen replete conditions. In the study reported here, nitrogen limitation enhanced production of both C16:0 during exponential growth of cultures bubbled with either flue gas or air. At stationary phase however, enhanced C16:0 content was only observed for cultures grown with flue gas. Specifically, the cultures grown with flue gas without added nitrate accumulated significantly more C16:0 at stationary phase than any of the other treatments. It should be noted that C16:0 and C18:0 are the final products of the fatty acid synthase cycle. High levels of these fatty acids indicated that they were not elongated or desaturated into longer chain and unsaturated fatty acids. As indicated by the decrease in protein concentration, the nitrate limitation may have lead to a decrease in desaturation and elongation enzymes that act on C16:0 and C18:0.

Many studies have investigated the links between fatty acid accumulation and

transcript abundance of enzymes involved in fatty acid synthesis in microalgae under nitrogen deprivation (Lei et al. 2012; Li et al. 2012; Rismani-Yazdi et al. 2012, Yang et al. 2013). Acetyl-CoA carboxylase (ACC) catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, which is the commitment step in *de novo* fatty acid synthesis. In this study, ACC transcript abundance was undetectable. Work reported in previous chapters here have also found that ACC transcript abundance in this species is typically in low abundance. Furthermore, RNA yield from this experiment was also low. During the stationary phase, RNA was not recovered from the culture bubbled with air and 0 μ M nitrate. According to Williams et al (2010), nucleic acids and proteins are the major determinates of growth rates. Thus, it is expected that the stationary phase nitrate-depleted cultures, would have low RNA content. Furthermore, nucleic acids contain 0.40 moles N for one mole C (reviewed by Williams et al. 2010), so a decrease in nucleic acid content as a result of nitrate limitation was expected.

KAS is a considered a key regulator of fatty acid chain length because it is a condensing enzyme responsible for the two carbon elongations of substrates from C4:0 to C14:0. As summarized in Figure 3.12, of the six genes investigated, only KAS transcript abundance was up-regulated in the cultures grown on flue gas during the exponential phase, which also had the highest C16:0 content compared to the air cultures. Similarly, Rismani-Yazdi et al. (2012) demonstrated that in *Neochloris oleoabundans*, the gene coding for KAS was up-regulated under nitrogen limitation resulting in a higher content of C16:0, C18:0 and C18:1 compared to nitrogen replete conditions.

Δ 6 elongase (FAE) transcript abundance was significantly higher in the culture with 0 μ M nitrate added to the medium and bubbled on air. FAE aids in the production of the PUFA, eicosapentaenoic fatty acid (C20:5, EPA), by catalyzing the elongation of C18:4 to form C20:4. As show in Figure 3.11, the culture with 0 μ M nitrate added to the medium and bubbled on air has a high C20:5 content. However, C20:4 was not present in the cultures bubbled with air. This indicates than the FAE may have been elongating C18:3 to form C20:3.

Cetane number (CN) is the primary indicator of diesel quality and measures the ignition delay when the diesel is injected into the combustion chamber. A high CN indicates a short ignition time. Unexpectedly, nitrate concentration did not have a significant effect on biodiesel quality of *H. akashiwo*. This may be because the FAME profiles of *H. akashiwo* were not altered drastically enough by nitrate concentration to alter the quality of the biodiesel. Biodiesel from the cultures grown on air in stationary phase had a lowest iodine value and a highest CN than the other treatments. These cultures are the only ones with iodine values below the European maximum of 120 (Nascimento et al. 2013). As previously mentioned, C20:5 significantly decreases biodiesel quality due to its high degree of unsaturation. In the stationary phase, the cultures bubbled on air had significantly less C20:5 than the cultures bubbled with flue gas, which contributed to its higher biodiesel quality. Additionally, if C20:5 were removed prior to biodiesel production, the biodiesel quality of all treatments significantly increases to between 51-65, which is above the US standard of 47.

3.6 Conclusion

In this chapter, the effects of both nitrate concentration and flue gas on the biodiesel potential of *H. akashiwo* were assessed. Results show that nitrate limitation caused an increase in lipid content and did not affect biodiesel quality. As previously determined, *H. akashiwo* grew faster when bubbled with flue gas compared to air. This flue gas-induced increase in growth rate is highly desirable in terms of biodiesel and may have been caused by the high levels of CO₂ and NO in the flue gas. This may indicate that the NO dissolved in the medium to form nitrous acid (HNO₂) and nitric acid (HNO₃), which in seawater are present as NO₂⁻ and NO₃⁻, respectively (reviewed by Van Den Hende et al. 2012). This would increase the availability of NO₃⁻ for *H. akashiwo*. Cultures grown with the flue gas and no added nitrate achieved the same growth rate as the cultures grown on air with 220 and 880 μM nitrate, while the cultures grown with air and no added nitrate had a significantly lower growth rate than all other treatments. Additionally, the cultures bubbled with flue gas and 0 μM nitrate reached

a higher cell density compared to the culture bubbled with air and 0 μM nitrate. After the trace amounts of nitrate in the seawater was used up, the cultures grown on air ceased growth while the cultures bubbled with the flue gas continued to grow.

In the cultures bubbled with air, during exponential and stationary phase, protein content decreased with a decrease in nitrate concentration. This trend was not observed in the cultures bubbled with flue gas; during the exponential phase the 0 μM nitrate has the same protein content as the 220 μM nitrate cultures. During the stationary phase, nitrate treatment did not affect protein content. These observations regarding protein content indicate that the culture bubbled on air were dependent on nitrate in the medium for their nitrogen source while the cultures bubbled on flue gas were not. Overall, the results suggest that *H. akashiwo* can use NO as a sole nitrogen source.

Table 3.1: Growth rate of *H. akashiwo* grown at varying nitrate concentrations (expressed as means \pm standard deviation, n= 4). Different letters denote significant differences between nitrate treatments using a one-way ANOVA ($p < 0.05$).

	Air			Flue Gas		
Nitrate Concentration (μM)	0	220	880	0	220	880
Growth Rate (day^{-1})	0.12 ± 0.01 (a)	0.20 ± 0.02 (b c)	0.23 ± 0.01 (c d)	0.18 ± 0.01 (b d)	0.19 ± 0.01 (c d)	0.33 ± 0.01 (e)

Table 3.2: Effect of gas treatment and nitrate concentration during the exponential growth phase on biodiesel quality of oil derived from *H. akashiwo* (expressed as means \pm standard deviation, n= 4). Different letters denote significant differences between groups using a one-way ANOVA (p<0.05).

	Control				Flue Gas			
	0	220	880	880	0	220	880	880
Nitrate Concentration								
Iodine Value	192 \pm 5 (a)	183 \pm 12 (a)	188 \pm 12 (a)	188 \pm 12 (a)	135 \pm 16 (b)	146 \pm 16 (b)	158 \pm 16 (b)	158 \pm 44 (b)
Saponification Number	216 \pm 4	198 \pm 6	199 \pm 6	199 \pm 6	196 \pm 1	193 \pm 1	191 \pm 3	191 \pm 3
Cetane Number	28 \pm 9 (a)	33 \pm 3 (a)	32 \pm 3 (a)	32 \pm 3 (a)	49 \pm 1 (b)	42 \pm 1 (b)	39 \pm 9 (b)	39 \pm 9 (b)
Cetane Number without 20:5(n3)	51 \pm 1	55 \pm 4	54 \pm 4	54 \pm 4	65 \pm 2	59 \pm 2	53 \pm 3	53 \pm 3

Table 3.3: Effect of gas treatment and nitrate concentration during the stationary growth phase on biodiesel quality of oil derived from *H. akashiwo* (expressed as means \pm standard deviation, n= 4). Different letters denote significant differences between groups using a one-way ANOVA ($p < 0.05$).

	Control			Flue Gas		
	0	220	880	0	220	880
Nitrate Concentration						
Iodine Value	131 \pm 5	124 \pm 12	127 \pm 12	135 \pm 16	142 \pm 16	149 \pm 44
Saponification Number	177 \pm 3 (a)	159 \pm 2 (a)	160 \pm 5 (a)	214 \pm 10 (b)	207 \pm 5 (b)	201 \pm 2 (b)
Cetane Number	48 \pm 2	53 \pm 3	52 \pm 3	41 \pm 1	41 \pm 1	39 \pm 4
Cetane Number without 20:5(n3)	60 \pm 2	65 \pm 4	64 \pm 2	64 \pm 2	60 \pm 3	55 \pm 3

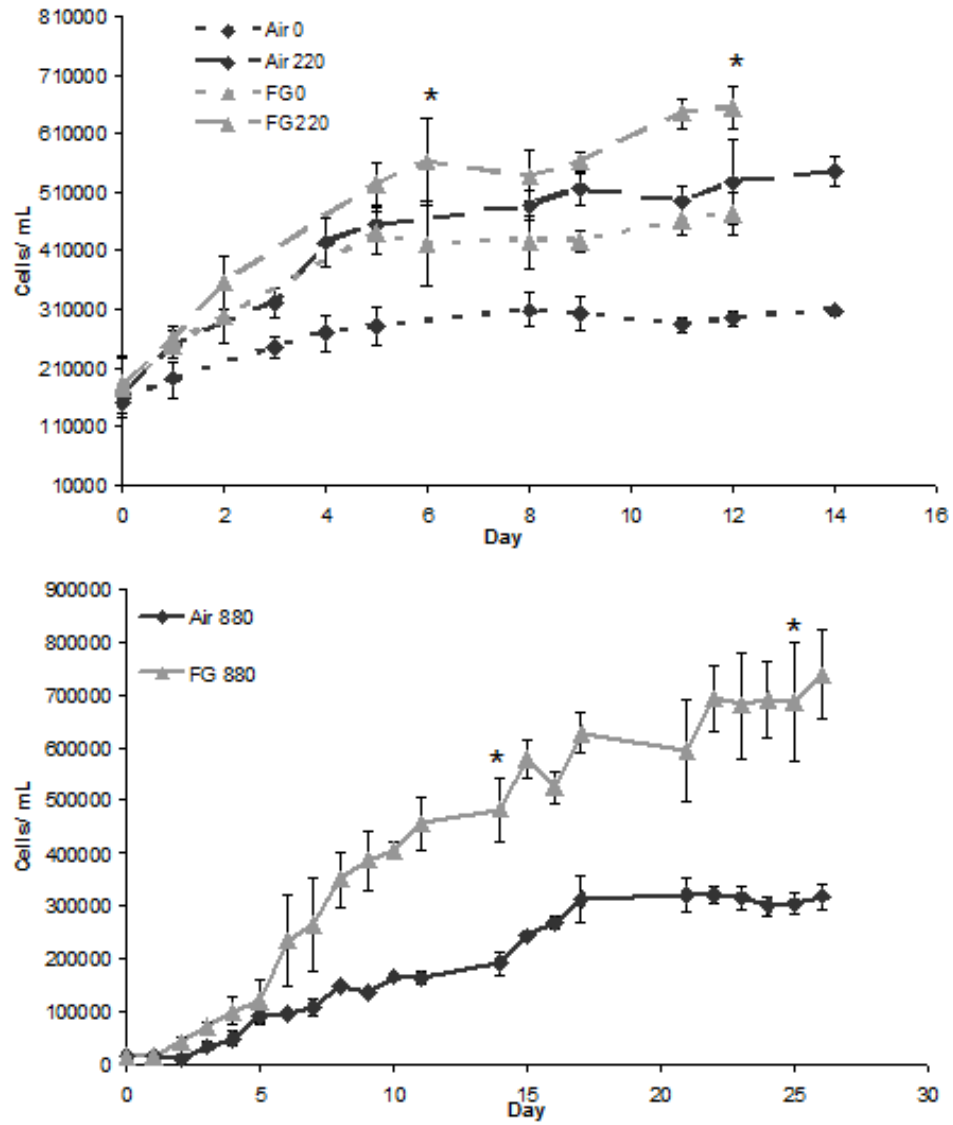


Figure 3.1: Growth curve of *H. akashiwo* for 0 μM and 220 μM (Upper) and 880 μM (lower) nitrate concentrations. Data are expressed as means and error bars represent one standard deviation ($n=4$). Samples were taken on days indicated with black asterisk.

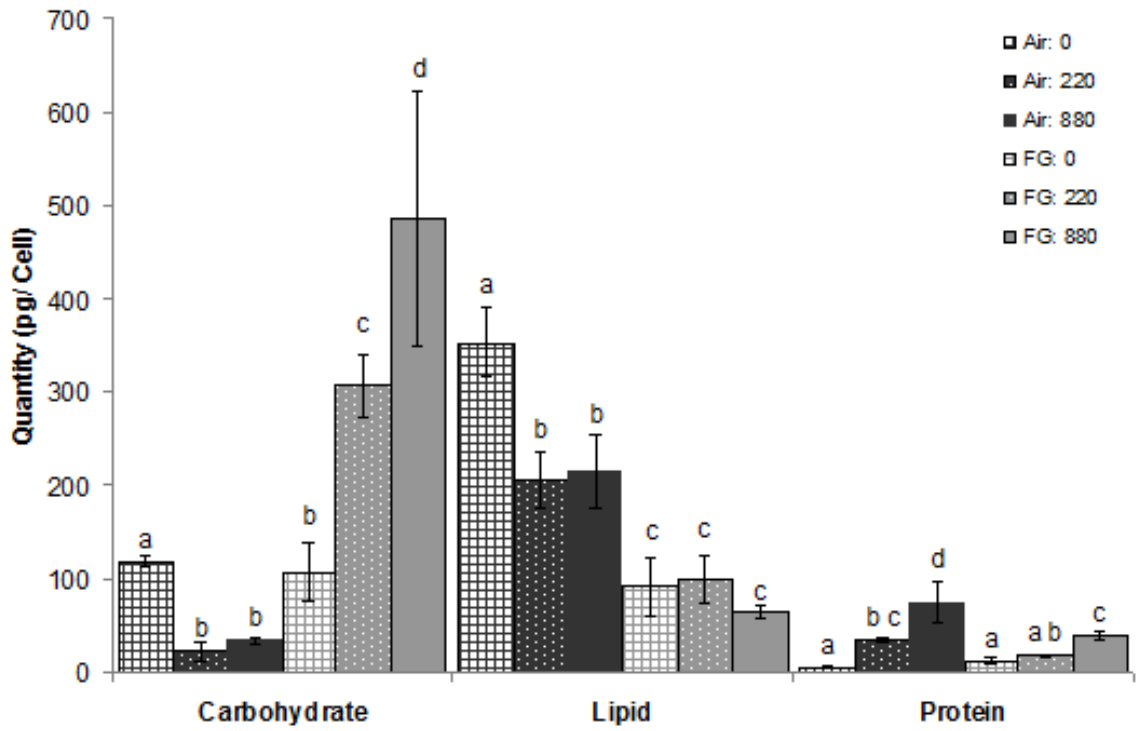


Figure 3.2: Effect of gas treatment and nitrate concentration during the exponential growth phase on carbohydrate, lipid and protein content of *H. akashiwo*. Data are expressed as means and error bars represent one standard deviation (n=4). FG: cultures bubbled with flue gas; Air: cultures bubbled with air. Numbers indicate the concentration of nitrate added to the algal medium. Different letters denote significant differences among treatments using a one-way ANOVA ($p < 0.05$).

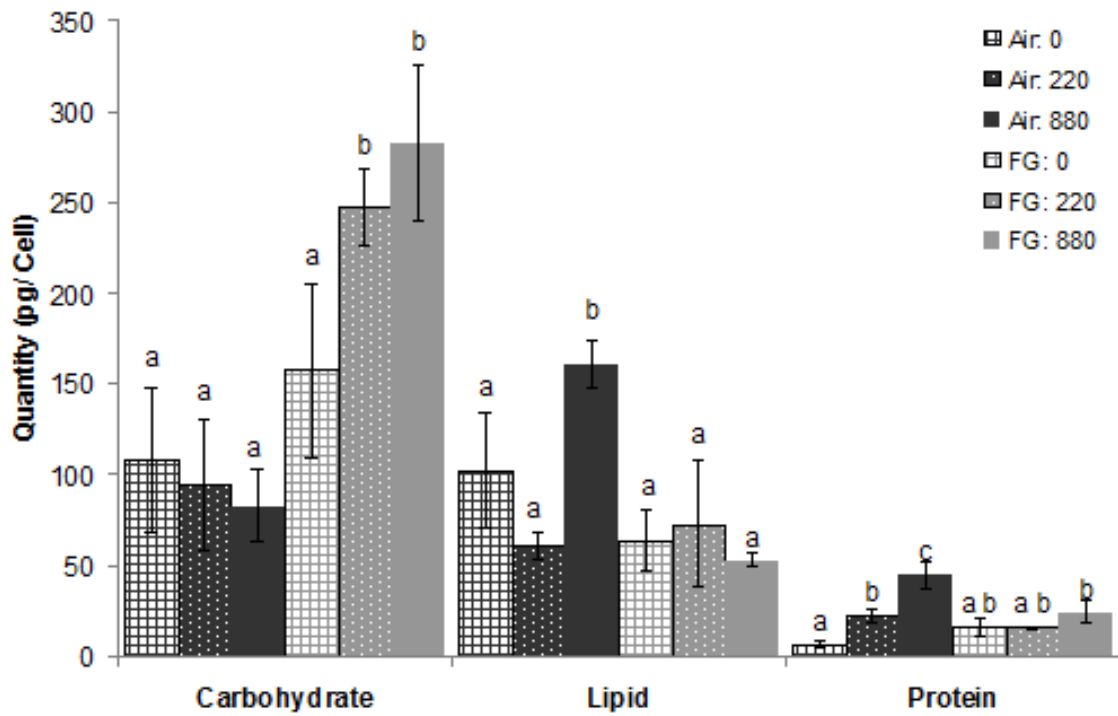


Figure 3.3: Effect of gas treatment and nitrate concentration during the stationary growth phase on carbohydrate, lipid and protein content of *H. akashiwo*. Data are expressed as means and error bars represent one standard deviation (n=4). FG: cultures bubbled with flue gas; Air: cultures bubbled with air. Numbers indicate the concentration of nitrate added to the algal medium. Different letters denote significant differences among treatments using a one-way ANOVA ($p < 0.05$).

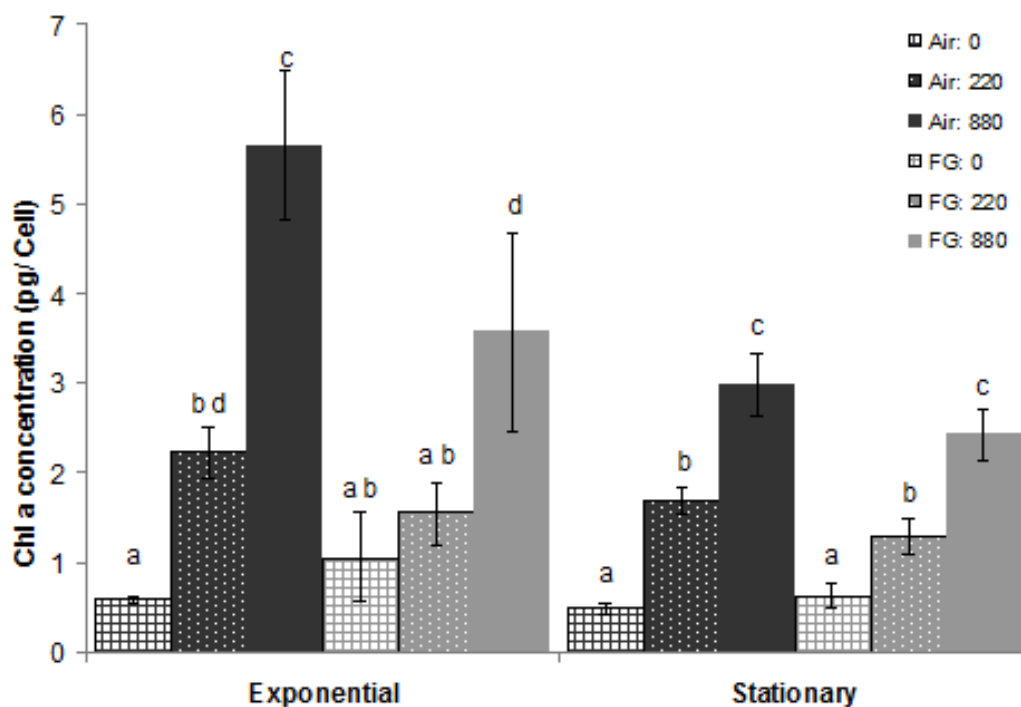


Figure 3.4: Effect of gas treatment and nitrate concentration on chlorophyll *a* content of *H. akashiwo*. Data are expressed as means and error bars represent one standard deviation (n=4). FG: cultures bubbled with flue gas; Air: cultures bubbled with air. Numbers indicate the concentration of nitrate added to the algal medium. Different letters denote significant differences among treatments using a one-way ANOVA (p<0.05).

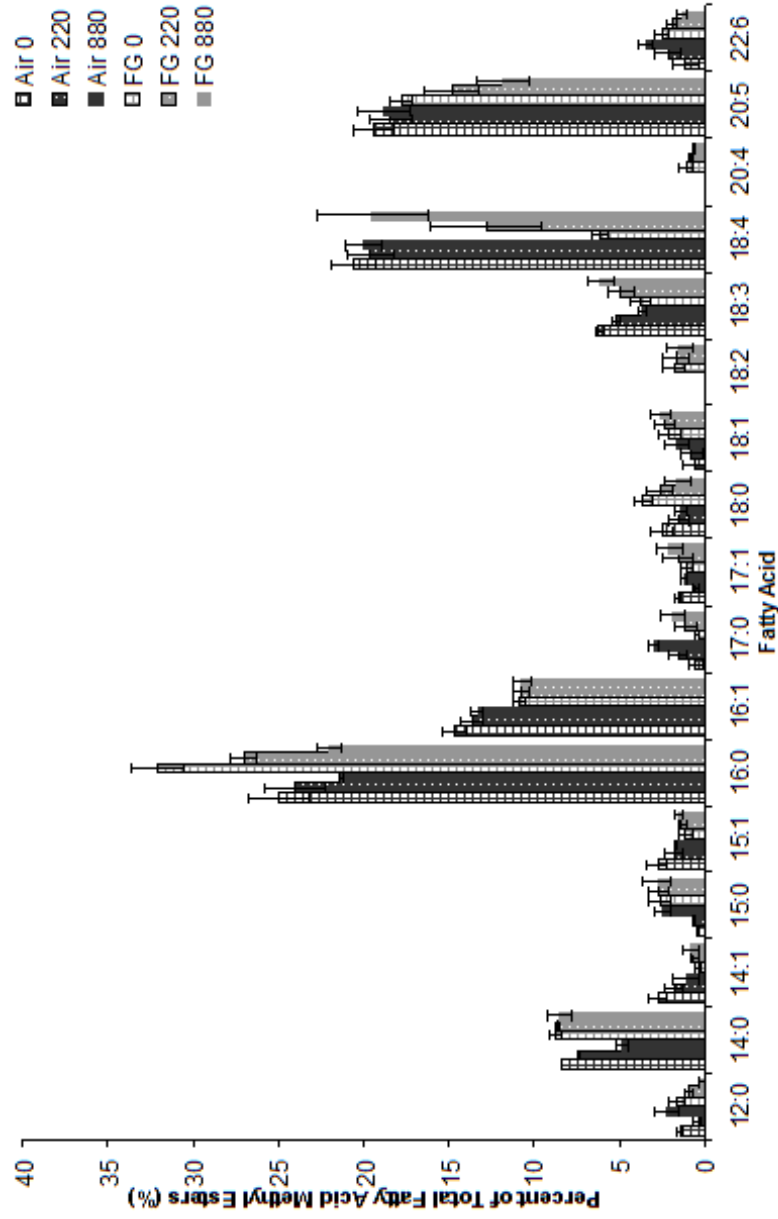


Figure 3.5: Effect of gas treatment and nitrate concentration during the exponential growth phase on the fatty acid profile of *H. akashiwo*. Data are expressed as means and error bars represent one standard deviation (n=4). FG: cultures bubbled with flue gas; Air: cultures bubbled with air. Numbers indicate the concentration of nitrate added to the algal medium.

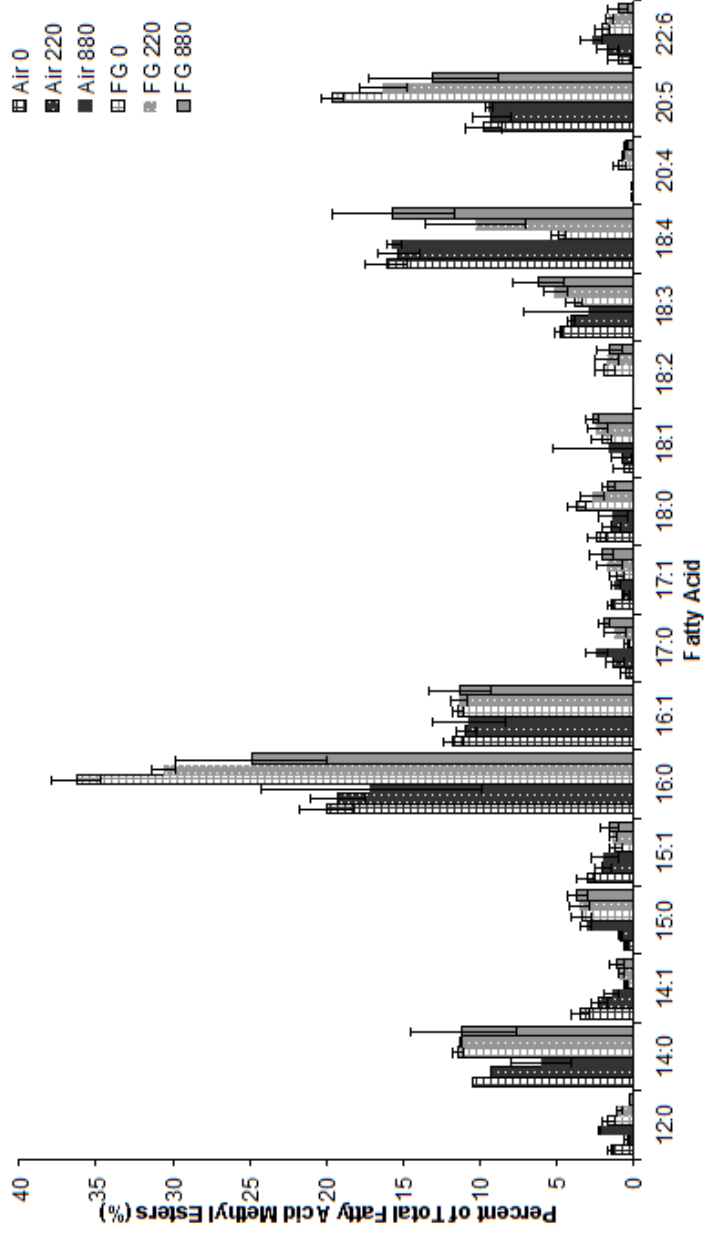


Figure 3.6: Effect of gas treatment and nitrate concentration during the stationary growth phase on the fatty acid profile of *H. akashiwo*. Data are expressed as means and error bars represent one standard deviation (n=4). FG: cultures bubbled with flue gas; Air: cultures bubbled with air. Numbers indicate the concentration of nitrate added to the algal medium.

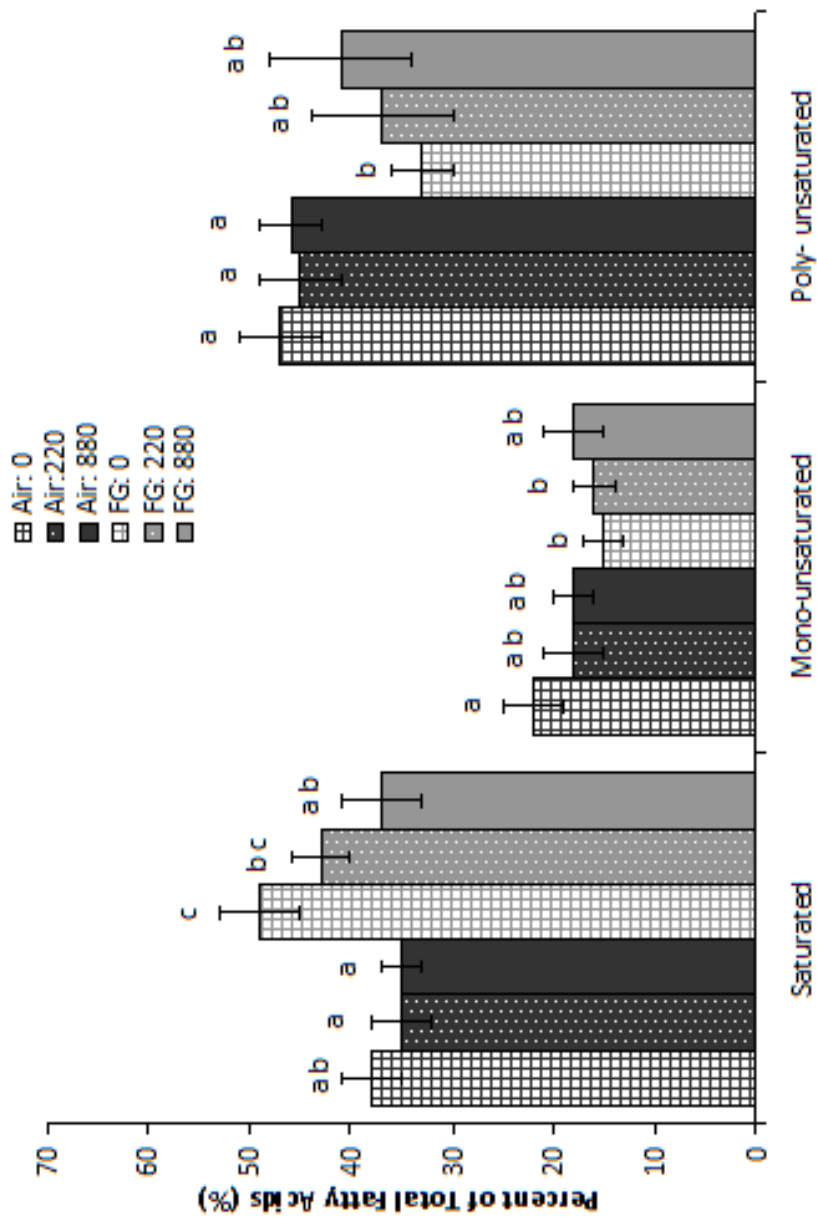


Figure 3.7: Fatty acid type as a percent of total fatty acid content in *H. akashiwo* during the exponential phase. Data are expressed as means and error bars represent one standard deviation (n=4). FG: cultures bubbled with flue gas; Air: cultures bubbled with air. Numbers indicate the concentration of nitrate added to the algal medium. Different letters denote significant differences among treatments groups using a one-way ANOVA ($p < 0.05$).

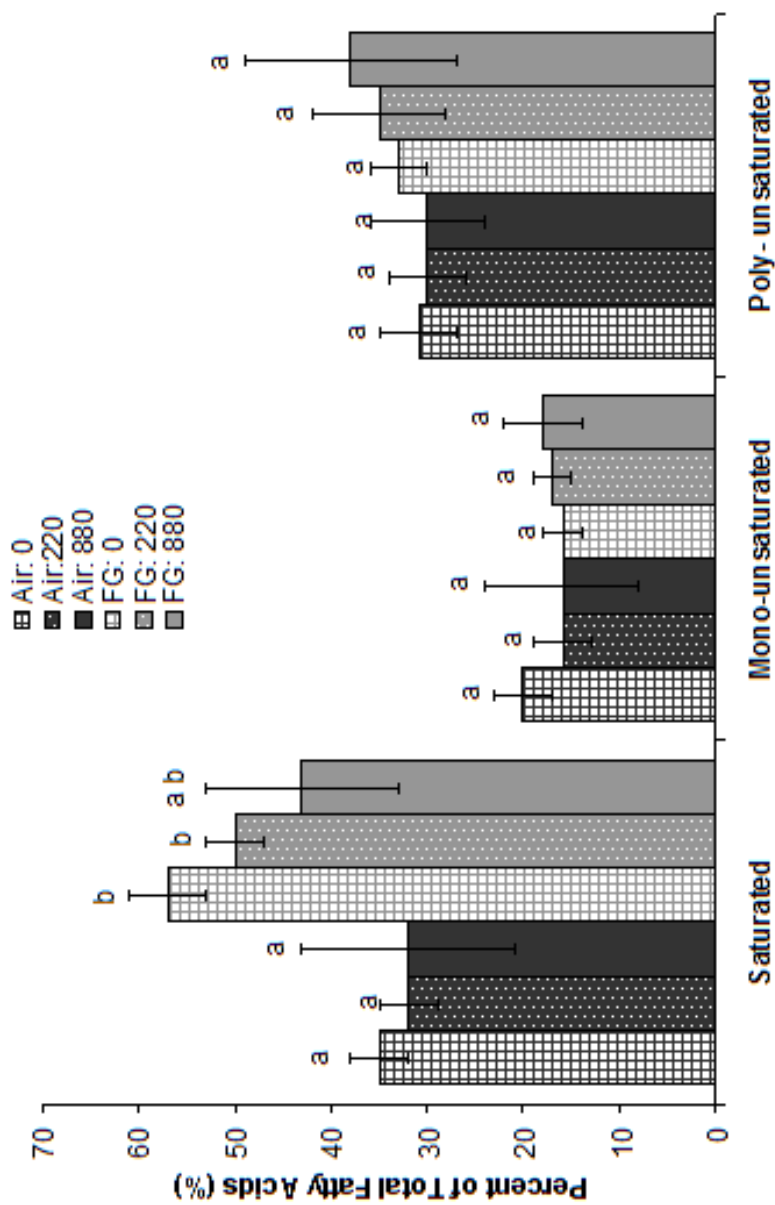


Figure 3.8: Fatty acid type as a percent of total fatty acid content in *H. akashiwo* during the stationary phase. Data are expressed as means and error bars represent one standard deviation (n=4). FG: cultures bubbled with flue gas; Air: cultures bubbled with air. Numbers indicate the concentration of nitrate added to the algal medium. Different letters denote significant differences among treatments groups using a one-way ANOVA ($p < 0.05$).

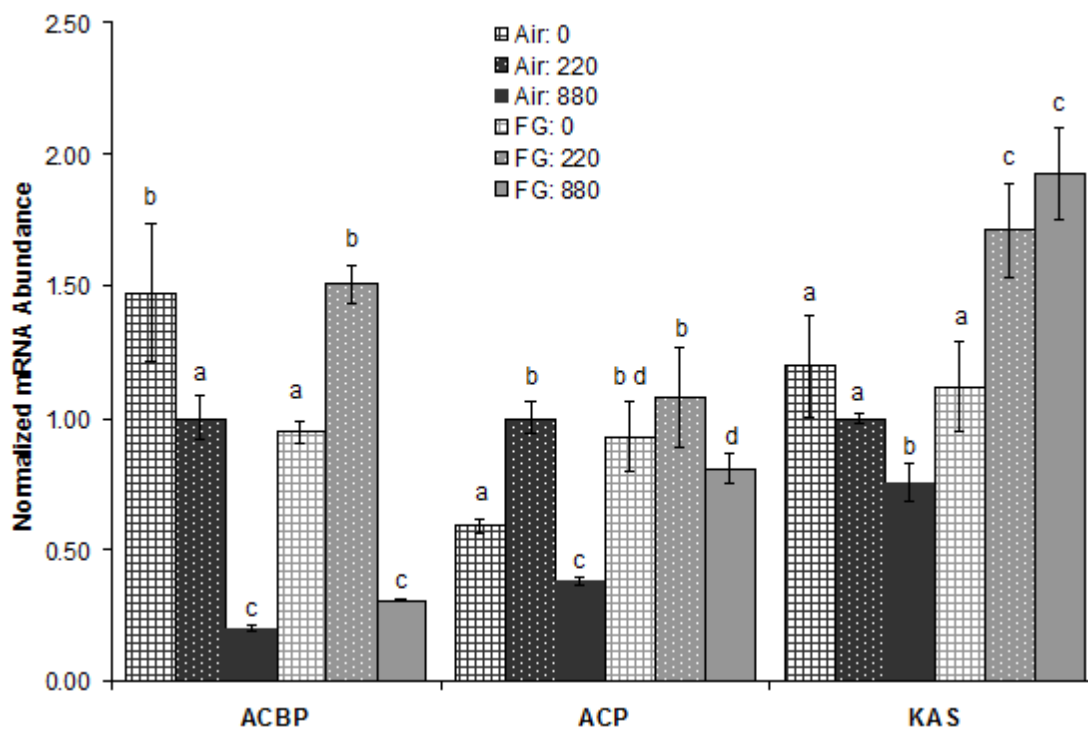


Figure 3.9: Effect of gas treatment and nitrate concentration during the exponential growth phase on the mRNA abundance of three key genes involved in fatty acid synthesis. ACBP: acyl CoA-binding protein, ACP: acyl carrier protein, KAS: beta-ketoacyl-ACP synthase. Gene expression was normalized to 18S rRNA abundance and relative to expression of each gene in the 220 μ M air treatment in exponential phase. FG: cultures bubbled with flue gas; Air: cultures bubbled with air. Numbers indicate the concentration of nitrate added to the algal medium. Data are expressed as means and error bars represent one standard deviation (n=4). Different letters denote significant differences among treatments groups using a one-way ANOVA ($p < 0.05$).

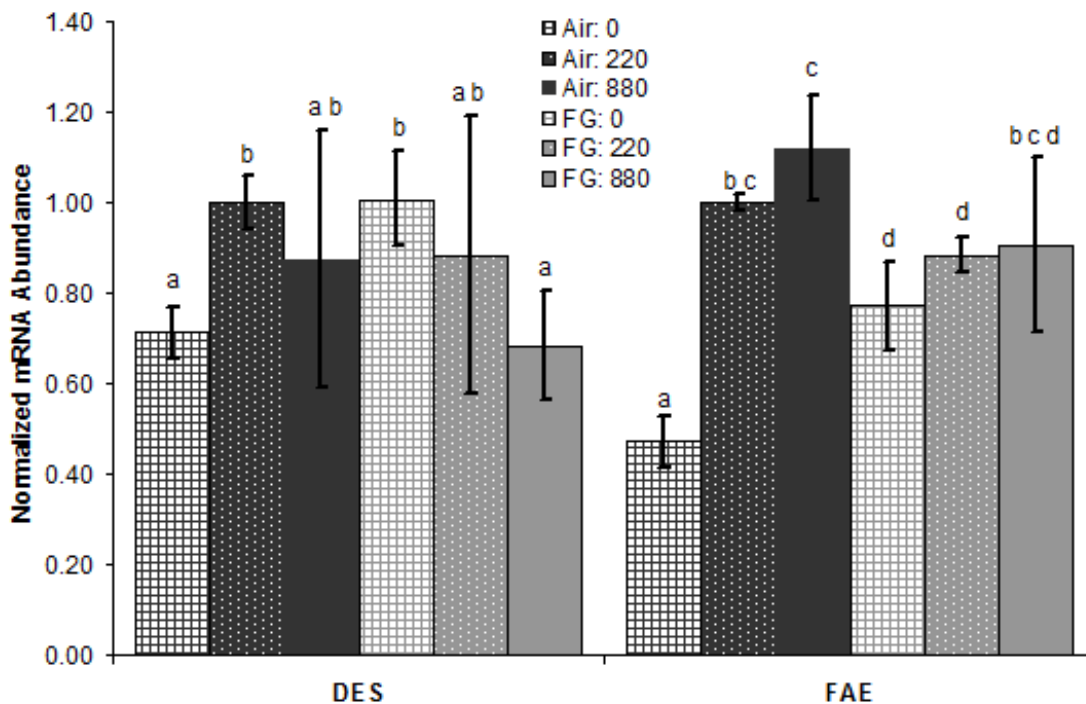


Figure 3.10: Effect of gas treatment and nitrate concentration during the exponential growth phase on the mRNA abundance of key genes involved in C20:5 synthesis. Δ 5 desaturase (DES) and Δ 6 elongase (FAE). Gene expression was normalized to 18S rRNA abundance and relative to expression of each gene in the 220 μ M air treatment in exponential phase. FG: cultures bubbled with flue gas; Air: cultures bubbled with air. Numbers indicate the concentration of nitrate added to the algal medium. Data are expressed as means and error bars represent one standard deviation (n=4). Different letters denote significant differences among treatments groups using a one-way ANOVA (p<0.05).

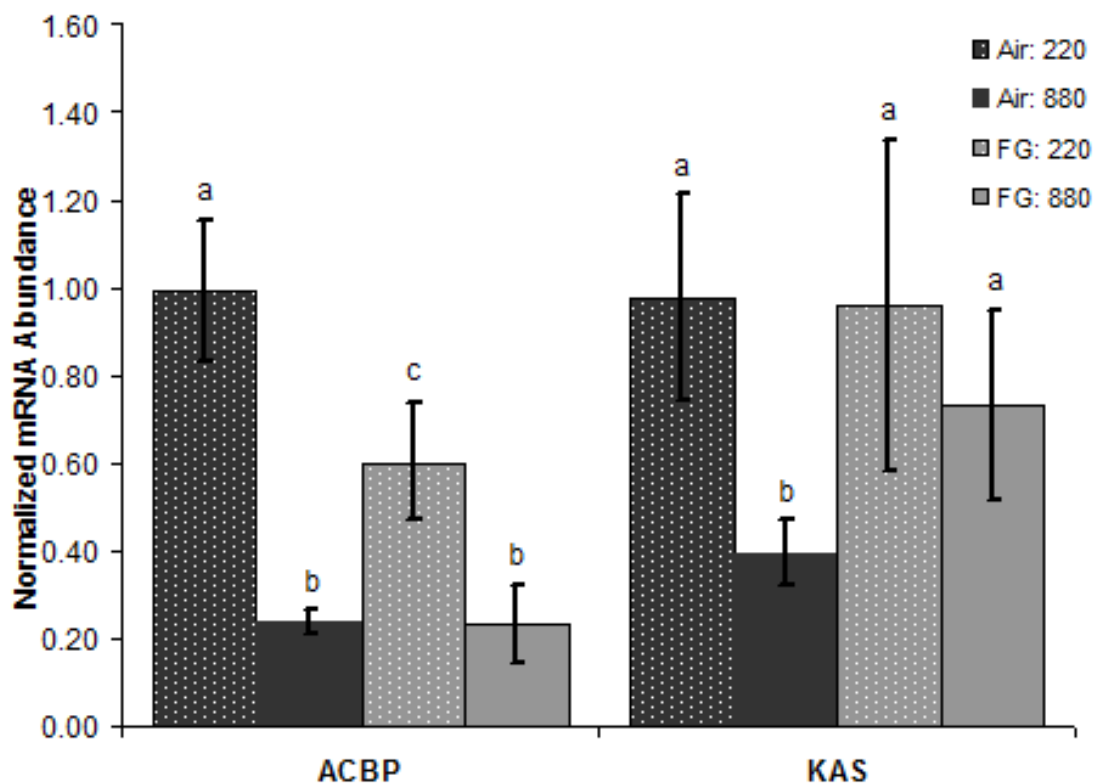


Figure 3.11: Effect of gas treatment and nitrate concentration during the stationary growth phase on the mRNA abundance of three key genes involved in fatty acid synthesis. ACBP: acyl CoA-binding protein, ACP: acyl carrier protein, KAS: beta-ketoacyl-ACP synthase. Gene expression was normalized to 18S rRNA abundance and relative to expression of each gene in the 220 μM air treatment in exponential phase. FG: cultures bubbled with flue gas; Air: cultures bubbled with air. Numbers indicate the concentration of nitrate added to the algal medium. Data are expressed as means and error bars represent one standard deviation (n=4). Different letters denote significant differences among treatments groups using a one-way ANOVA ($p < 0.05$).

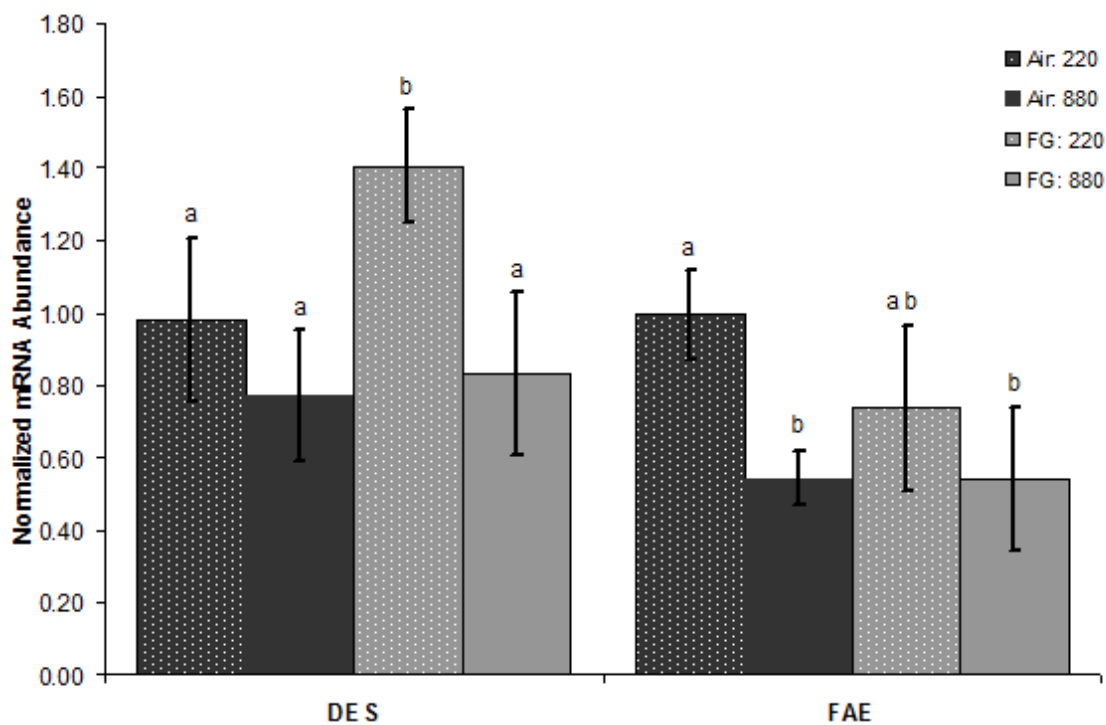


Figure 3.12: Effect of gas treatment and nitrate concentration during the stationary growth phase on the mRNA abundance of key genes involved in C20:5 synthesis. Δ 5 desaturase (DES) and Δ 6 elongase (FAE). Gene expression was normalized to 18S rRNA abundance and relative to expression of each gene in the 220 μ M air treatment in exponential phase. FG: cultures bubbled with flue gas; Air: cultures bubbled with air. Numbers indicate the concentration of nitrate added to the algal medium. Data are expressed as means and error bars represent one standard deviation (n=4). Different letters denote significant differences among treatments groups using a one-way ANOVA (p<0.05).

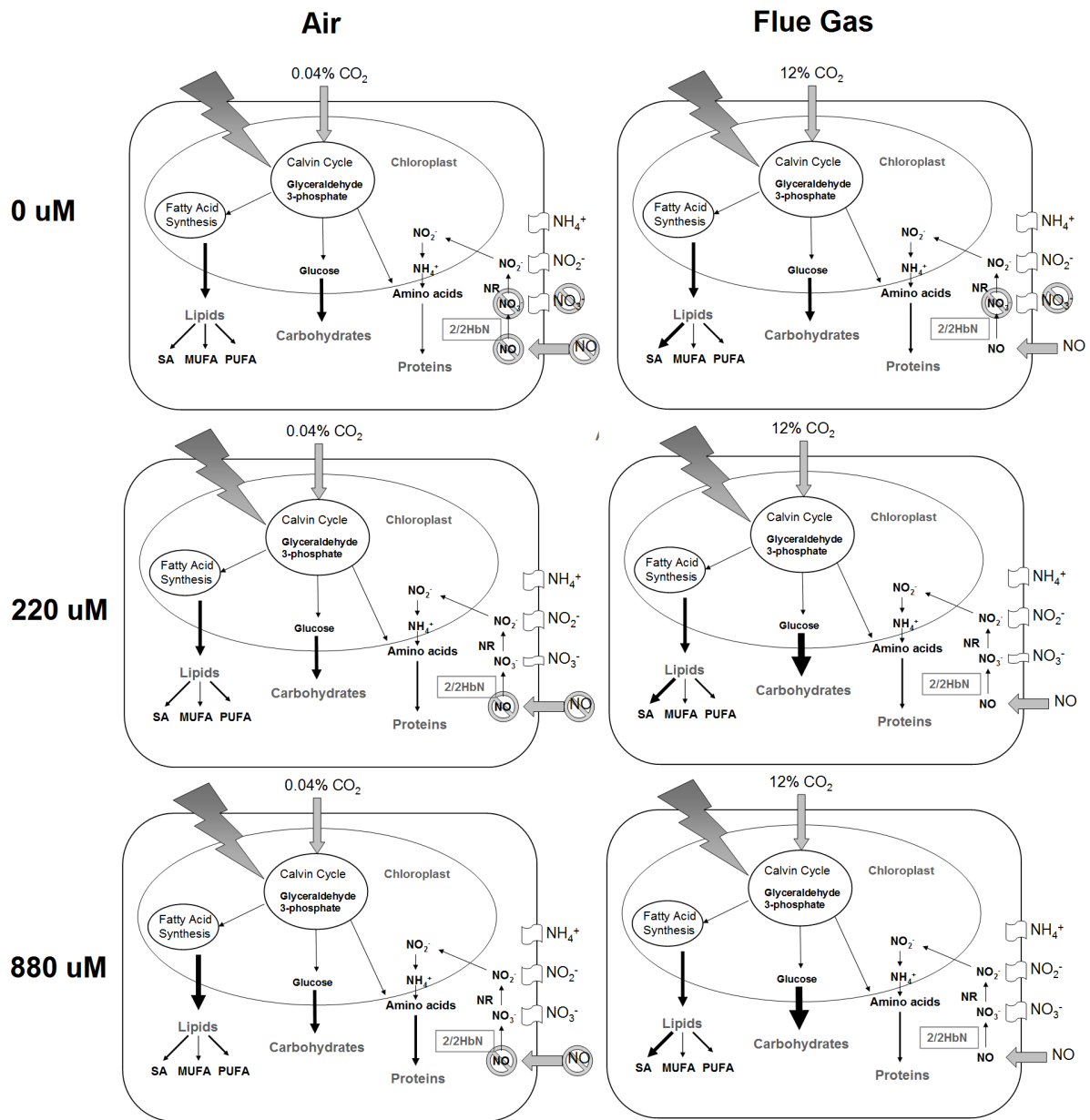


Figure 3.13: Effects of nitrate concentration and flue gas on carbon partitioning in *H. akashiwo* during stationary phase. Numbers indicate the concentration of nitrate added to the algal medium. SA: saturate; MUFA: monounsaturated; PUFA: polyunsaturated fatty acid. Arrows indicate content that was significantly different among treatments. The heavier the arrow, the more abundant the molecule.

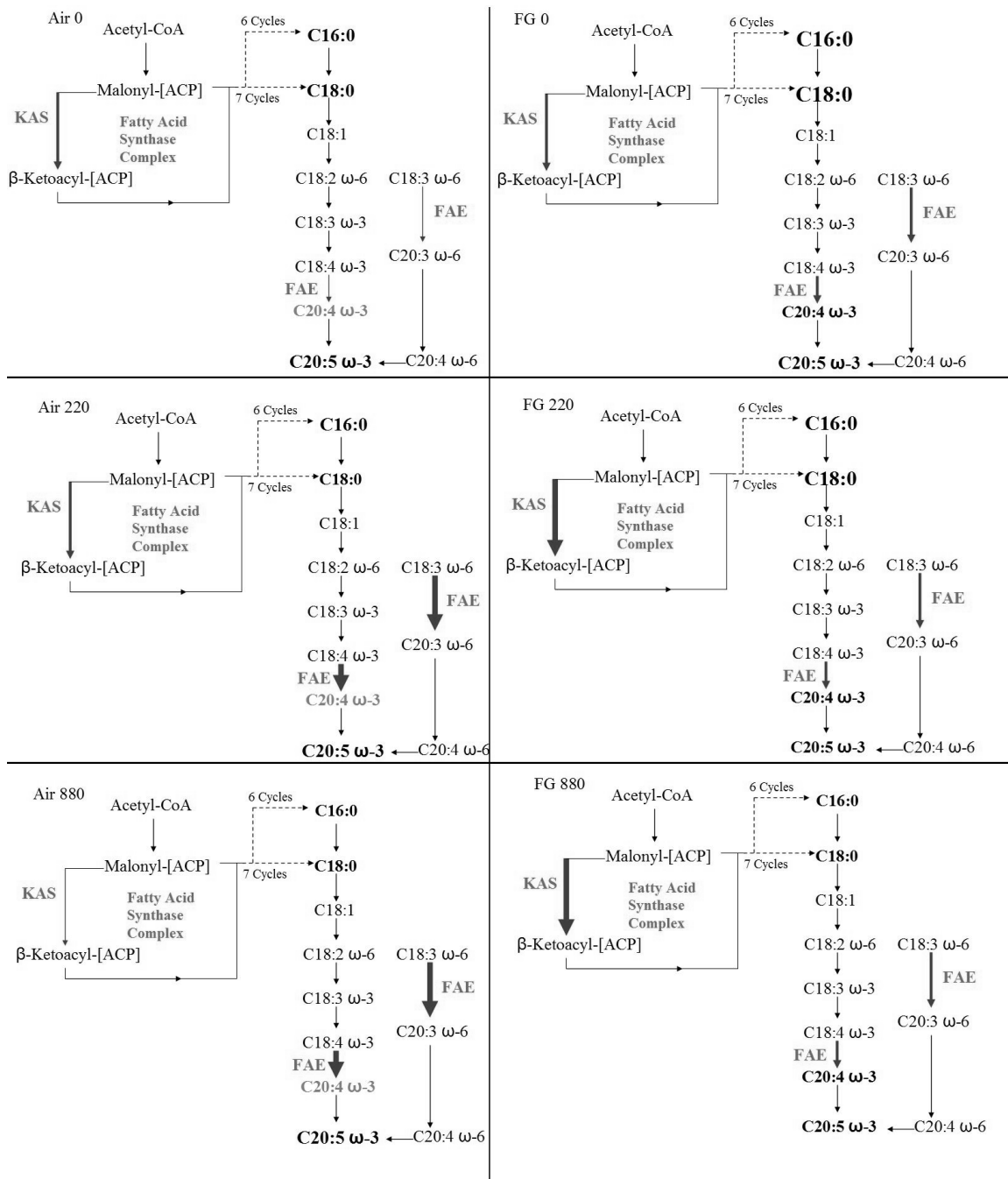


Figure 3.14: Effects of nitrate concentration and flue gas on fatty acid synthesis in *H. akashiwo* during exponential phase. Numbers indicate the concentration of nitrate added to the algal medium. Arrows indicate gene transcripts that were significantly different among treatments. The heavier the arrow, the higher the transcript abundance of the indicated enzyme. The large fatty acid indicates that the fatty acid was high in concentration. Gray fatty acids indicate that they were not detected in the treatment. Modified from: Rismani-Yazdi et al. (2012) and Wen et al. (2003).

3.7 References

Brown, L.M. 1996, "Uptake of carbon dioxide from flue gas by microalgae", *Energy Conversion and Management*, vol. 37, no. 6-8, pp. 1363-1367.

Chisti, Y. 2008, "Biodiesel from microalgae beats bioethanol", *Trends in biotechnology*, vol. 26, no. 3, pp. 126-131.

Ciubota-Rosie, C., Ramon Ruiz, J., Jesus Ramos, M. Perez, A. 2013, "Biodiesel from *Camelina sativa*: A comprehensive characterisation", *Fuel*, vol. 105, pp. 572-577.

Fuentes-Grünewald, C., Garces, E., Alacid, E., Rossi, S. Camp, J. 2013, "Biomass and Lipid Production of Dinoflagellates and Raphidophytes in Indoor and Outdoor Photobioreactors", *Marine Biotechnology*, vol. 15, no. 1, pp. 37-47.

Fuentes-Grünewald, C., Garces, 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*, vol. 39, no. 1, pp. 207-216.

Halim, R., Danquah, M.K. Webley, P.A. 2012, "Extraction of oil from microalgae for biodiesel production: A review", *Biotechnology Advances*, vol. 30, no. 3, pp. 709-732.

Hogue, C. 2011, *Clearing Skies*.

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", *Plant Journal*, vol. 54, no. 4, pp. 621-639.

Lei, A., Chen, H., Shen, G., Hu, Z., Chen, L. Wang, J. 2012, "Expression of fatty acid synthesis genes and fatty acid accumulation in *Haematococcus pluvialis* under different stressors", *Biotechnology for Biofuels*, vol. 5, pp. 18.

Li, Y., Han, D., Sommerfeld, M. Hu, Q. 2011, "Photosynthetic carbon partitioning and lipid production in the oleaginous microalga *Pseudochlorococcum* sp (Chlorophyceae) under nitrogen-limited conditions RID D-2553-2010", *Bioresource technology*, vol. 102, no. 1, pp. 123-129.

Liang, C., Cao, S., Zhang, X., Zhu, B., Su, Z., Xu, D., Guang, X. Ye, N. 2013, "De Novo Sequencing and Global Transcriptome Analysis of *Nannochloropsis* sp (Eustigmatophyceae) Following Nitrogen Starvation", *Bioenergy Research*, vol. 6, no. 2, pp. 494-505.

Lopes, D.C., Baron Maurer, J.B., Stevan-Hancke, F.R., de Oliveira Proenca, L.A. Zawadzki-Baggio, S.F. 2012, "Chemical analysis of exopolysaccharide fractions and lipid compounds of the microalga *Heterosigma akashiwo* grown in vitro", *Botanica Marina*, vol. 55, no. 6, pp. 565-580.

Nascimento, I.A., Izabel Marques, S.S., Dominguez Cabanelas, I.T., Pereira, S.A., Druzian, J.I., de Souza, C.O., Vich, D.V., de Carvalho, G.C. Nascimento, M.A. 2013, "Screening Microalgae Strains for Biodiesel Production: Lipid Productivity and Estimation of Fuel Quality Based on Fatty Acids Profiles as Selective Criteria", *Bioenergy Research*, vol. 6, no. 1, pp. 1-13.

Negoro, M., Shioji, N., Miyamoto, K. Miura, Y. 1991, "Growth of Microalgae in High CO₂ Gas and Effects of Sox and Nox", *Applied Biochemistry and Biotechnology*, vol. 28-9, pp. 877-886.

Rawat, I., Kumar, R.R., Mutanda, T. Bux, F. 2013, "Biodiesel from microalgae: A critical evaluation from laboratory to large scale production", *Applied Energy*, vol. 103, pp. 444-467.

Rismani-Yazdi, H., Haznedaroglu, B.Z., Hsin, C. Peccia, J. 2012, "Transcriptomic analysis of the oleaginous microalga *Neochloris oleoabundans* reveals metabolic insights into triacylglyceride accumulation", *Biotechnology for Biofuels*, vol. 5, pp. 74.

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*, vol. 77, no. 6, pp. 565-575.

Van den Hende, S., Vervaeren, H. Boon, N. 2012, "Flue gas compounds and microalgae: (Bio-)chemical interactions leading to biotechnological opportunities", *Biotechnology Advances*, vol. 30, no. 6, pp. 1405-1424.

Wen, Z.Y. Chen, F. 2003, "Heterotrophic production of eicosapentaenoic acid by microalgae", *Biotechnology Advances*, vol. 21, no. 4, pp. 273-294.

Williams, P.J.L.B. Laurens, L.M.L. 2010, "Microalgae as biodiesel biomass feedstocks: Review analysis of the biochemistry, energetics economics", *Energy Environmental Science*, vol. 3, no. 5, pp. 554-590.

Yang, Z., Niu, Y., Ma, Y., Xue, J., Zhang, M., Yang, W., Liu, J., Lu, S., Guan, Y. Li, H. 2013, "Molecular and cellular mechanisms of neutral lipid accumulation in diatom following nitrogen deprivation.", *Biotechnology for biofuels*, vol. 6, no. 1, pp. 67-67.

Chapter 4

IMPACT OF SATURATING LIGHT INTENSITIES ON FATTY ACID COMPOSITION AND BIODIESEL SOURCE POTENTIAL OF *HETEROSIGMA AKASHIWO* GROWN ON MODEL FLUE GAS

4.1 Abstract

The microalga, *Heterosigma akashiwo*, is a promising candidate for biodiesel feedstock due to its high productivity, high lipid yields and short growth period. Triacylglyceride concentration and fatty acid content are important biodiesel qualities that can be altered with environmental stress, such as low nutrient concentration and high irradiance. The aim of this study was to evaluate the biodiesel potential of *H. akashiwo* when grown under three light intensities and bubbled with model flue gas. There were no significant differences in growth rates among the light intensities in this study, indicating that *H. akashiwo* was not photoinhibited at $1200\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$. Carbohydrate content was higher than lipid and protein content in all treatments. While the medium light treatment produced the highest lipid content (26% of its dry weight), the high light treatment produced the most desirable fatty acid profile. Palmitic acid (C16:0) accounted for 24.5% of total fatty acid content in high light treatment algae, which was significantly more than the C16:0 content of the low and medium light treatment. Polyunsaturated fatty acids made up 32% of fatty acids in the high light cultures, which was significantly less than in low light cultures. Beta-ketoacyl-ACP synthase, a key regulator of fatty acid chain length, was up-regulated in the high light cultures. Biodiesel from the high light treatment was calculated to have a cetane number of 43, indicating the potential biodiesel is of high quality. The fatty acid profiling reported in this study suggests that *H. akashiwo* is an ideal candidate for biodiesel production when grown under high light.

4.2 Introduction

Previous studies (Fuentes-Grünewald et al., 2012), along with work presented in Chapter Two of this thesis, have identified *Heterosigma akashiwo* (Raphidophyceae) as a promising biodiesel feedstock candidate due to its high productivity, high lipid yields and fast growth rate. Raphidophytes contain high proportions of the saturated palmitic acid (C16:0), making them ideal candidates for biodiesel production (Nascimento et al., 2013; reviewed by Hu et al., 2008; work presented in Chapter Two). *H. akashiwo* also produces high levels of eicosapentaenoic fatty acid (C20:5, EPA), a precursor to many lipid regulators and complex lipid molecules (Wen et al., 2003; work presented in Chapter Two). However, changes in fatty acid profiles with culture age and environmental conditions are not well understood for raphidophytes.

Biodiesel is produced by the transesterification of triacylglycerol (TAG) with methanol to yield fatty acid methyl esters (FAMES). The structure of these resulting fatty acid methyl esters largely determines the properties of the biodiesel (reviewed by Hu et al., 2008). FAMES from TAGs are desirable because they lack highly unsaturated (more than four double bonds) polyunsaturated fatty acids (PUFA), which are often responsible for poor volatility, low oxidation stability and gum formation in biofuels (reviewed by Hu et al., 2008).

Manipulation of light intensities can result in changes in lipid content and alter fatty acid composition of microalgae. Under high light irradiance, many microalgae alter their lipid biosynthetic pathways to accumulate saturated and mono-unsaturated fatty acids, which make up neutral lipids (reviewed by Hu et al., 2008; Li et al., 2011). Li et al. (2011) observed that moderate photoinhibition of photosynthesis induced by high light intensity was needed for maximum formation and accumulation of neutral lipids, but further increased light intensities inhibited growth. In addition, high levels of polyunsaturated fatty acids, which are typically incorporated into membrane lipids, are produced under low light intensities (reviewed by Hu et al., 2008)

Microalgal biodiesel can potentially decrease fossil fuel dependence and also reduce greenhouse gases, such as carbon dioxide CO₂, present in flue gas. Flue gas also

contains 100-300 ppm NO_x (>95% as NO), which inhibits the growth of most microalgae (Negoro et al., 1991). However, *H. akashiwo* contains a hybrid nitrate reductase, NR2-2/2HbN, that may detoxify and convert NO to nitrate (Stewart and Coyne, 2011). This enzyme may harness NO from flue gas as a free nitrogen source, effectively reducing the cost of biodiesel production from microalgae. The research presented in Chapter Two of this thesis demonstrates higher growth rates for *H. akashiwo* when grown on model flue containing NO.

The goal of the study presented here was to evaluate the biodiesel potential of *H. akashiwo* under varying light conditions when grown on flue gas. Fatty acid composition was evaluated by gas chromatography and used to calculate biodiesel quality. To better understand lipid metabolism in microalgae, the expression patterns of six genes involved in fatty acid synthesis were investigated under each light level and gas treatment.

4.3 Materials and Methods

4.3.1 Experimental Design

Heterosigma akashiwo (Delaware Inland Bay isolate, CCMP 2393) was grown in 20 psu f/2 medium buffered with 20 mM HEPES (pH 7.4) at 25°C and bubbled with model flue gas (nitrogen gas containing 12% carbon dioxide and 150 ppm nitric oxide) at 2 mL minute⁻¹. Replicate cultures (N=4) were acclimated to varying light intensities; low light (160 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$), medium light (560 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$), high light (1200 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$) on a 12:12 hour light: dark cycle. After six weeks of growth at the respective light intensity, each set of replicate cultures were maintained semi-continuously in exponential growth phase. During semi-continuous growth, cell counts, in vivo chlorophyll *a* (chl *a*), extracted chl *a*, and photosynthetic efficiency (Fv/Fm) were measured every other day before cultures were diluted to monitor the cultures for steady state growth. Cultures were diluted to 150 000 cells/mL every other day. Steady state growth was reached when the cell counts of the cultures and extracted chl *a* did not vary between dilution days by 10% . Samples were collected for analysis

of total lipid content, lipid composition, carbon partitioning and gene expression after 10 days of steady state growth. Sampling and analytical methods are described in Appendix A.

4.3.2 Statistical Analysis

The experiment was performed in four replicates for each light level. Data represents means \pm standard deviations. One-Way Analysis of Variance (ANOVA) was used to determine statistical significance among the light levels followed by Tukey's HSD Test. A p-value of 0.05 was used as the standard for statistical significance. When a Kolmogorov-Smirnov test indicated the data were not normally distributed, a Kruskal-Wallis ANOVA was performed.

4.4 Results

4.4.1 Growth, Particulate Nitrogen and Particulate Carbon

There was no significant difference in growth or doubling rates among the light treatments (Table 4.1), which suggests that *H. akashiwo* may not have been photoinhibited at the high light treatment. The particulate carbon to particulate nitrogen ratio (C/N) was significantly different between the low and high light treatments. C/N in the low light algae was 5.48 ± 0.06 (mean \pm standard deviation), which is significantly lower than the C/N ratio of the high light treatment (6.3 ± 0.2 ; Tukey's HSD, $p < 0.01$).

4.4.2 Carbon Partitioning

Light intensity affected the carbon partitioning between lipids, carbohydrates and proteins in *H. akashiwo*. Carbohydrate content was higher than lipid and protein content in all light treatment cultures (Figure 4. 1). Total carbohydrate content was significantly higher in the cultures grown at a high light intensity compared to the cultures grown at low and a medium light intensities, which were not significantly different from each other (Tukey's HSD, $p < 0.01$ for high light vs. low light and high light vs. medium light). In contrast, total lipid quantity was significantly higher in

the cultures grown at the low light intensity compared to the cultures grown at the medium light intensity (Tukey's HSD, $p < 0.05$). Protein content was not significantly different among all treatments.

4.4.3 Fatty Acid Profile

The fatty acid profiles of *H. akashiwo* were similar for the three light treatments (Figure 4.2). The major fatty acids in all three treatments were myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), linolenic (C18:3), stearidonic (C18:4) and eicosapentaenoic (C20:5, EPA). Saturated fatty acid profiles varied with light treatment but the total saturated fatty acid content as a proportion of total fatty acids was not significantly different among treatments (Table 4.2). The largest difference in the saturated fatty acid profiles among treatments was in the proportion of C16:0 (ANOVA, $p = 0.007$). C16:0 accounted for $24.5 \pm 0.5\%$ of total fatty acid content in high light algae, which was significantly more than the C16:0 content of the low and medium light algae ($19 \pm 2\%$ and $19 \pm 2\%$ respectively; Tukey's HSD, $p < 0.05$). Cultures grown at the low light intensity contained significantly less C14:0 ($10.5 \pm 0.8\%$; Tukey's HSD, $p < 0.05$) than the medium and high light treatments ($16 \pm 1\%$ and $16 \pm 1\%$ respectively).

The total monounsaturated fatty acid (MUFA) and total polyunsaturated fatty acid (PUFA) content varied with light level. Total MUFA as a proportion of total fatty acid concentration was significantly less in the algae grown under low light ($15 \pm 3\%$ compared to the medium ($17 \pm 1\%$) and high ($21 \pm 3\%$) light treatments (Tukey's HSD, $p < 0.05$). C16:1 content was significantly higher in the high light treatment compared to the low and medium light treatment (Tukey's HSD, $p < 0.05$). C16:1 was $14 \pm 1\%$ of the total fatty acid content in the high light treatment and $8 \pm 2\%$ and $9.7 \pm 0.8\%$ in the low and medium light treatment, respectively. PUFA content was significantly lower in the high light treatment ($32 \pm 4\%$; Tukey's HSD, $p < 0.05$) compared to PUFA content in low light cultures ($44 \pm 5\%$). The algae grown under the high light intensity

contained $9 \pm 2\%$ C18:4, which is significantly less than C18:4 content in medium ($13.3 \pm 0.4\%$) and low ($16 \pm 3\%$) light treatments (Tukey's HSD, $p < 0.01$).

4.4.4 Transcript Abundance

Relative messenger RNA levels of six key genes involved in fatty acid synthesis were investigated (Figure 4.4 and 4.5). Acetyl-CoA carboxylase (ACC) catalyzes the conversion of acetyl-CoA to malonyl-CoA, which is the commitment step in *de novo* fatty acid synthesis. Malonyl-CoA then enters the fatty acid cycle, where acyl carrier protein (ACP) and beta-ketoacyl-ACP synthase (KAS) aid in fatty acid chain growth to ultimately produce C16:0. Acyl CoA-binding protein (ACBP) is involved in the transport of fatty acids. Of these four enzymes, the transcript abundance of ACP and KAS were up-regulated in the high light treatment. In the high light treatment, ACP transcript abundance was 3.3 fold higher than in the low light algae (Tukeys HSD, $p < 0.001$) and 2.9 fold higher than in the medium light algae (Tukey's HSD, $p < 0.001$). The high light cultures also had significantly higher KAS transcript abundance compared to the other treatments (ANOVA, $p < 0.003$). KAS transcript abundance was 2.9 fold higher in the high light algae than in the low light (Tukeys HSD, $p < 0.001$) and 3.2 fold higher than in the medium light algae (Tukey's HSD, $p < 0.001$). Δ 6 fatty acid elongase (FAE) and Δ 5 desaturase (DES) catalyze the formation of eicosapentaenoic fatty acid (C20:5, EPA) which was the third most abundant fatty acid in each light treatment. There were no significant differences in FAE transcript abundance among the light treatment cultures. Gene expression of DES, which catalyzes the desaturation of C20:4 to form EPA, was significantly higher under the high light treatment compared to the low and medium light algae (Kruskal-Wallis ANOVA, $p < 0.001$). The fatty acid C20:4 was not detectable in the high light cultures, which suggests that DES immediately converted C20:4 to EPA.

4.4.5 Biodiesel Quality

The light treatments had a significant effect on biodiesel quality. Biodiesel from cultures grown at the high light intensity had a lower iodine value (130 ± 10) and a higher cetane number (43 ± 2) than the other treatments (Table 4.2). This iodine value suggests that the high light treatment culture a good candidate for biodiesel production. However, due to the high EPA concentration in all treatment cultures, the cetane number of biodiesel from each treatment culture is below the US minimum for diesel (47, American Society for Testing and Materials). If EPA were removed from the algal biodiesel, all three treatments would have cetane numbers between 53-54. These cetane numbers would make the biodiesel from *H. akashiwo* competitive with US premium diesel, which has a cetane value of 55 (American Society for Testing and Materials).

4.5 Discussion

In this study, *H. akashiwo* was cultured with model flue gas under light intensities ranging from 160 to 1200 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$. Previous studies on algae show that irradiance above the level of light saturation will cause a decrease in growth rate due to photoinhibition (Rawat et al. 2013). There were no significant differences in growth rates among the light intensities in this study, indicating that *H. akashiwo* was not photoinhibited at 1200 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$. Furthermore, the equal growth rates at all three light levels suggest that large-scale production of biodiesel from *H. akashiwo* may be commercially viable. Open raceway ponds are commonly used during the mass cultivation of microalgae for biodiesel (Rawat et al. 2013). However, low algal productivities in open ponds occur as a result of a number of factors, the main one being light limitation due to high culture densities. Our study indicates that *H. akashiwo* can be productive at the pond surface where the irradiance is very high as well as below the surface where the irradiance is low.

Light intensity had a significant effect on carbohydrate content of *H. akashiwo*. The high light algae had the highest carbohydrate content per cell of all treatments.

This agrees with Li et al. (2011), who determined that starch synthesis was up-regulated in *Pseudochlorococcum* sp. under high light. This suggests that starch is used for primary carbon and energy storage. As discussed in Chapter Two, cultures grown on flue gas used carbohydrates rather than lipids to store the high amount of CO₂ in the flue gas. Because simple carbohydrates are produced through photosynthesis, the accumulation of carbohydrates at the high light intensity, where more photons are available for photosynthesis, supports this hypothesis. Protein content did not vary with light intensity while lipid content varied slightly with light treatment. The cultures grown under low light had a slightly higher lipid content than the cultures grown under medium light.

Fatty acid profiling suggests that *H. akashiwo* is a good candidate for biodiesel production when grown under high light. C16:0 was the principle fatty acid under all treatments and is considered desirable for biodiesel due to its chain length and degree of unsaturation (Nascimento et al. 2013). The high light treatment cultures had significantly more C16:0 content compared to the low and medium light cultures, which contributed to its higher cetane number (CN).

CN is the primary indicator of diesel quality and measures the ignition delay when the diesel is injected into the combustion chamber. A high CN indicates a short ignition time. The estimated CN for the microalgal biodiesel studied here were all below the US standard of 47 (Nascimento et al. 2013). However, CN increases with increasing fatty acid chain length and decreases with increasing degree of unsaturation. EPA (C20:5), which is the third most abundant fatty acid in all three treatments, significantly decreases the estimated CN. EPA is an economically valuable omega-3 fatty acid and if it were removed from the biodiesel, all treatments would have a CN value between 55-58, which is well above the US standard. These estimated CNs are higher than the 50.9 CN of soybeans, which the United States is currently using for biodiesel (Hu et al. 2008; Nascimento et al. 2013).

Transcript abundances of the six genes investigated were not significantly different between cultures grown under low light intensity and cultures grown under medium

light intensity. However, the only significant difference in the fatty acid profiles of these two light treatments was C14:0 content (which was lower in cultures grown under low light). C14:0 production is not regulated by the enzymes studied here. The lack of difference in transcript abundance and in fatty acid composition between the cultures grown under low light intensity and the cultures grown under medium light intensity, are in agreement.

ACC transcript abundance decreased as light intensity increased, although these differences were not statistically significant. As described in Chapter One, ACC is a major determinant of the overall rate of fatty acid synthesis (Baud et al. 2003). Cultures grown under low light had a significantly higher lipid content per cell than the cultures grown under medium light, which may correspond to the higher ACC transcript abundance observed in the culture grown under low light.

In this study, the only significant differences in gene expression were KAS and ACP, which were both up-regulated in the cultures grown under high light (Figure 4.5). These cultures also had the highest C16:0 content compared to the other treatments. High KAS and high ACP transcript abundances may be linked to high C16:0 content because KAS and ACP play key roles in fatty acid synthesis, of which C16:0 (and C18:0) is the major product (Lei et al. 2012). KAS is responsible for the two carbon elongations of substrates from C4:0 to C14:0, which results in C16:0 as the end product. Throughout fatty acid synthesis, ACP carries the acyl groups of the fatty acid intermediates. High gene expression for ACP may correlate to higher levels of ACP activity, which would ultimately yield more C16:0.

Δ 5 desaturase (DES) gene expression was also significantly higher in the cultures grown in high light compared to the other light treatments. However, there was no difference in C20:5 concentration as a proportion of total fatty acids. As shown in Figure 4.5, Δ 5 desaturase also desaturates C20:3 to form C20:4. The increase in DES gene expression observed in the high light treatment may have been due to this reaction, not the desaturation of C20:4 to form C20:5. Irradiance can alter the fatty acid composition of microalgae (reviewed by Hu et al. 2008). High levels of

polyunsaturated fatty acids, which are typically incorporated into membrane lipids, are produced under low light intensities (reviewed by Hu et al. 2008). In this study, polyunsaturated fatty acids content was significantly higher in the low light cultures compared to the high light cultures. This high level of polyunsaturated fatty acids may have contributed to the low biodiesel quality of the low light. Hu et al. (2008) also found that saturated and monounsaturated fatty acids are predominantly synthesized under high light intensities. This is consistent with the results of this study, in which total monounsaturated fatty acids as a proportion of total fatty acid concentration was significantly higher in the algae grown under high light compared to the low light treatment. The higher monounsaturated fatty acid content contributed to the high quality of the biodiesel from the high light cultures.

4.6 Conclusion

In this chapter, the effects of low, medium and high light intensity on the carbon partitioning and the biodiesel potential of *H. akashiwo* were assessed. *H. akashiwo* was not limited in growth by the low light intensity because there were no differences in growth rate among the light intensities. However, each light treatment culture partitioned carbon differently. Carbohydrate content increased under the high light treatment, while lipid content was highest under the low light treatment. Furthermore, cultures grown under high light increased saturated fatty acid content while cultures grown under low light increased PUFA content.

The apparent link between ACP and KAS gene expression and C16:0 should be explored further because, due to its saturation and long chain length, C16:0 is a good fatty acid for biodiesel. C16:0 was the most abundant fatty acid in all light treatments and accounted for 24.5% of total fatty acid content in high light treatment algae, which was significantly more than the C16:0 content of the low and medium light treatments. Biodiesel from the high light treatment was calculated to have a cetane number of 43, which was the highest of all light treatments.

Table 4.1: Growth rate and cellular carbon and nitrogen (*mol/mol) of *H. akashiwo* at low light (160 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$), medium light (560 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$), high light (1200 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$). Data are expressed as means \pm standard deviation, n= 4. Different letters denote significant differences among treatments using a one-way ANOVA followed by a Tukey's HSD means comparison test ($p < 0.05$).

	Low	Medium	High
Growth rate (day^{-1})	0.20 \pm 0.04 (a)	0.22 \pm 0.04 (a)	0.18 \pm 0.02 (a)
Doubling time (days)	3.5 \pm 0.6 (a)	3.3 \pm 0.5 (a)	4.0 \pm 0.4 (a)
C/N*	5.48 \pm 0.06 (a)	6.0 \pm 0.6 (a b)	6.3 \pm 0.2 (b)

Table 4.2: Biodiesel quality of oil derived from *H. akashiwo* under light treatment conditions (expressed as means \pm standard deviation, n= 4)

	Low	Medium	High
Iodine Number	172 \pm 18	148 \pm 24	131 \pm 11
Saponification Number	204 \pm 2	207 \pm 3	210 \pm 1
Cetane Number	34 \pm 4	39 \pm 5	43 \pm 2
Cetane Number without 20:5(n3)	55 \pm 1	57 \pm 2	58 \pm 2

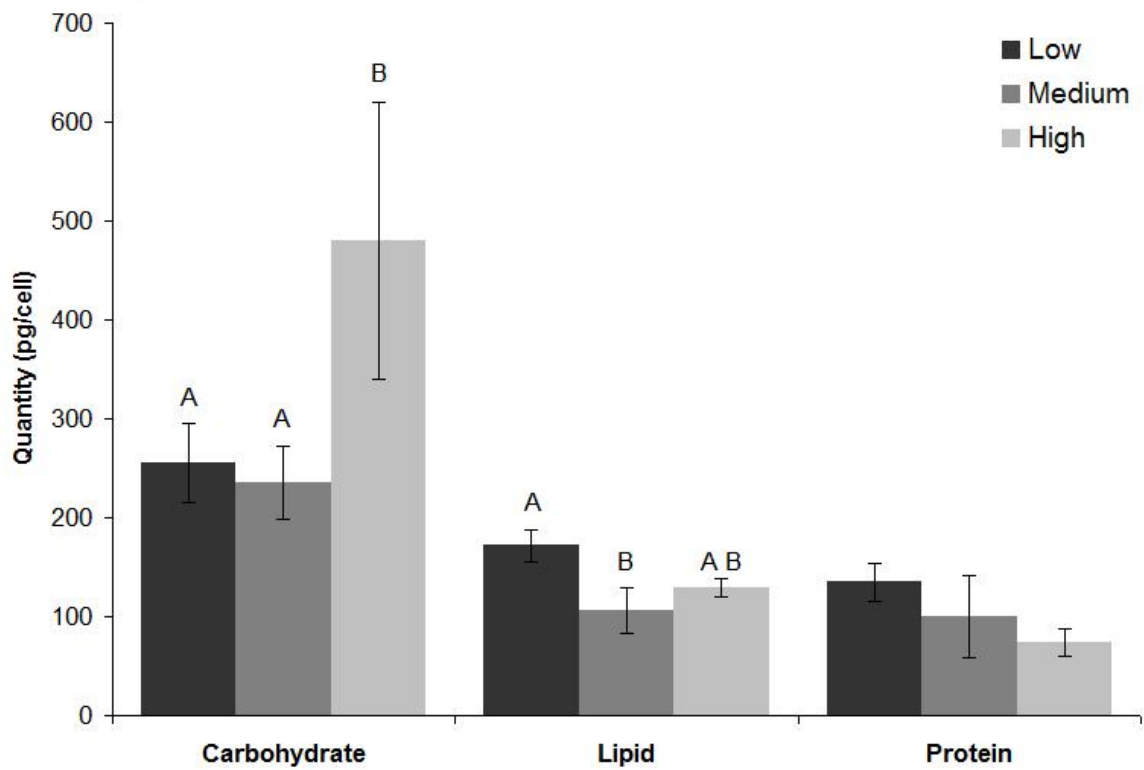


Figure 4.1: Effect of light intensity on carbohydrate, lipid and protein content of *H. akashiwo*. Low: $160 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$, medium: $560 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$, high: $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$. Data are expressed as means and error bars represent one standard deviation ($n=4$). Different letters denote significant differences between groups using Tukey's HSD means comparison test ($p<0.05$). There were no significant differences in protein quantity among light treatments.

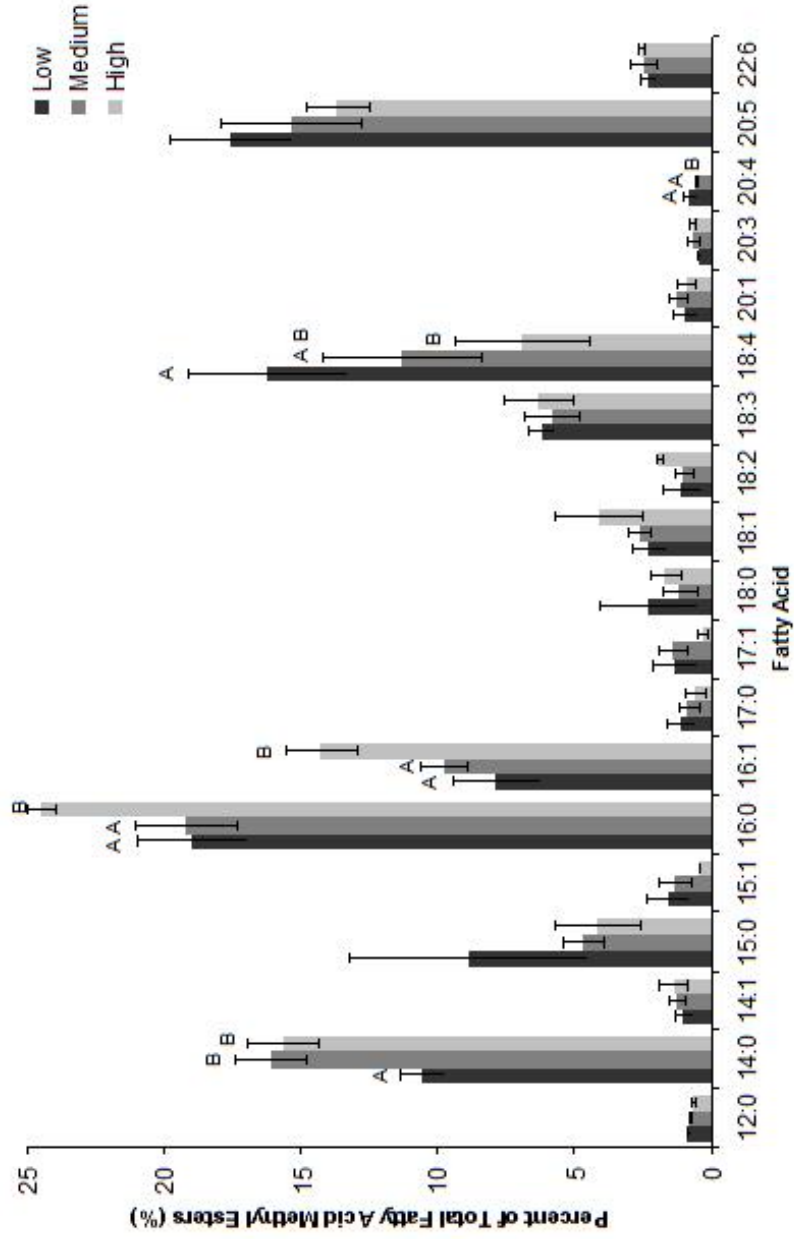


Figure 4.2: Fatty acid profile of *H. akashiwo* under three light treatments (Low, Medium, High). Data are expressed as means and error bars represent one standard deviation (n=4). Different letters denote significant differences between treatments using Tukeys HSD means comparison test ($p < 0.01$). A lack of letters indicates that there was no significant difference among treatments.

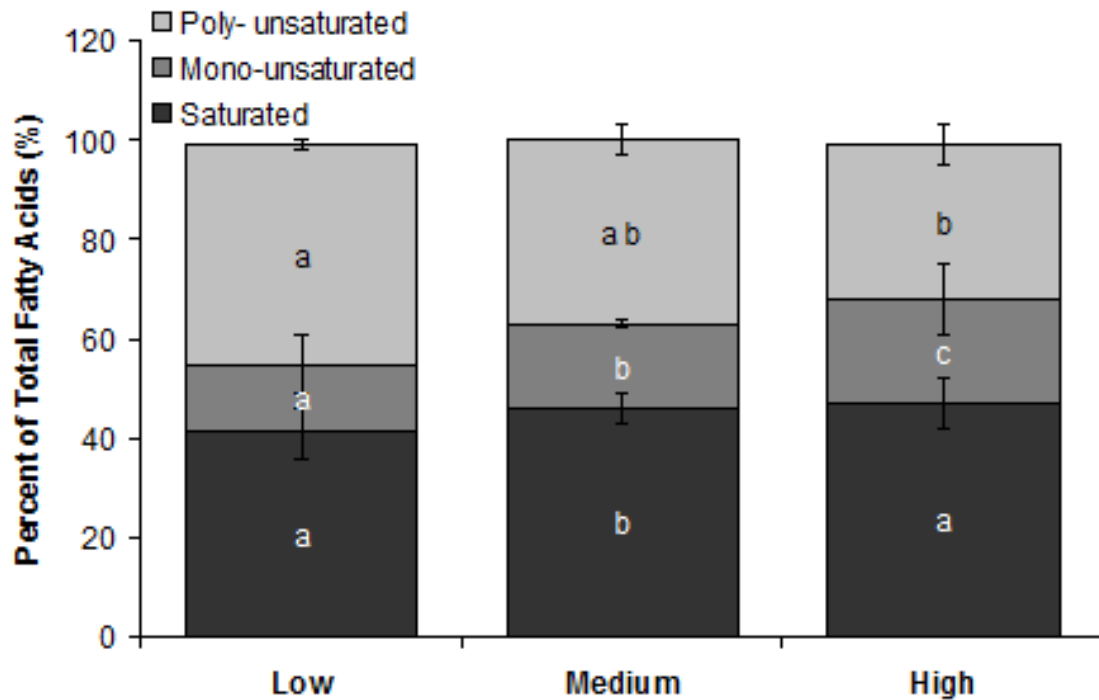


Figure 4.3: Fatty acid type as a percent of total fatty acid content in *H. akashiwo* under gas treatment at low, medium and high light intensities (expressed as means \pm standard deviation, n= 4). Different letters denote significant differences between groups using a one-way ANOVA ($p < 0.05$).

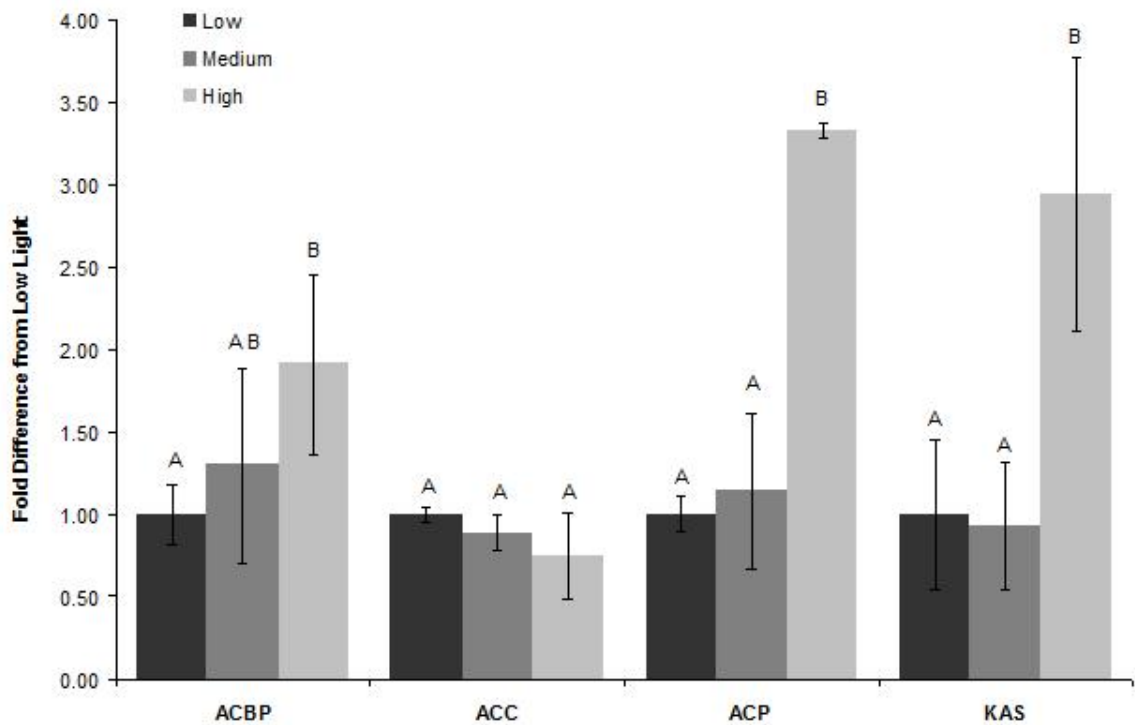


Figure 4.4: Effects of light level (Low, Medium, High) on the mRNA abundance of four key genes involved in fatty acid synthesis. ACBP: acyl CoA-binding protein, ACC: acetyl-CoA carboxylase, ACP: acyl carrier protein, KAS: beta-ketoacyl-ACP synthase. Gene expression was normalized to 18S rRNA abundance and relative to expression of each gene in the low light treatment. Data are expressed as means and error bars represent one standard deviation (n=4). Different letters denote significant differences between treatments ($p < 0.01$).

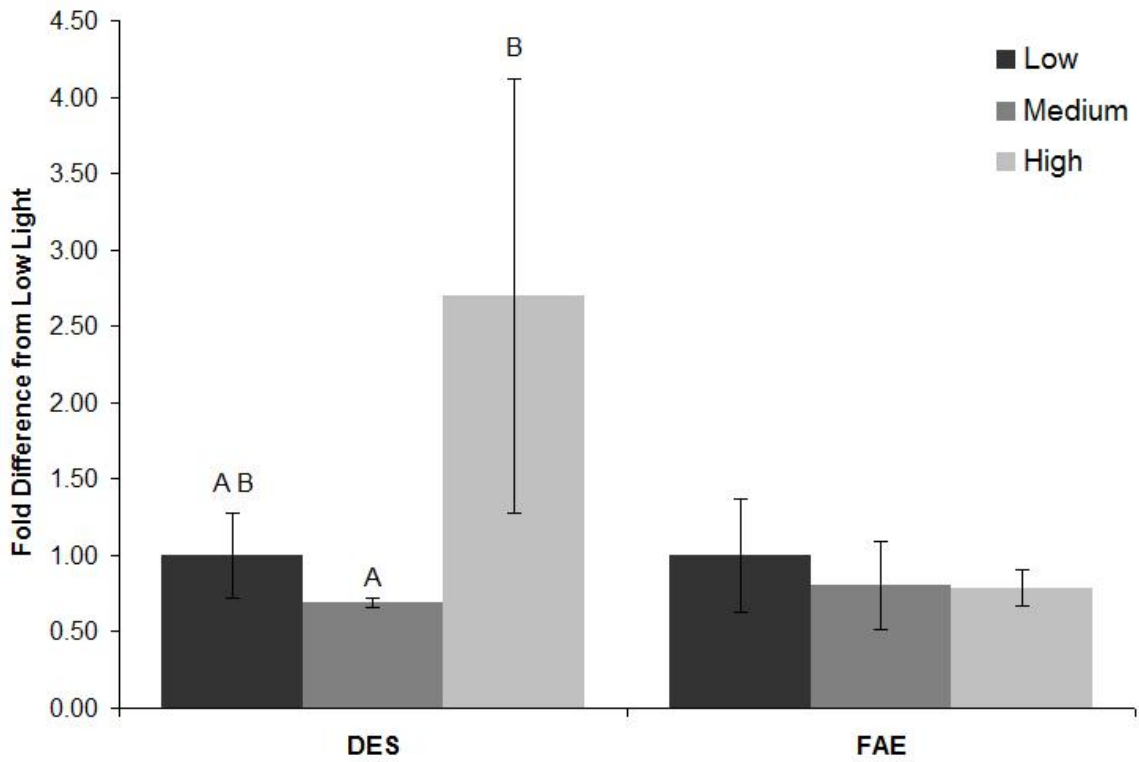


Figure 4.5: Effects of light level on $\Delta 5$ desaturase (DES) and $\Delta 6$ elongase (FAE) mRNA abundance. Gene expression was normalized to 18S rRNA abundance and relative to expression of each gene in the low light treatment. Data are expressed as means and error bars represent one standard deviation (n=4). Different letters denote significant differences between treatments ($p < 0.05$). There were no significant differences in FAE among light treatments.

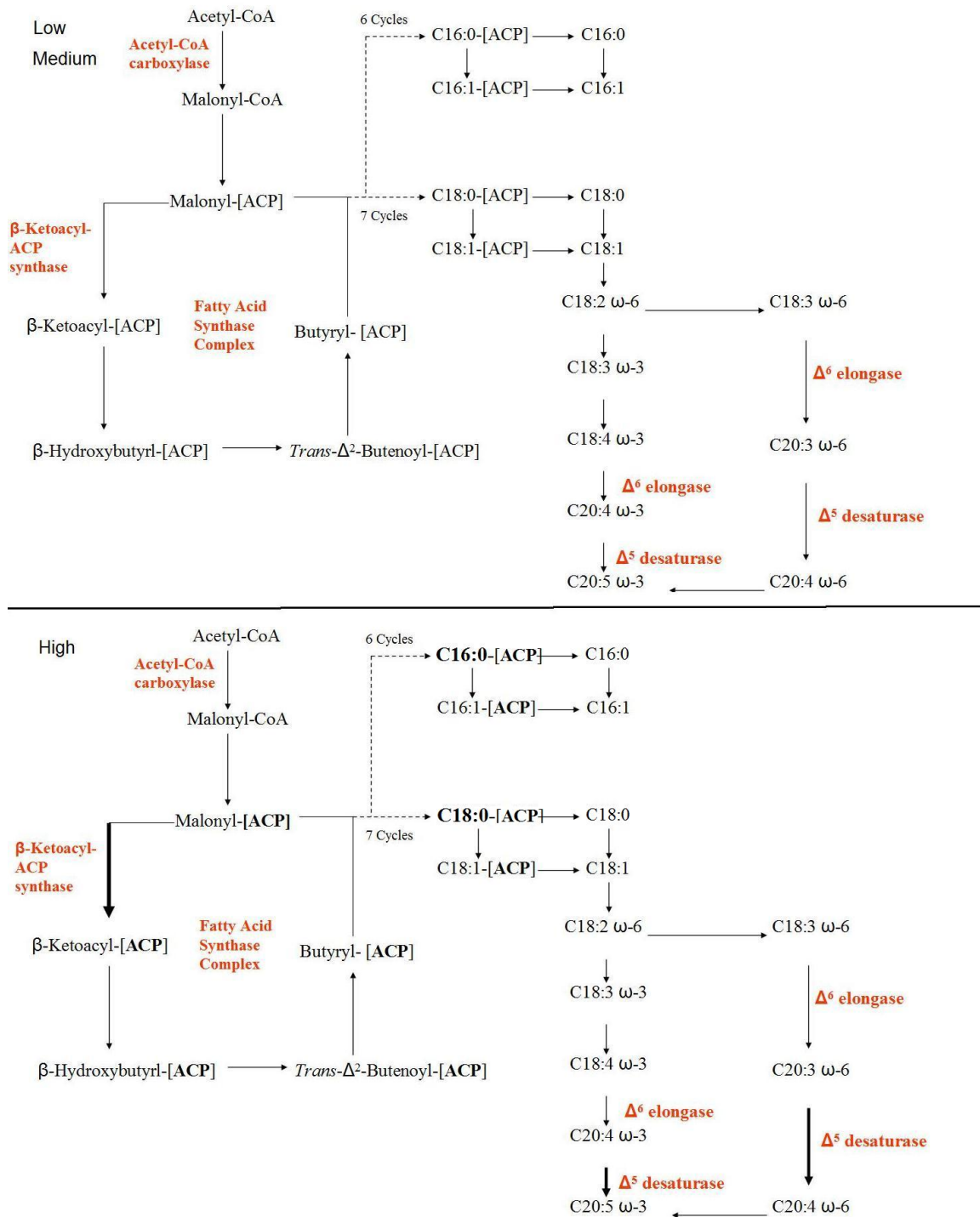


Figure 4.6: Summary of differences in gene expression between culture grown on high light (High) and culture grown on low (Low) and medium (Medium) light. An increase in C16:0 content may be caused by an increase in ACP gene expression (Bold). When up-regulated, KAS may increase C16:0 and C18:0 content. In the culture grown under high light, DES may have converted C20:3 to C20:4. Modified from: Rismani-Yazdi et al. (2012) and Wen et al. (2003).

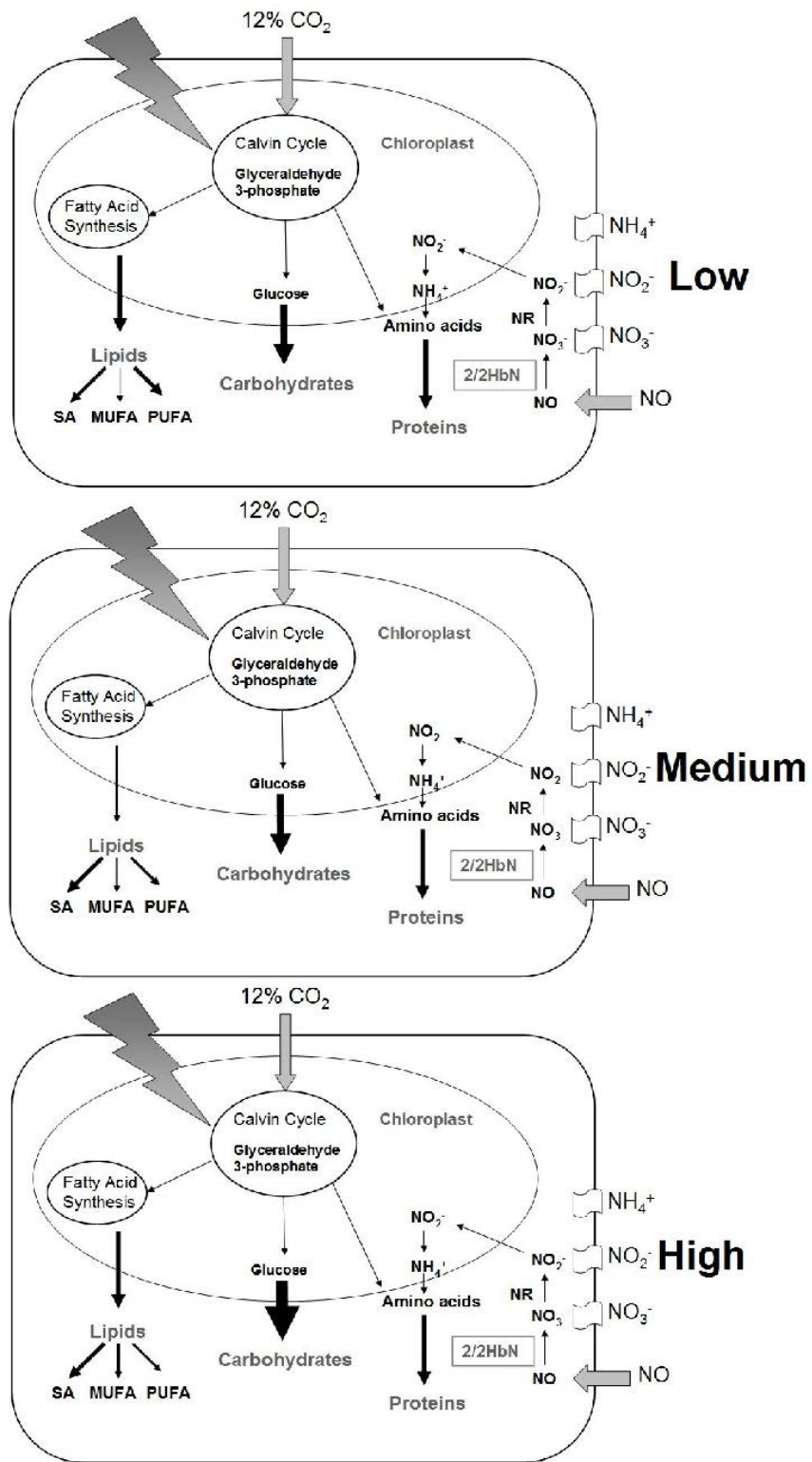


Figure 4.7: Effects of light intensity on carbon partitioning in *H. akashiwo*. SA: saturate; MUFA: monounsaturated; PUFA: polyunsaturated fatty acid. Arrows indicate content that was significantly different among treatments. The heavier the arrow, the more abundant the molecule.

4.7 References

Baud, S., Guyon, V., Kronenberger, J., Wuilleme, S., Miquel, M., Caboche, M., Lepiniec, L. Rochat, C. 2003, "Multifunctional acetyl-CoA carboxylase 1 is essential for very long chain fatty acid elongation and embryo development in Arabidopsis", *Plant Journal*, vol. 33, no. 1, pp. 75-86.

Chisti, Y. 2008, "Biodiesel from microalgae beats bioethanol", *Trends in biotechnology*, vol. 26, no. 3, pp. 126-131.

Ciubota-Rosie, C., Ramon Ruiz, J., Jesus Ramos, M. Perez, A. 2013, "Biodiesel from *Camelina sativa*: A comprehensive characterisation", *Fuel*, vol. 105, pp. 572-577.

Fuentes-Grünewald, C., Garces, E., Alacid, E., Rossi, S. Camp, J. 2013, "Biomass and Lipid Production of Dinoflagellates and Raphidophytes in Indoor and Outdoor Photobioreactors", *Marine Biotechnology*, vol. 15, no. 1, pp. 37-47.

Fuentes-Grünewald, C., Garces, 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*, vol. 39, no. 1, pp. 207-216.

Halim, R., Danquah, M.K. Webley, P.A. 2012, "Extraction of oil from microalgae for biodiesel production: A review", *Biotechnology Advances*, vol. 30, no. 3, pp. 709-732.

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", *Plant Journal*, vol. 54, no. 4, pp. 621-639.

Lei, A., Chen, H., Shen, G., Hu, Z., Chen, L. Wang, J. 2012, "Expression of fatty acid synthesis genes and fatty acid accumulation in *Haematococcus pluvialis* under different stressors", *Biotechnology for Biofuels*, vol. 5, pp. 18.

Li, Y., Han, D., Sommerfeld, M. Hu, Q. 2011, "Photosynthetic carbon partitioning and lipid production in the oleaginous microalga *Pseudochlorococcum* sp (Chlorophyceae) under nitrogen-limited conditions RID D-2553-2010", *Bioresource technology*, vol. 102, no. 1, pp. 123-129.

Lopes, D.C., Baron Maurer, J.B., Stevan-Hancke, F.R., de Oliveira Proenca, L.A. Zawadzki-Baggio, S.F. 2012, "Chemical analysis of exopolysaccharide fractions and lipid compounds of the microalga *Heterosigma akashiwo* grown in vitro", *Botanica Marina*, vol. 55, no. 6, pp. 565-580.

Marshall, J.A., Nichols, P.D. Hallegraeff, G.M. 2002, "Chemotaxonomic survey of sterols and fatty acids in six marine raphidophyte algae", *Journal of Applied Phycology*, vol. 14, no. 4, pp. 255-265.

Misra, N., Patra, M.C., Panda, P.K., Sukla, L.B. Mishra, B.K. 2013, "Homology modeling and docking studies of FabH (beta-ketoacyl-ACP synthase III) enzyme involved in type II fatty acid biosynthesis of *Chlorella variabilis*: a potential algal feedstock for biofuel production.", *Journal of Biomolecular Structure Dynamics*, vol. 31, no. 3, pp. 241-257.

Nascimento, I.A., Izabel Marques, S.S., Dominguez Cabanelas, I.T., Pereira, S.A., Druzian, J.I., de Souza, C.O., Vich, D.V., de Carvalho, G.C. Nascimento, M.A. 2013, "Screening Microalgae Strains for Biodiesel Production: Lipid Productivity and Estimation of Fuel Quality Based on Fatty Acids Profiles as Selective Criteria", *Bioenergy Research*, vol. 6, no. 1, pp. 1-13.

Radakovits, R., Jinkerson, R.E., Darzins, A. Posewitz, M.C. 2010, "Genetic Engineering of Algae for Enhanced Biofuel Production", *Eukaryotic Cell*, vol. 9, no. 4, pp. 486-501.

Rawat, I., Kumar, R.R., Mutanda, T. Bux, F. 2013, "Biodiesel from microalgae: A critical evaluation from laboratory to large scale production", *Applied Energy*, vol. 103, pp. 444-467.

Rismani-Yazdi, H., Haznedaroglu, B.Z., Hsin, C. Peccia, J. 2012, "Transcriptomic analysis of the oleaginous microalga *Neochloris oleoabundans* reveals metabolic insights into triacylglyceride accumulation", *Biotechnology for Biofuels*, vol. 5, pp. 74.

Roessler, P. 1988, "Effects of Silicon Deficiency on Lipid-Composition and Metabolism in the Diatom *Cyclotella-Cryptica*", *Journal of Phycology*, vol. 24, no. 3, pp. 394-400.

Schwenzfeier, A., Wierenga, P.A. Gruppen, H. 2011, "Isolation and characterization of soluble protein from the green microalgae *Tetraselmis* sp.", *Bioresource technology*, vol. 102, no. 19, pp. 9121-9127.

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*, vol. 77, no. 6, pp. 565-575.

Van den Hende, S., Vervaeren, H. Boon, N. 2012, "Flue gas compounds and microalgae: (Bio-)chemical interactions leading to biotechnological opportunities", *Biotechnology Advances*, vol. 30, no. 6, pp. 1405-1424.

Wen, Z.Y. Chen, F. 2003, "Heterotrophic production of eicosapentaenoic acid by

microalgae”, *Biotechnology Advances*, vol. 21, no. 4, pp. 273-294.

Williams, P.J.I.B. Laurens, L.M.L. 2010, ”Microalgae as biodiesel biomass feedstocks: Review analysis of the biochemistry, energetics economics”, *Energy Environmental Science*, vol. 3, no. 5, pp. 554-590.

Chapter 5

CONCLUSIONS AND FUTURE PERSPECTIVES

This work aimed to evaluate *Heterosigma akashiwo* and *Chattonella subsalsa* as biodiesel feedstocks. Specific objectives were to:

1. Evaluate the long-term impacts of model flue gas on carbon metabolism of *H. akashiwo* and *C. subsalsa* and the ability of *H. akashiwo* to use NO present in flue gas as a nitrogen source
2. Determine the environmental conditions (light intensity and nitrogen stress) that produce the highest quantity of lipids and the highest quality of fatty acids in *H. akashiwo*
3. Explore the transcript abundance of key fatty acid synthesis genes to further understand fatty acid synthesis in microalgae and target candidate genes for metabolic engineering

5.1 Evaluate the long-term impacts of model flue gas on carbon metabolism of *H. akashiwo* and *C. subsalsa* and the ability of *H. akashiwo* to use NO present in flue gas as a nitrogen source

This study determined that both *H. akashiwo* and *C. subsalsa* grew faster when bubbled with flue gas compared to when bubbled with air. The flue gas treatment also caused a shift in carbon partitioning to carbohydrates in both species. This is probably due to the high amount of CO₂ (12%) in the flue gas, which is converted into simple carbohydrates through photosynthesis. Carbohydrate content also significantly

increased into the stationary phase for both species. The mechanism by which this occurs warrants further investigation. Furthermore, this significant accumulation of carbohydrates indicates that, when grown on flue gas, *H. akashiwo* and *C. subsalsa* may be candidates for bioethanol production. In the future, the bioethanol feedstock of both species should be investigated. This investigation should include carbohydrate profiling because carbohydrates are used to produce bioethanol.

On a per cell basis, *C. subsalsa* had a higher lipid content than *H. akashiwo*, which indicates that *C. subsalsa* may be a better biodiesel feedstock. The highest lipid content in all the treatments investigated in this study was obtained when *C. subsalsa* was grown on air. The culture bubbled with air also had the best fatty acid profile when it was in stationary growth. In *C. subsalsa*, saturated fatty acid content as a percent of total fatty acid content was higher during the stationary phase compared to the exponential phase. Because saturated fatty acids are ideal for biodiesel, this increase in the stationary phase makes the air cultures when bubbled with air, the best biodiesel candidate of the treatments investigated in *C. subsalsa*.

Similar to *C. subsalsa*, the major fatty acid in *H. akashiwo* was palmitic (C16:0). The most notable change with culture age was during the stationary phase, when the air cultures had a sharp decrease in polyunsaturated fatty acids (PUFA) accompanied by an increase in monounsaturated fatty acids (MUFA). Similar to *C. subsalsa*, *H. akashiwo* cultures bubbled with air and in stationary phase had the best fatty acid profiles for biodiesel production.

Regardless of treatment, both species contained a high eicosapentaenoic (C20:5, EPA) content. C20:5 significantly decreases biodiesel quality due to its high degree of unsaturation. If C20:5 were removed prior to biodiesel production, the quality of the biodiesel significantly increases to between 48-63, which is above the US standard of 47.

The ability of *H. akashiwo* to use nitric oxide (NO) in model flue gas as a nitrogen source was also evaluated. Cultures grown with the flue gas and no added nitrate achieved the same growth rate as the cultures grown on air with 880 μ M nitrate

and reached higher cell density than no nitrate added cultures when grown on air. This suggests that NO supplied in the flue gas was used as a nitrogen source. Additionally, the concentration of the nitrogen rich molecules, protein and chl *a*, in exponential phase cultures grown with flue gas without added nitrate was not significantly different from cultures grown with 220 μM nitrate added.

5.2 Determine the environmental conditions (light intensity and nitrogen stress) that produce the highest quantity of lipids and the highest quality of fatty acids in *H. akashiwo*

It was determined that light intensities did not significantly affect the growth rate of *H. akashiwo*. These equal growth rates demonstrate the commercial viability of large-scale production of biodiesel from *H. akashiwo* in open raceway ponds. During large-scale production of algal biomass, light limitation due to high culture densities causes low algal productivity. However, our study indicates that *H. akashiwo* can be productive at the pond surface where the irradiance is very high as well as below the surface where the irradiance is low.

However, *H. akashiwo* had the best fatty acid profile when grown under high light. When grown under high light, the cultures had significantly more C16:0 content compared to when grown under low and medium light, which contributed to its higher cetane number due to this fatty acid's chain length and degree of unsaturation.

Nitrate concentration did not have a significant effect on biodiesel quality of *H. akashiwo*. This may be because the FAME profiles of *H. akashiwo* were not altered drastically enough by nitrate concentration to alter the quality of the biodiesel. Overall, the highest predicted cetane number was obtained from the 220 and 880 μM nitrate air cultures in the stationary phase despite the observation that the 0 μM nitrate culture produced the highest lipid concentration.

Overall, *H. akashiwo* should be bubbled on flue gas and processed for biodiesel during the stationary phase. Flue gas should be used because it increased the growth rate of *H. akashiwo* 2.3- fold. Flue gas can also be a nitrogen source for *H. akashiwo*.

This study has established that *H. akashiwo* generally decreases PUFA content in stationary phase, which is beneficial for biodiesel. Lastly, because *H. akashiwo* was not light limited or photoinhibited in this study, this species should be able to thrive in an open race way pond.

5.3 Understand fatty acid synthesis in microalgae and target candidate genes for metabolic engineering

Metabolic engineering is believed to be an effective solution to the high production cost of microalgal biodiesel and may be key to obtaining a favorable fatty acid composition. Although the lipid metabolic pathway of higher plants has been characterized extensively, the process in microalgae has yet to be thoroughly investigated. For each experiment, the gene transcript abundance of six proteins involved in fatty acid synthesis was investigated. Figure 5.1 summarized the changes in gene expression for these proteins.

Acetyl CoA carboxylase (ACC):

Generally, the activity of acetyl CoA carboxylase (ACC) is considered to be highly regulated and to be a major determinant of the overall rate of fatty acid synthesis. ACC catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, the first committed step in fatty acid synthesis. Throughout this study, ACC transcript abundance was low. The only significant difference in ACC transcript abundance was when the effects of flue gas and culture age were being evaluated. For *H. akashiwo*, the flue gas cultures had significantly more ACC transcript abundance during the stationary phase compared to the exponential phase. Meanwhile, in *C. subsalsa*, the air cultures had significantly more ACC transcript abundances compared to the flue gas treatment. In *C. subsalsa*, this increased ACC gene expression may be correlated to the higher lipid content observed in the air cultures. However, an increase in lipid content was not observed in the exponential phase of the flue gas culture of *H. akashiwo*. This may be due to the ACC subunit that was targeted. ACC contains three subunits:

biotin carboxylase, biotin carboxyl carrier protein and carboxyl transferase. The *C. subsalsa* primer pair targeted the carboxyl transferase subunit while the *H. akashiwo* primer pair targeted the biotin carboxyl carrier protein. Furthermore, Lei et al. (2012) targeted the biotin carboxylase subunit of ACC in *Haematococcus pluvialis* and determined that ACC transcript abundance was highly correlated to C12:0 concentration. In the future, ACC primers for *H. akashiwo* should be designed to target the biotin carboxylase or the carboxyl transferase subunit.

Acyl carrier protein (ACP):

Following the reaction involving ACC, fatty acid synthesis from malonyl-CoA occurs through a series of reactions catalyzed by multiple enzymes. The malonyl group is transferred from malonyl-CoA to the thiol group of acyl carrier protein (ACP) by malonyl-CoA-ACP transferase (MCT) to form malonyl-ACP. Through out fatty acid synthesis, ACP carries the acyl groups of the fatty acid intermediates through a thioester linkage.

The *H. akashiwo* cultures grown under high light had significantly more ACP gene expression compared to the low and medium light treatments. Because ACP is present throughout the fatty acid cycle, it is difficult to pin point a specific result of this increase. Total lipid content of the high light treatment did not differ from the other treatments. However, the high light treatment has significantly more C16:0 than the other treatments. C16:0 one of the final products of the fatty acid synthase cycle. High levels of C16:0 and ACP gene expression may indicate that the fatty acid synthase cycle was up regulated when *H. akashiwo* was grown under the high light intensity.

When grown with 880 μM nitrate, *H. akashiwo* had significantly less ACP gene expression during the exponential phase compared to the other treatments. The 880 μM nitrate treatment also had less C16:0 compared to the other treatments. This agrees with the observation made with the high light cultures: less C16:0 and less ACP gene expression may indicate that the fatty acid synthase cycle was down regulated under nitrate replete conditions (880 μM nitrate).

Malonyl-CoA-ACP transferase (MCT):

Following the reaction involving ACC, malonyl-CoA-ACP transferase (MCT) transfers the malonyl group from malonyl-CoA to the thiol group of acyl carrier protein (ACP) to form malonyl-ACP. MCT gene abundance was only measured in *C. subsalsa*. MCT transcript abundance was up-regulated during the exponential phase when *C. subsalsa* was bubbled with flue gas. While the formation of malonyl-ACP adds a two-carbon acetyl unit to the growing fatty acyl chain and initiates the fatty acid synthase cycle, there was no increase in C16:0 or C18:0 abundance. However, C16:0 and C18:0 are converted into long chain fatty acids and polyunsaturated fatty acids (PUFA) through a series of desaturation and elongation steps. During exponential phase, flue gas culture had a high abundance of C18:3, which is the desaturated product of C18:0. This indicates that the results of the up-regulated MCT during the exponential phase when *C. subsalsa* was bubbled with flue gas, may be seen downstream, in the high abundance of C18:3.

Ketoacyl-ACP synthase (KAS):

The formation of malonyl-ACP adds a two-carbon acetyl unit to the growing fatty acyl chain. This step is followed by six or seven cycles of condensation, reduction, dehydration and another reduction to produce C16:0 or C18:0, respectively. These reactions are catalyzed by β -ketoacyl-ACP synthase (KAS), β -ketoacyl-ACP reductase, β -hydroxyacyl-ACP dehydrase and enoyl-ACP reductase, respectively. KAS is considered a key regulator of fatty acid chain length because it is a condensing enzyme responsible for the two carbon elongations of substrates from C4:0 to C14:0 (Lei et al. 2012). The role of KAS in fatty acid synthesis ceases after C16:0 or C18:0 is produced.

A high KAS gene abundance in *H. akashiwo* grown with flue gas (Chapter Three) coincided with high C16:0 content. Similar to the observation made with ACP, high C16:0 content and high KAS gene expression may indicate that the fatty acid

synthase cycle was up-regulated. The apparent link between ACP and KAS gene expression and C16:0 should be explored further because, due to its saturation and long chain length, C16:0 contributes to higher quality biodiesel.

Δ 6 fatty acid elongase (FAE) and Δ 5 desaturase (DES):

The end products of fatty acid synthesis, C16:0 and C18:0, are converted into long chain fatty acids and polyunsaturated fatty acids (PUFA) through a series of desaturation and elongation steps. Two enzymes, Δ 6 fatty acid elongase (FAE) and Δ 5 desaturase (DES), are responsible for the production of the PUFA, eicosapentaenoic fatty acid (C20:5, EPA). However, it is important to note that neither FAE nor DES is specific for EPA production. For example, DES also desaturates C20:3 to form C20:4 and FAE also elongates C18:3 to form C20:3.

In the light experiment (Chapter Four) DES gene expression was significantly higher under the high light treatment, compared to the other light treatments. However, there was no difference in C20:5 concentration as a percent of total fatty acids. Furthermore, it can be hypothesized that an increase in DES gene expression would increase the percent of PUFA. However, the high light culture had the lowest percentage of PUFA. Long chain fatty acids could have been produced but were not measured in this study.

In Chapter Two, when evaluating the long-term impacts of model flue gas on carbon metabolism of *H. akashiwo*, FAE gene expression was significantly higher in the air controls compared to the flue gas treatment. However, there was no difference in C20:5 content between the gas treatments. Furthermore, C20:4 (a product of FAE) was not detected in the air treatment, indicating that FAE may have worked on a substrate other than C18:4.

Overall, C20:4, which is a product of FAE and a substrate of DES, was not detected in any air cultures and only detected in trace amounts in the flue gas cultures through out this study. This indicates that DES immediately converted C20:4 to C20:5. Another possibility is that C20:5 is produced through the ω -6 pathway via

Δ 17 desaturase. Further investigation into the gene abundance of Δ 17 desaturase is needed to fully understand C20:5 synthesis in *H. akashiwo*.

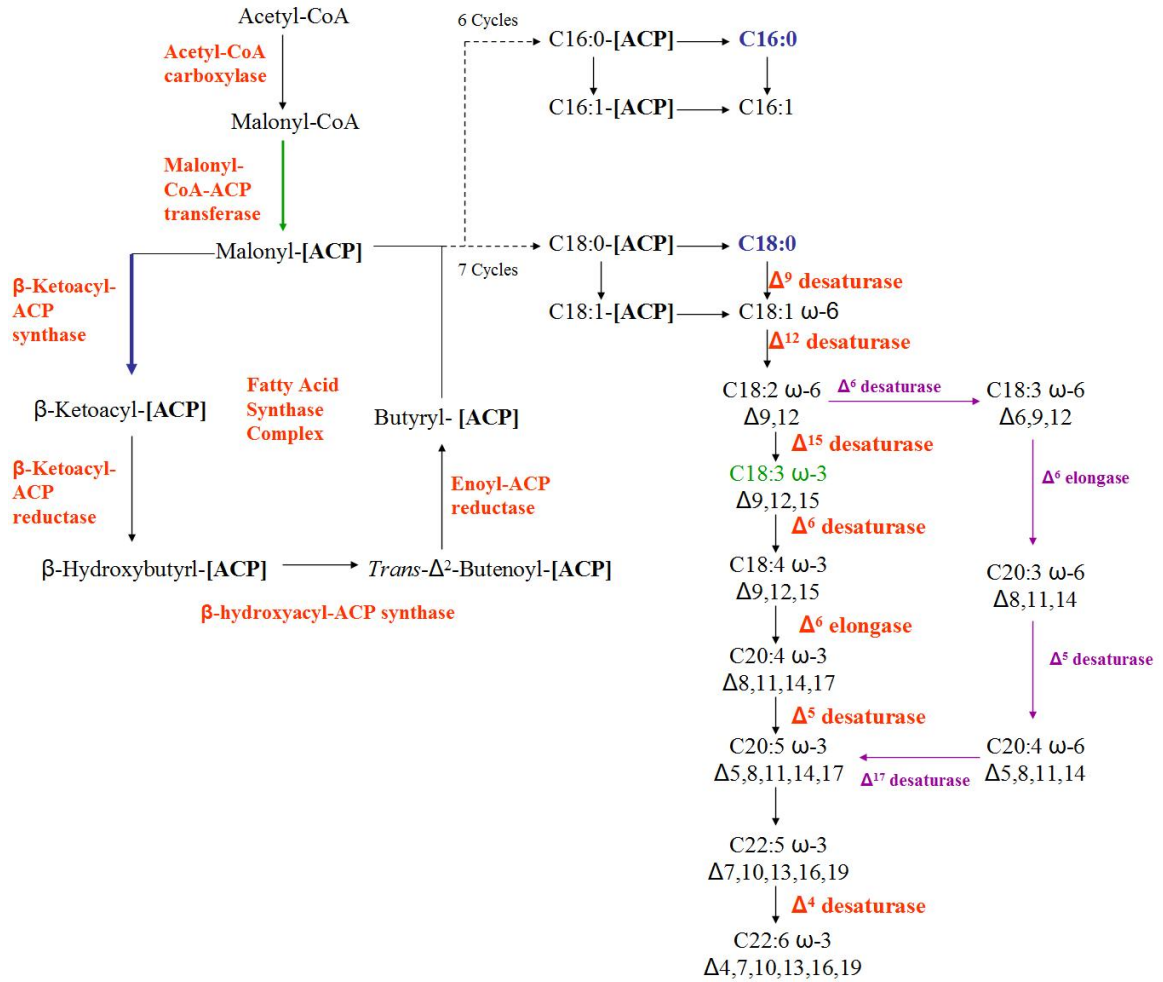


Figure 5.1: Summary of gene expression in the pathway of fatty acid biosynthesis. An increase in C16:0 content may be caused by an increase in ACP gene expression (Bold). When up-regulated, MCT (green arrow) may increase C16:0 and C18:0 content, which can be seen in a increase in C18:3, which is the desaturated product of C18:0. KAS (blue) was up-regulated under nitrogen limitation which may lead to a higher content of C16:0 and C18:0. C20:5 can be produced through the ω -6 pathway (purple) via Δ 17 desaturase, which does not involve DES or FAE. Modified from: Rismani-Yazdi et al. (2012) and Wen et al. (2003).

REFERENCES

- Baud, S., Guyon, V., Kronenberger, J., Wulleme, S., Miquel, M., Caboche, M., Lepiniec, L. Rochat, C. 2003, "Multifunctional acetyl-CoA carboxylase 1 is essential for very long chain fatty acid elongation and embryo development in Arabidopsis", *Plant Journal*, vol. 33, no. 1, pp. 75-86.
- Cheng, Y., Zheng, Y. VanderGheynst, J.S. 2011, "Rapid Quantitative Analysis of Lipids Using a Colorimetric Method in a Microplate Format", *Lipids*, vol. 46, no. 1, pp. 95-103.
- Chisti, Y. 2008, "Biodiesel from microalgae beats bioethanol", *Trends in biotechnology*, vol. 26, no. 3, pp. 126-131. Ciubota-Rosie, C., Ramon Ruiz, J., Jesus Ramos, M. Perez, A. 2013, "Biodiesel from *Camelina sativa*: A comprehensive characterisation", *Fuel*, vol. 105, pp. 572-577.
- Coyne, K.J., Handy, S.M., Demir, E., Whereat, E.B., Hutchins, D.A., Portune, K.J., Doblin, M.A. Cary, S.C. 2005, "Improved quantitative real-time PCR assays for enumeration of harmful algal species in field samples using an exogenous DNA reference standard", *Limnology and Oceanography-Methods*, vol. 3, pp. 381-391.
- Dubois, M., Gilles, K., Hamilton, J., Rebers, P. Smith, F. 1956, "Colorimetric Method for Determination of Sugars and Related Substances", *Analytical Chemistry*, vol. 28, no. 3, pp. 350-356.
- Folch, J., Lees, M. Stanley, G.H.S. 1957, "A Simple Method for the Isolation and Purification of Total Lipides from Animal Tissues", *Journal of Biological Chemistry*, vol. 226, no. 1, pp. 497-509.
- Fuentes-Grünewald, C., Garces, E., Alacid, E., Rossi, S. Camp, J. 2013, "Biomass and Lipid Production of Dinoflagellates and Raphidophytes in Indoor and Outdoor Photobioreactors", *Marine Biotechnology*, vol. 15, no. 1, pp. 37-47.
- Fuentes-Grünewald, C., Garces, 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*, vol. 39, no. 1, pp. 207-216.
- Halim, R., Danquah, M.K. Webley, P.A. 2012, ”Extraction of oil from microalgae for biodiesel production: A review”, *Biotechnology Advances*, vol. 30, no. 3, pp. 709-732.
- Hogue, C. 2011, *Clearing Skies*.
- 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”, *Plant Journal*, vol. 54, no. 4, pp. 621-639.
- Hutchins, D.A., Hare, C.E., Weaver, R.S., Zhang, Y., Firme, G.F., DiTullio, G.R., Alm, M.B., Riseman, S.F., Maucher, J.M., Geesey, M.E., Trick, C.G., Smith, G.J., Rue, E.L., Conn, J. Bruland, K.W. 2002, ”Phytoplankton iron limitation in the Humboldt Current and Peru Upwelling”, *Limnology and Oceanography*, vol. 47, no. 4, pp. 997-1011.
- Ichihara, K. Fukubayashi, Y. 2010, ”Preparation of fatty acid methyl esters for gas-liquid chromatography”, *Journal of lipid research*, vol. 51, no. 3, pp. 635-640.
- Lei, A., Chen, H., Shen, G., Hu, Z., Chen, L. Wang, J. 2012, ”Expression of fatty acid synthesis genes and fatty acid accumulation in *Haematococcus pluvialis* under different stressors”, *Biotechnology for Biofuels*, vol. 5, pp. 18.
- Li, Y., Han, D., Sommerfeld, M. Hu, Q. 2011, ”Photosynthetic carbon partitioning and lipid production in the oleaginous microalga *Pseudochlorococcum* sp (Chlorophyceae) under nitrogen-limited conditions RID D-2553-2010”, *Biore-source technology*, vol. 102, no. 1, pp. 123-129.
- Lopes, D.C., Baron Maurer, J.B., Stevan-Hancke, F.R., de Oliveira Proenca, L.A. Zawadzki-Baggio, S.F. 2012, ”Chemical analysis of exopolysaccharide fractions and lipid compounds of the microalga *Heterosigma akashiwo* grown in vitro”, *Botanica Marina*, vol. 55, no. 6, pp. 565-580.
- Marshall, J.A., Nichols, P.D. Hallegraeff, G.M. 2002, ”Chemotaxonomic survey of sterols and fatty acids in six marine raphidophyte algae”, *Journal of Applied Phycology*, vol. 14, no. 4, pp. 255-265.
- Misra, N., Patra, M.C., Panda, P.K., Sukla, L.B. Mishra, B.K. 2013, ”Homology modeling and docking studies of FabH (beta-ketoacyl-ACP synthase III) enzyme involved in type II fatty acid biosynthesis of *Chlorella variabilis*: a potential algal

- feedstock for biofuel production.”, *Journal of Biomolecular Structure Dynamics*, vol. 31, no. 3, pp. 241-257.
- Nascimento, I.A., Izabel Marques, S.S., Dominguez Cabanelas, I.T., Pereira, S.A., Druzian, J.I., de Souza, C.O., Vich, D.V., de Carvalho, G.C. Nascimento, M.A. 2013, ”Screening Microalgae Strains for Biodiesel Production: Lipid Productivity and Estimation of Fuel Quality Based on Fatty Acids Profiles as Selective Criteria”, *Bioenergy Research*, vol. 6, no. 1, pp. 1-13.
- Radakovits, R., Jinkerson, R.E., Darzins, A. Posewitz, M.C. 2010, ”Genetic Engineering of Algae for Enhanced Biofuel Production”, *Eukaryotic Cell*, vol. 9, no. 4, pp. 486-501.
- Rawat, I., Kumar, R.R., Mutanda, T. Bux, F. 2013, ”Biodiesel from microalgae: A critical evaluation from laboratory to large scale production”, *Applied Energy*, vol. 103, pp. 444-467.
- Rismani-Yazdi, H., Haznedaroglu, B.Z., Hsin, C. Peccia, J. 2012, ”Transcriptomic analysis of the oleaginous microalga *Neochloris oleoabundans* reveals metabolic insights into triacylglyceride accumulation”, *Biotechnology for Biofuels*, vol. 5, pp. 74.
- Roessler, P. 1988, ”Effects of Silicon Deficiency on Lipid-Composition and Metabolism in the Diatom *Cyclotella-Cryptica*”, *Journal of Phycology*, vol. 24, no. 3, pp. 394-400.
- Schwenzfeier, A., Wierenga, P.A. Gruppen, H. 2011, ”Isolation and characterization of soluble protein from the green microalgae *Tetraselmis* sp.”, *Bioresource technology*, vol. 102, no. 19, pp. 9121-9127.
- Sharp, J.H., Beuregard, A.Y., Burdige, D., Cauwet, G., Curless, S.E., Lauck, R., Nagel, K., Ogawa, H., Parker, A.E., Primm, O., Pujo-Pay, A., Savidge, W.B., Seitzinger, S., Spyres, G. Styles, R. 2004, ”A direct instrument comparison for measurement of total dissolved nitrogen in seawater”, *Marine Chemistry*, vol. 84, no. 3-4, pp. 181-193.
- 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*, vol. 77, no. 6, pp. 565-575.
- Van den Hende, S., Vervaeren, H. Boon, N. 2012, ”Flue gas compounds and microalgae: (Bio-)chemical interactions leading to biotechnological opportunities”, *Biotechnology Advances*, vol. 30, no. 6, pp. 1405-1424.

Wen, Z.Y. Chen, F. 2003, "Heterotrophic production of eicosapentaenoic acid by microalgae", *Biotechnology Advances*, vol. 21, no. 4, pp. 273-294.

Williams, P.J.I.B. Laurens, L.M.L. 2010, "Microalgae as biodiesel biomass feedstocks: Review analysis of the biochemistry, energetics economics", *Energy Environmental Science*, vol. 3, no. 5, pp. 554-590.

Appendix A

MATERIALS AND METHODS

A.1 Isolates and culture conditions

Heterosigma akashiwo (CCMP 2393) and *Chattonella subsalsa* (CCMP 2191) were originally isolated from Torquay Canal and Love Creek, respectively, in Rehoboth Beach, Delaware. Both cultures were deposited in the National Center for Marine Algae and Microbiota (formerly the Center for the Culture of Marine Phytoplankton). Stock cultures were maintained at 25°C in f/2 medium, made with filtered and autoclaved 20 psu seawater. Stock cultures had an irradiance of 100 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ provided by cool white fluorescent lamps on a 12:12 light dark cycle.

A.2 Dry weight

To determine dry weight, duplicate samples of each culture were filtered onto pre-combusted and pre-weighed glass filters (Whatman GF/F 25 mm). Filters were dried at 90°C for 5 hours then weighed on a Sartorius SE balance (Data Weighing Systems, Illinois).

A.3 Growth rate

Growth rates were determined during the exponential phase of all experiments. Cells were fixed using a 2% glutaraldehyde solution and counted using a hemocytometer. Growth rates were calculated using the following equation:

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

where N_1 and N_2 are cell concentrations at time 1 (t_1) and time 2 (t_2), respectively.

A.4 Chlorophyll *a*

Chlorophyll *a* (Chl *a*) was measured by filtering duplicate samples of each culture onto 25 mm Whatman GF/F glass filters and extracted in 10 mL of 90% acetone for 12 hours at -20°C. Chl *a* concentration was measured using a Turner 10 AU fluorometer (Turner Designs, USA).

A.5 Particulate carbon and particulate nitrogen

Particulate carbon and particulate nitrogen were quantified using a particulate auto-analyzer (Costech Elemental Analyzer, Costech Analytical Technologies) and standard techniques described by Hutchins et al. (2002). Briefly, 5 mL of algae were filtered onto precombusted GF/F Whatman glass-fiber filters, stored at -80°C then dried in an 80°C oven prior to analysis. Phenylalanine and ethylenediaminetetraacetic acid were used as standards.

A.6 RNA extraction

Prior to RNA extraction, 50 mL of algae were filtered onto a polycarbonate 3 μ M membrane (Millipore, USA). Total RNA was extracted with the RNeasy Kit (Qiagen, USA) according to manufacture's instructions. About one μ g of the extracted RNA was treated with DNase then reverse transcribed with random hexamers using the Superscript III First Strand Synthesis Kit (Life Technologies, USA) according to manufacture's instructions.

A.7 Cloning of fatty acid synthesis genes

The transcriptomes of *H. akashiwo* and *C. subsalsa* along with the NCBI database were used to identify gene sequences of proteins involved in fatty acid synthesis. The sequences identified for *H. akashiwo* were: 3-keto acyl-acyl carrier protein synthase (KAS); Acyl carrier protein (ACP); Acyl-CoA-binding protein (ACBP); Δ 6 fatty acid elongase (FAE); Δ 5 fatty acid desaturase (DES); and acetyl-CoA carboxylase (ACC). The sequences identified for *C. subsalsa* were: ACC, ACP, ACBP, FAE, DES and malonyl-CoA transferase (MCT). Specific primers (Table A.1 and A.2) were designed and gene sequences were amplified by PCR in a 20 μ L reaction containing 125 ng template, reverse transcribed cDNA (1:20 dilution in LoTE), 0.2 mM dNTPs, 0.25 μ M each primer, 2.5 mM MgCl₂, 1 Taq polymerase buffer and 0.1 units Taq Polymerase. The PCR reaction consisted of 2 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C, followed by a 5 min extension at 72°C. The PCR products were

cloned into TOPO II vector using TA Cloning Kit with One Shot TOP 10 chemically competent *E. coli* (Life Technologies, USA). *E. coli* was spread on a LB containing 50 $\mu\text{g} / \text{mL}$ Kanamycin plate and incubated overnight at 37°C. PCR was performed on the colonies using the 10 μM of each primer (M13F/ M13R), using the same reaction conditions as above. The PCR products were evaluated on a 2% agarose gel to ensure that the plasmid contained the correct size insert. The PCR products were then purified with a GenElute PCR Clean-up Kit (Sigma) and sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit and ABI Prism 310 Genetic Analyzer. The colonies used for PCR, were inoculated in LB and incubated overnight at 37°C. Plasmid DNA was extracted using a QIAprep Spin Miniprep Kit (Qiagen, USA).

A.8 Transcript abundance

Gene abundance was evaluated using quantitative reverse transcription real time PCR (QRT-PCR) with an Applied Biosystems 7500 Real Time PCR System. DNA was amplified in triplicate- 10 μL reactions containing 1 μL of 1:20 diluted template cDNA, 5.0 μL SYBR Green I Master Mix (Applied Biosystems, USA), 9 μM of each specific primer (Table A1 and A2) with the following reaction conditions: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at 56 °C, then 72°C for 1 min. The fluorescence signal was measured at the end of each cycle. The dissociation step consisted of one cycle of 15 s at 95°C, 1 min at 60°C, and 15 s at 95°C. Primer concentrations were optimized according to Coyne et al. (2005). Standard curves were prepared using previously cloned plasmid DNA. Gene expression was normalized to 18S rRNA using species- specific primers as described by Coyne et al. (2005).

A.9 Transesterification and analysis of fatty acids

About 15 mL of algal culture were centrifuged for 5 minutes at 4 000 RPM using a swinging bucket rotor centrifuge. Fatty acid methyl esters (FAME) were prepared according to Ichihara and Fukubayashi (2010) with no modifications. Briefly, the lyophilized cell was re-suspended in toluene (0.2 mL), methanol (1.5 ml) and an 8% HCl in methanol solution (0.3 mL). The re-suspended cell solution was incubated at 45°C overnight. FAMES were extracted in 1 mL of hexane and tridecanoic acid (C13:0; final concentration of 0.19 mM) was added as the internal standard. Extracted FAMES were stored at -20 °C until analysis.

FAMES were analyzed using gas chromatography (Hewlett Packard HP 5890 Series) equipped with a Zebron ZB-1 wax (60 m x 0.32 mm x 0.25 μm , Phenomenex) column and a flame ionization detector. Helium was the carrier gas with a flow rate of 1.0 mL/min. The injector and detector temperatures were 190 °C and 250 °C, respectively. A splitless injection was used for 4 μL of FAME samples. The oven temperature for FAME

analysis was initially at 190 °C then increased 15 °C min⁻¹ to 250 °C and then held for 20 min. Peaks were initially identified using gas chromatography- mass spectrometry (HP 5890 GC interfaced to a HP 5972 Mass Spectrometer). Mass spectra of the sample peaks were compared to known spectra in the National Institute of Standards and Technology database. Subsequent sample chromatograms were compared to Supelco 37 Component FAME Mix (Sigma-Aldrich).

A.10 Total lipid, protein and carbohydrate quantification

About 10 mL of algal culture were centrifuged for 5 minutes at 4 000 RPM using a swinging bucket rotor centrifuge. Total lipid content was determined using the colorimetric sulfo-phospho-vanillin (SPV) assay for microalgae as described by Cheng et al. (2010) and optimized during this study for *Heterosigma akashiwo* and *Chattonella subsalsa*. Lipids were extracted from centrifuged algal pellet using the method developed by Folch et al. (1956). Briefly, the frozen algal pellet was homogenized with a 2:1 chloroform-methanol mixture then washed with 0.2 volumes of 0.05 M sodium chloride in deionized water, making a final critical ratio of 2:1:0.8 chloroform-methanol- sodium chloride solution. For the SPV assay, 100 μ L of the lower phase containing the pure lipid extract and corn oil standards containing 5- 160 μ g lipids in chloroform were added directly to a 96- well PCR plate. Methanol was added to each well to obtain a 2:1 chloroform-methanol ratio. The PCR plate was gently heated in a water bath. After the solvent evaporated, 100 μ L of concentrated sulfuric acid was added to each well. Using a thermo cycler, the PCR plate was then incubated at 90°C for 20 min then cooled on ice for 2 min. Equal volumes of samples and standards were transferred to a Costar 96- well polypropylene microplate and background absorbance was read at 540 nm. Vanillin- phosphoric acid reagent (0.2mg/mL vanillin in 17% phosphoric acid) was immediately added to obtain a final vanillin concentration of 0.06 mg/mL. After 5 min of color development, the absorbance was read at 540 nm.

Total proteins were extracted from the centrifuged algal cells by sonicating the cells in 200 mM KPi buffer (dipotassium phosphate and potassium hydroxide) then measured using the BCA Protein Assay Kit (Pierce, Rockford, IL) according to manufacturer instructions (Schwenzfeier et al. 2011). Total carbohydrate content of the algal cells was quantified using the Phenol - Sulfuric Acid colorimetric method described by Dubois et al. (1956) and a standard curve of known glucose concentrations (range 0-3mM). Algal cells were re-suspended in deionized water, then phenol and sulfuric acid were added to give a final concentration of 0.66% and 13.0M, respectively. Samples were incubated in a room temperature water bath for 30 minutes and the absorbance was measured at 482 nm.

A.11 Estimation of biodiesel parameters

The saponification number (SN), iodine number (IV), and cetane number (CN) were estimated using the following equations from Lei et al. (2012).

$$SN = \sum(560xP_i) \div MW_i$$

$$IV = \sum(254xDxP_i) \div MW_i$$

$$CN = (46.3 + 5458) \div (SN - 0.225 \times IV)$$

where P_i is the weight percent of each FAME, MW_i is the molecular weight of each FAME and D is the number of double bonds in each FAME.

Table A.1: Sequences of oligonucleotide primers for *H. akashiwo* real time RT-qPCR

Target Gene	Sequence Name	Sequence (5'-3')
Acyl-CoA-binding protein	HaACBP 3F	AAC GAC CAT AAG CTG GCA CT
	HaACBP 117R	TCC AGG CAT CCC ATT TTG CT
Acetyl-CoA carboxylase	HaACC 283 F	GCA CGA CTG GGC GGT ATT C
	HaACC 363 R	TCC TGG CTG GTG GGA TCG
Acyl carrier protein	HaACP 105F	CAT GAC CAC CTC GAC AGC AT
	HaACP 235R	GCG AGG TCA CGG AGA GAA TC
Δ 5 desaturase	Ha5DES 173F	GTT GAA CAT CAG GAG GGG CA
	Ha5DES 378R	GCT GTG GCT GGT TAA TGC AC
Δ 6 elongase	HaFAE 48F	ACT CGA GGG AAA GCC AGT TG
	HaFAE 176R	ACG GCC TCC ATC TTG AAC AG
3-keto acyl-acyl carrier protein synthase	HaKAS 749F	CCG GTA GAG ACA ACC AC
	HaKAS 860R	AGT TCC GTG AGA AGG CTC TG

Table A.2: Sequences of oligonucleotide primers for *C. subsalsaa* real time RT-qPCR

Target Gene	Sequence Name	Sequence (5'-3')
Acyl-CoA-binding protein	ACBP 90F	TAG GGA GTA ATG GCA GAG CAG
Acyl-CoA-binding protein	ACBP 182R	TTT TGG TGA GTC CCA ACT CCT
Acetyl-CoA carboxylase	ACC 4948F	CTC GGT GGG TAT TGG TGC TT
Acetyl-CoA carboxylase	ACC 5099R	TCT GAG GTC CCC CAA GTT GA
Acyl carrier protein	ACP 952F	CTT TCG TCG CAA CAA CTC CA
Acyl carrier protein	ACP 1023R	AAA CTA ATA CTC CCG CCG AAC T
Δ 5 desaturase	DES 14F	GGC TCC ACA CGA CGG ATT AT
Δ 5 desaturase	DES 236R	CCA ACA ATC GTT TGC GGT GT
Δ 6 elongase	FAE 267F	AAG GTG ATG GCT AAA CGG GA
Δ 6 elongase	FAE 369R	GGA CAA CTC TGA TGA CCC CA
Malonyl- CoA Transferase	MCT 200F	TGG TCC GTC ATC ATG GCA AG
Malonyl- CoA Transferase	MCT 342R	GAC CTC CTC CAC CAC AGA AC

A.12 References

- Cheng, Y., Zheng, Y. VanderGheynst, J.S. 2011, "Rapid Quantitative Analysis of Lipids Using a Colorimetric Method in a Microplate Format", *Lipids*, vol. 46, no. 1, pp. 95-103.
- Ciubota-Rosie, C., Ramon Ruiz, J., Jesus Ramos, M. Perez, A. 2013, "Biodiesel from *Camelina sativa*: A comprehensive characterisation", *Fuel*, vol. 105, pp. 572-577.
- Coyne, K.J., Handy, S.M., Demir, E., Whereat, E.B., Hutchins, D.A., Portune, K.J., Doblin, M.A. Cary, S.C. 2005, "Improved quantitative real-time PCR assays for enumeration of harmful algal species in field samples using an exogenous DNA reference standard", *Limnology and Oceanography-Methods*, vol. 3, pp. 381-391.
- Dubois, M., Gilles, K., Hamilton, J., Rebers, P. Smith, F. 1956, "Colorimetric Method for Determination of Sugars and Related Substances", *Analytical Chemistry*, vol. 28, no. 3, pp. 350-356.
- Folch, J., Lees, M. Stanley, G.H.S. 1957, "A Simple Method for the Isolation and Purification of Total Lipides from Animal Tissues", *Journal of Biological Chemistry*, vol. 226, no. 1, pp. 497-509.
- Hutchins, D.A., Hare, C.E., Weaver, R.S., Zhang, Y., Firme, G.F., DiTullio, G.R., Alm, M.B., Riseman, S.F., Maucher, J.M., Geesey, M.E., Trick, C.G., Smith, G.J., Rue, E.L., Conn, J. Bruland, K.W. 2002, "Phytoplankton iron limitation in the Humboldt Current and Peru Upwelling", *Limnology and Oceanography*, vol. 47, no. 4, pp. 997-1011.
- Ichihara, K. Fukubayashi, Y. 2010, "Preparation of fatty acid methyl esters for gas-liquid chromatography", *Journal of lipid research*, vol. 51, no. 3, pp. 635-640.
- Lei, A., Chen, H., Shen, G., Hu, Z., Chen, L. Wang, J. 2012, "Expression of fatty acid synthesis genes and fatty acid accumulation in *Haematococcus pluvialis* under different stressors", *Biotechnology for Biofuels*, vol. 5, pp. 18.
- Li, Y., Han, D., Sommerfeld, M. Hu, Q. 2011, "Photosynthetic carbon partitioning and lipid production in the oleaginous microalga *Pseudochlorococcum* sp (Chlorophyceae) under nitrogen-limited conditions RID D-2553-2010", *Bioresource technology*, vol. 102, no. 1, pp. 123-129.

Appendix B

SUPPLEMENTAL DATA

B.1 Optimization of Lipid Extraction

To determine the most efficient lipid extraction method, four methods were compared (Table B.1). Lipids were extracted from a *H. akashiwo* culture in stationary growth, when nitrogen was limited. The Bligh Dyer method (Bligh et al. 1959) was performed using the originally specified chloroform/methanol/water ratio of 1:2:0.8. The Folch method (Folch et al. 1956) was performed using the originally specified chloroform/methanol/sodium chloride solution ratio of 2:1:0.8. For the Folch Emulsion method, an emulsion of all solvents in the chloroform/methanol/sodium chloride solution ratio of 2:1:0.8 was used. The Wahlen method (Wahlen et al. 2011) was performed using a chloroform/methanol ratio of 2:1. The algal lipids were quantified using the colorimetric sulfo- phospho- vanillin assay for microalgae as optimized by Cheng et al. (2010). A one- way ANOVA was performed to determine that there is a significant difference between the methods ($p=0.036$). A LSD post hoc test confirmed that the Emulsion Folch method extracted the highest percent of lipids.

Table B.1: A comparison of algal lipid extraction methods. Error represents one standard deviation among replicates (N=2)

Method	% Dry Weight
Bligh Dyer	9.6 ± 0.7
Folch	8.7 ± 0.7
Wahlen	8.2 ± 1.5
Emulsion Folch	15.4 ± 2.8

B.2 Supplemental Data: Chapter Three

B.2.1 Particulate Carbon and Nitrogen

Table B.2: Particulate carbon and nitrogen of *H. akashiwo* during exponential growth expressed as means \pm standard deviation, n= 4. C/N = moles carbon/ moles nitrogen.

	Control		Flue Gas			
Nitrate Concentration	0	220	880	0	220	880
C/N	3.44 ± 0.02	6.5 ± 0.2	9.0 ± 0.2	3.61 ± 0.03	7.3 ± 0.5	10.4 ± 0.5

Table B.3: Particulate carbon and nitrogen of *H. akashiwo* during stationary growth expressed as means \pm standard deviation, n= 4. C/N = moles carbon/ moles nitrogen.

	Control		Flue Gas			
Nitrate Concentration	0	220	880	0	220	880
C/N	ND	5.3 ± 0.3	7.2 ± 0.4	ND	6.6 ± 0.4	9.0 ± 0.4

B.3 Statistics: Chapter Three

B.3.1 Growth Rate

Table B.4: Two- way ANOVA table for growth rate

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Gas	1	0.01	0.01	16.22	0.0008
Nitrate	1	0.06	0.06	79.62	0.0000
Gas:Nitrate	1	0.00	0.00	5.46	0.0313
Residuals	18	0.01	0.00		

B.3.2 Carbon Partitioning

Table B.5: Two- way ANOVA table for carbohydrate content in exponential phase

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Gas	1	174441.52	174441.52	40.06	0.0000
Nitrate	1	38694.53	38694.53	8.89	0.0093
Gas:Nitrate	1	83823.70	83823.70	19.25	0.0005
Residuals	15	65325.11	4355.01		

Table B.6: Two- way ANOVA table for lipid content in exponential phase

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Gas	1	168103.65	168103.65	64.74	0.0000
Nitrate	1	17693.78	17693.78	6.81	0.0177
Gas:Nitrate	1	5382.92	5382.92	2.07	0.1671
Residuals	18	46737.53	2596.53		

Table B.7: Two- way ANOVA table for protein content in exponential phase

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Gas	1	1022.18	1022.18	13.62	0.0017
Nitrate	1	7894.07	7894.07	105.15	0.0000
Gas:Nitrate	1	1318.55	1318.55	17.56	0.0005
Residuals	18	1351.36	75.08		

Table B.8: Two- way ANOVA table for carbohydrate content in stationary phase

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Gas	1	70307.98	70307.98	41.38	0.0000
Nitrate	1	6303.92	6303.92	3.71	0.0732
Gas:Nitrate	1	12748.02	12748.02	7.50	0.0152
Residuals	15	25485.80	1699.05		

Table B.9: Two- way ANOVA table for lipid content in stationary phase

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Gas	1	8473.37	8473.37	9.63	0.0061
Nitrate	1	3105.61	3105.61	3.53	0.0766
Gas:Nitrate	1	6603.62	6603.62	7.51	0.0135
Residuals	18	15837.85	879.88		

Table B.10: Two- way ANOVA table for protein content in stationary phase

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Gas	1	97.34	97.34	4.26	0.0537
Nitrate	1	1786.99	1786.99	78.21	0.0000
Gas:Nitrate	1	676.56	676.56	29.61	0.0000
Residuals	18	411.25	22.85		

B.3.3 Transcript Abundance

Table B.11: Two- way ANOVA table for acyl CoA- binding protein (ACBP) in exponential phase

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Gas	1	0.00	0.00	0.00	0.9694
Nitrate	1	7.61	7.61	13.11	0.0040
Gas:Nitrate	1	0.27	0.27	0.47	0.5066
Residuals	11	6.38	0.58		

Table B.12: Two- way ANOVA table for Acyl carrier protein (ACP) in exponential phase

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Gas	1	0.87	0.87	3.92	0.0676
Nitrate	1	0.33	0.33	1.50	0.2411
Gas:Nitrate	1	0.00	0.00	0.01	0.9321
Residuals	14	3.10	0.22		

Table B.13: Two- way ANOVA table for beta-ketoacyl-ACP synthase (KAS) in exponential phase

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Gas	1	7.19	7.19	14.69	0.0016
Nitrate	1	0.17	0.17	0.34	0.5675
Gas:Nitrate	1	6.49	6.49	13.26	0.0024
Residuals	15	7.34	0.49		

Table B.14: Two- way ANOVA table for Δ 6 elongase (FAE) in exponential phase

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Gas	1	0.05	0.05	0.69	0.4225
Nitrate	1	1.05	1.05	13.95	0.0025
Gas:Nitrate	1	0.21	0.21	2.81	0.1178
Residuals	13	0.98	0.08		

Table B.15: Two- way ANOVA table for Δ 5 desaturase (DES) in exponential phase

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Gas	1	0.08	0.08	0.43	0.5214
Nitrate	1	0.00	0.00	0.00	0.9806
Gas:Nitrate	1	0.39	0.39	1.98	0.1796
Residuals	15	2.92	0.19		

Table B.16: Two- way ANOVA table for acyl CoA- binding protein (ACBP) in stationary phase

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Gas	1	0.05	0.05	3.68	0.0912
Nitrate	1	0.92	0.92	63.26	0.0000
Gas:Nitrate	1	0.10	0.10	7.06	0.0290
Residuals	8	0.12	0.01		

Table B.17: Two- way ANOVA table for beta-ketoacyl-ACP synthase (KAS) in stationary phase

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Gas	1	0.01	0.01	0.15	0.7105
Nitrate	1	0.43	0.43	7.11	0.0372
Gas:Nitrate	1	0.07	0.07	1.20	0.3148
Residuals	6	0.37	0.06		

Table B.18: Two- way ANOVA table for Δ 5 desaturase (DES) in stationary phase

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Gas	1	0.13	0.13	2.73	0.1425
Nitrate	1	0.32	0.32	6.62	0.0368
Gas:Nitrate	1	0.08	0.08	1.72	0.2308
Residuals	7	0.34	0.05		

Table B.19: Two- way ANOVA table for Δ 6 elongase (FAE) in stationary phase

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Gas	1	0.09	0.09	2.81	0.1322
Nitrate	1	0.28	0.28	9.01	0.0170
Gas:Nitrate	1	0.04	0.04	1.41	0.2685
Residuals	8	0.25	0.03		