Reevaluating the Global Warming Potential of Algae-Derived Biofuels: Accounting for Nitrogen

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Abstract

As the world's population and energy demand continue to increase, causing a shortage of fossil fuels, the need for a domestically-sourced, climate-neutral fuel is of growing concern. Across the world, biofuels have been gaining traction as an energy source and are predicted to continue to be a significant component of the world's energy. Algae-derived biofuels are anticipated to be especially promising due to their purported sustainability benefits, which position them as one of the most promising alternative energies under development.

The environmental impacts, specifically greenhouse gas (GHG) emissions, of algae-derived biofuels have been evaluated by life cycle assessment (LCA). We hypothesize that previous LCAs have overlooked a possible key contributor to the overall global warming potential (GWP) of algae-derived biofuels; namely, nitrous oxide (N_2O) emissions during cultivation. Scientific studies have observed significant N_2O emissions from lakes exhibiting eutrophication, causing algal blooms. These lakes can be compared to the shallow, open cultivation ponds used in algae-derived biofuel production, and, as such, failing to account for N_2O emissions during cultivation could lead to an underestimation of GWP. Under the EPA's *Renewable Fuel Standard* (RFS2), life-cycle GHG emissions must be 50% less than the average life-cycle GHG emissions for gasoline or diesel used as transportation fuel in 2005. Because the GWP of N_2O is 298 times greater than that of CO_2 on a per mass basis, even small quantities of N_2O emissions could pose a severe threat to the environment and could possibly prevent approval of an algae-derived biofuel process under RFS2.

The primary objective of this research was to determine if accounting for N_2O production during algae cultivation could substantively change previous LCA-based estimates of the life-cycle GWP. This research was conducted in three parts: (1) thorough examination of existing literature on N_2O emissions from eutrophic lakes similar to algae cultivation ponds, (2) bench-scale experiments for measurement of N_2O emissions during simulated algae cultivation, and (3) integrating literature and laboratory results into an existing LCA framework for algae-derived gasoline.

Eutrophic lakes similar to algae cultivation ponds are estimated to produce fluxes in the range of 7-99 μg $N_2O/m^2/hr$ from portions of the lakes containing dense algae cultures, making eutrophic lakes a significant contributor of N_2O into the atmosphere. Bench-scale experiments simulating algae cultivation under different growth conditions (i.e., air- or CO_2-sparging) were conducted. Higher N_2O emissions were produced when cultivated under a headspace of CO_2, which is anticipated to be the preferred method for commercial algae cultivation. N_2O emissions produced under these conditions were up to 32 μg N_2O/g algae biomass, suggesting that the previously undocumented contribution of N_2O could substantially increase the life-cycle GWP of algae-derived biofuels. Literature and laboratory results were integrated into an existing LCA-based framework which reveals that accounting for N_2O could dramatically increase the life-cycle GWP of algae-derived gasoline by as much as a factor of 10, depending on system boundaries and parameter selection. These results will provide a better understanding of how algae-derived biofuels can contribute to achievement of national energy and climate change goals, as laid out in RFS2.

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1.0 Introduction

Energy is one of the most critical resources of our time. The bulk of energy used across the globe comes from a finite supply of fossil fuels, and in the U.S., energy demand is so high that the population must rely on foreign sources for approximately 30% of its energy needs (EIA, 2012). These fossil fuels are also a primary source of harmful greenhouse gas (GHG) emissions, which significantly contribute to climate change. As the world's population and energy demand continue to increase, causing a shortage of fossil fuels, the need for a domestically-sourced, climate-neutral fuel is of growing concern.

Across the world, biofuels have been gaining traction as a theoretically carbon-neutral and seemingly environmentally preferable alternative to traditional fossil fuels. They are predicted to be a significant component of the world's energy as seen in Figure 1. Ethanol, a biofuel derived from corn and originally thought to be carbon neutral, has faced concerns that its GHG performance is marginal at best. This is largely because ethanol requires more energy to produce than gasoline (EIA, 2012). More recently, algae-derived bioenergy sources have emerged as a promising new alternative.

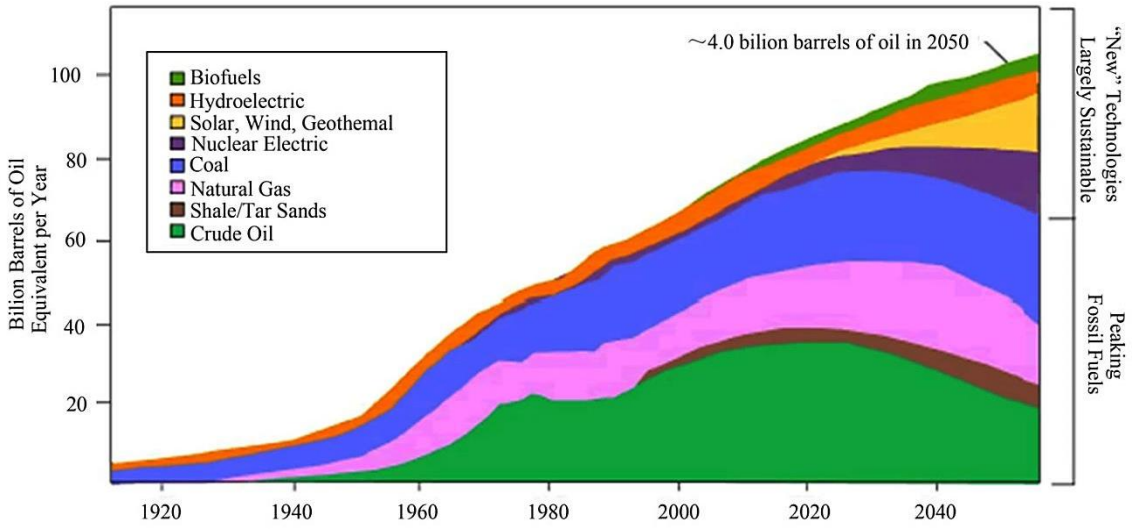

Figure 1: Long-term Energy Sources: The emergence of biofuel technology with the world's increasing demand for energy (Orr, 2006).

The purported sustainability benefits of algae-derived biofuels are tri-fold, with respect to global climate change, land use intensity, and freshwater consumption. First, microalgae have the potential to be a carbon-neutral fuel source. Every gallon of gasoline from fossil fuels produces 8.9 kg of CO_2, which is a known GHG, with no counteracting uptake method (EPA, 2012). In contrast, uptake of CO_2 during photosynthetic growth of algae biomass offsets the emission of GHG, alleviating some of the burden. Second, algae do not compete with agricultural resources that could be otherwise devoted to food production. This is in contrast to biofuel feedstocks that are comprised of major food supplies; e.g., the increased demand for corn to produce ethanol has had severe ramifications for worldwide food prices (Clarens et al., 2010). Microalgae also use less land than other terrestrial crops, such as corn or switchgrass, and can utilize non-arable land (Liu et al., 2012; Peccia et al., 2013). With available farmland acreage

steadily decreasing over the last decade, extensive allocation of an already limited supply is undesirable (Menetrez, 2012; EPA, 2013). Finally, microalgae can grow in salty or brackish water, relieving pressure on already strained freshwater sources (Murphy, 2011; Rogers, 2008). Together, these advantages position an algae-derived biofuel as one of the most promising alternative energies currently under development.

There are, however, concerns about algae-derived biofuels which are similar to those of corn ethanol, mainly whether or not algae biofuels truly provide a net decrease in GHG emissions as compared to gasoline. Recent studies have suggested that current algae-derived biofuels may not perform as well as anticipated, despite all of the purported potential advantages (Clarens et al., 2010**;** Singh and Olsen, 2010). Therefore, it is important to provide a quantitative analysis of the global warming potential (GWP) of the technology before it is implemented.

2.0 Motivation & Significance

The environmental impacts, specifically GHG emissions, of hypothetical algae-derived biofuels have been previously evaluated by life cycle assessment (LCA). Recent results suggest that the GHG emission of algae-derived diesel produced at pilot scale through the process of hydrothermal liquefaction (HTL) is 56 g CO_2 equivalents (eq) per MJ (Liu et al., 2013). The authors proposed that "a shift to algae-derived gasoline could have immediate climate benefits even using existing technologies" (Liu et al., 2013). It should be emphasized that all existing LCAs on algae-derived biofuels are based on theoretical engineering calculations and extrapolation from bench- and pilot-scale studies,

because there are currently very few, if any, full-scale algae cultivation facilities in operation without heavy subsidy.

When determining the overall GWP of each of these production processes, Liu et al. (2012, 2013) and others (Stephenson et al. 2010; Frank et al. 2013) have consistently overlooked a possible key contributor to the overall GWP of algae biofuels produced via HTL; namely, nitrous oxide (N_2O) emissions during algae cultivation. Existing literature documents the production of N_2O in eutrophic lakes, which suggests that it may also be emitted during algae cultivation in large, uncovered outdoor cultivation ponds as shown in Figure 2 (Wang et. al., 2006).

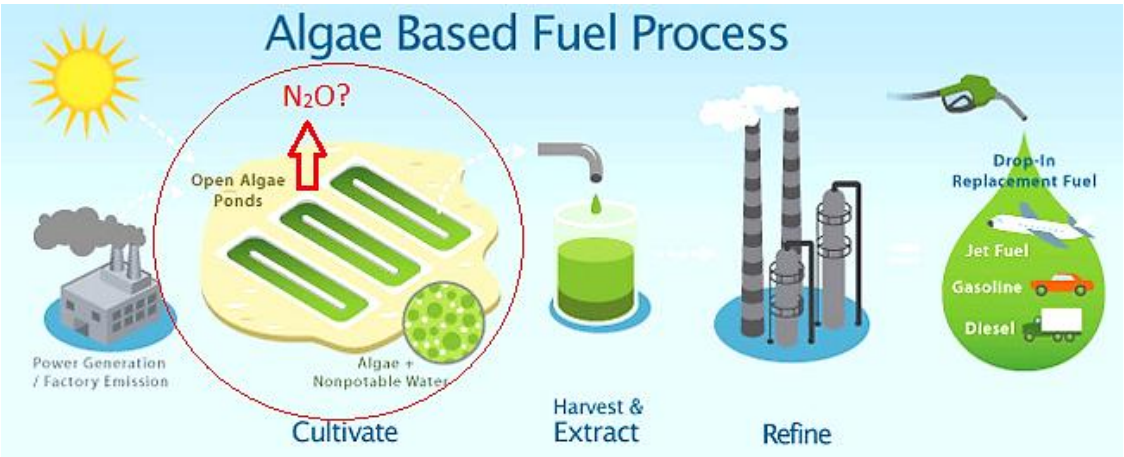

Figure 2: Previous LCAs have overlooked N_2O as a possible key contributor to GWP during production of algae-derived biofuels (Modified from Sapphire Energy Inc., 2010).

In producing biofuels derived from microalgae, it is desirable to maximize the efficiency of nitrogen uptake during algae cultivation in order to minimize the environmental and economic costs of producing chemical fertilizers. It is also desirable

to minimize environmentally damaging nitrogen emissions. Scientific studies have observed significant N_2O emissions from lakes exhibiting severe eutrophication, which occurs when nutrients are released into a lake, causing algal blooms (Wang et al., 2009; Skeffington et al., 1988). The zones of these lakes that are emitting N_2O can be compared to the shallow, open cultivation ponds used in algae-derived biofuel production, such as the ponds shown in Figure 3. As such, failing to account for N_2O emissions during cultivation could lead to a significant underestimation of the GWP arising from production of algae-derived biofuels.

Figure 3: Approximately 100 acre open microalgae cultivation pond, typically comprising mixed communities of microalgae and bacteria, located in New Mexico, USA (Sapphire Energy Inc., 2013).

The Intergovernmental Panel on Climate Change (IPCC) assumes that only 1% of applied nitrogen is converted to N_2O during terrestrial agriculture (Fagerstone et al., 2011). With regard to aquatic emissions, IPCC methodologies do not account for N_2O

production, because complicated spatial variation of N_2O emission limits accurate assessment (Wang et al., 2006; Hashimoto et al., 1993). Still, it has been estimated that aquatic systems account for up to 33% of global anthropogenic N_2O emissions (Wang et al., 2006). Eutrophication (i.e., algae blooms) in lakes contributes to these emissions.

A 2006 study of a hyper-eutrophic lake in China found significant emissions of N_2O, identifying algae as a possible source of these emissions (Wang et al., 2006). Though the report connected algae to N_2O production, it did not directly measure emissions from the algae itself. Figure 4 depicts the aquatic environment of a eutrophic lake, which is similar to that of an algae cultivation pond. Research shows that several classes of microbes are known to produce N_2O as a by-product of nitrogen transformations, such as nitrification and denitrification, as seen by the marked stars in Figure 4 (Bouwman et al., 2013; Wrage et al., 2001). Nitrification occurs under primarily aerobic conditions in two distinct steps. The first stage is oxidation of ammonia (NH_3) to nitrite (NO_2^-) by aerobic ammonia-oxidizing microbes (Robertson and Groffman, 2007; Wrage et al., 2001). The second stage is nitrite oxidation (Wrage et al., 2001). This reaction converts NO_2^- to nitrate (NO_3^-). For complete nitrification, both ammonia oxidation and nitrite oxidation must occur (Durand et al., 2011).

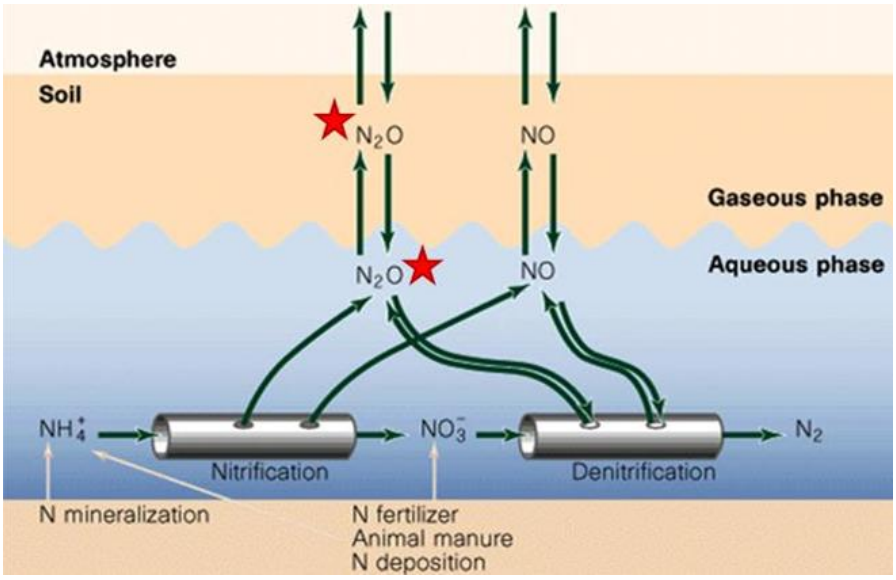

Figure 4: The formation and release of N_2O in an aquatic environment as a by-product of transformation processes: nitrification and denitrification (Modified from Bouwman et al., 2013).

Denitrification is the biochemical reduction of NO_3^- coupled with the oxidation of organic matter to produce dinitrogen gas (N_2). Denitrification occurs under mostly anaerobic conditions, specifically in anoxic the hypolimnia and sediments of lake systems, but can also occur in aerobic environments (Durand et al., 2011). However, denitrification is sensitive to specific oxygen levels. It has been recently suggested that denitrification accounts for a significant percentage of fertilizer losses in terrestrial agriculture (Wrage et al., 2001).

If algae and/or synergistic bacteria are emitting the GHG N_2O during commercial algae cultivation, not only would this have damaging environmental effects, but it could also have serious implications for the algae-to-energy industry as a whole, undermining

its possible ability to produce a commercially viable product. The *Energy Independence and Security Act* (EISA) was adopted in 2007 with the following goals: reducing the U.S.'s reliance on imported fuels; increasing the efficiency of products, buildings, and vehicles; and increasing the production of renewable fuels, among other goals (EPA, 2014; RFA, 2007). EISA pushed for research and innovation in various types of biofuels, specifically with the creation of the *Renewable Fuel Standard* (RFS2) program enacted by the Environmental Protection Agency (EPA). RFS2 sets targets for production of alternative fuels and oversees compliance among biofuel producers. Ethanol was the first biofuel to gain national attention and was promoted by the government via RFS2, as a means to reduce GHG emissions (EPA, 2007); however, there are growing concerns that ethanol's GHG emissions are more severe than originally reported (Cappiello et al., 2013). These concerns have highlighted the need to thoroughly catalogue the life-cycle impacts of biofuels before they become widely commercialized. This is relevant to algae-derived biofuels, as both public and private sectors are investing in emerging algae technologies.

Under RFS2, life-cycle GHG emissions for an "advanced biofuel" must be 50% less than the average life-cycle GHG emissions for gasoline or diesel used as transportation fuel in 2005 (RFA, 2007). According to the *Greenhouse Gases, Regulated Emissions, and Energy Use in Transportation* (GREET) developed by Argonne National Laboratory (2013), 2005 transportation fuel has a GWP of 93 g CO_2 eq/MJ for petroleum-based gasoline, making the target GWP equal to 46.5 g CO_2 eq/MJ in order to be certified as a renewable fuel under RFS2 (Han et al., 2011; Zhang et al., 2013). With

a 100-year global warming potential (GWP) 298 times greater than that of CO_2, N_2O emissions pose a severe threat to the environment and could possibly prevent approval of an algae-derived biofuel process by the EPA (IPCC, 2007). Eligibility for certification under RFS2 could be in jeopardy if previously undocumented N_2O emissions are proven to make up a substantial contribution to the overall GWP of the algae-derived biofuel production process.

3.0 Research Objectives

The primary objective of this thesis was to determine if accounting for N_2O production during algae cultivation could substantively change previous LCA-based estimates of life-cycle GWP for algae-derived gasoline. The research was conducted in three parts:

Objective 1 - Thorough examination of existing literature on N_2O emissions from eutrophic lakes to estimate possible N_2O fluxes

Objective 2 - Bench-scale experiments for measurement of N_2O emissions during various simulated algae cultivation to determine if algae produce substantive quantities of N_2O under commercially-relevant conditions, and

Objective 3 - Integrating literature and laboratory results into an existing LCA-based framework for algae-derived biofuels as modeled by Liu et al. (2013), to determine if N_2O fluxes contribute to meaningful

changes in the likely certifiability of algae-derived gasoline under EPA's RFS2.

The information arising from these tasks sheds light on whether algae-derived biofuels can contribute to the achievement of national energy and climate change goals, as laid out in RFS2. More specifically, information gathered from this research will be inserted into current LCAs to provide a more complete assessment of the potential global warming impacts from algae-derived biofuel processes. An underlying hypothesis is that eligibility for certification under RFS2 could be in jeopardy if previously undocumented N_2O emissions constitute an appreciable contribution to overall GWP of these processes.

4.0 Methodology

In order to evaluate the ability of microalgae to produce N_2O during cultivation, two methods were employed. First, an extensive literature review was conducted to acquire data from previously published studies related to GHG emissions from lakes exhibiting severe eutrophication. Second, bench-scale experiments were conducted in order to track the nitrogen mass balance within a simulated algae cultivation system. Results from both the literature review and bench-scale cultivation experiments were then integrated into the existing LCA-based model of Liu et al. (2013) to calculate the total GWP for both the cultivation and production of algae-derived biofuels.

4.1 Examination of Existing Literature

An extensive literature review was conducted on previously published studies of shallow lakes exhibiting severe eutrophication, and thus, possessing large algal blooms.

Studies chosen included lakes that most closely resemble the shallow algae cultivation ponds used for algae-derived biofuels. Relevant studies were conducted by Hashimoto et al. (1993), Mengis (1997), Huttunen wt al. (2003), Wang et al. (2006), Wang et al. (2009), Fagerstone et al. (2011), and Ferrón et al. (2012). From each study, measurements of average N_2O fluxes were collected and analyzed. These data were used to help quantify N_2O flux emissions as normalized per unit area per time or per mass of algae per time, depending on available information. These emissions were then converted to GWP emissions, in units of CO_2 mass equivalents (eq), based on the GWP impact factor put forth by the IPCC (2007), which states the GWP of N_2O to be 298 times greater than that of CO_2.

4.2 Bench-Scale Cultivation Experiments

A series of bench-scale experiments was conducted in order to track total nitrogen mass and transformation among the aqueous, gaseous, and solid (algae biomass) phases during algae cultivation. Total nitrogen was measured at the beginning and end of each experiment.

4.2.1 Algae Cultivation Methods

The model alga *Scenedesmus dimorphus* was used for bench-scale cultivation experiments. *Scenedesmus sp*. are widely prevalent in temperate freshwater systems, and they have potential application to wastewater treatment and commercial algae-to-energy production (Oron et al., 1981; Chisti, 2007). A culture was purchased from the UTEX culture collection at the University of Texas in Austin, TX. Pure cultures of *S. dimorphus*

were pre-incubated aseptically in capped tubes containing 10 mL of Protease-Peptone medium (PPM) for five days, and then transferred into sealed 250-mL flasks containing 60 mL PPM for another five days. The sealed 250-mL flasks were incubated in a shaker illuminated by a cool white fluorescent growth lamp (125-W 6500-K) for a period of 12 hours of light and 12 hours of darkness. More detailed information for the incubation of the algae and preparation of various growth media is included in Appendix A. All culture chemicals were purchased from Fisher Scientific Inc. (Waltham, MA).

Pure cultures were then cultivated over 7-day intervals in Modified Bold 3N (MB3N) medium (Fisher Scientific, Waltham, MA). Growth reactors comprised duplicate 500-mL glass flasks with wide-mouth screw tops. Reactors were subject to 12 hours of light and 12 hours of darkness per day as during the incubation period. They were constantly agitated by a stir plate stirring at 400 revolutions per minute (rpm). Temperature was maintained at 27°C ± 2°C and measured with a thermometer. The pH of the system was maintained at 7 ± 1 and monitored with a digital pH meter (Fisher Scientific, Pittsburgh, PA). Dissolved oxygen (DO) was monitored over time using an YSI 550A meter. CO_2 was provided in the form of filter-sterilized air flowing through an air diffusion stone, or as pure CO_2 from a pressurized tank. Reactors were plugged with glass wool to enable gas exchange while limiting particle access. Figure 5 shows the laboratory set up for cultivation of axenic *S. dimorphus*. The light source and timer, in-line filter, flow meters, and stir plate are labeled as such. Not shown in Figure 5 is the air diffusion stone or pressured CO_2 tank.

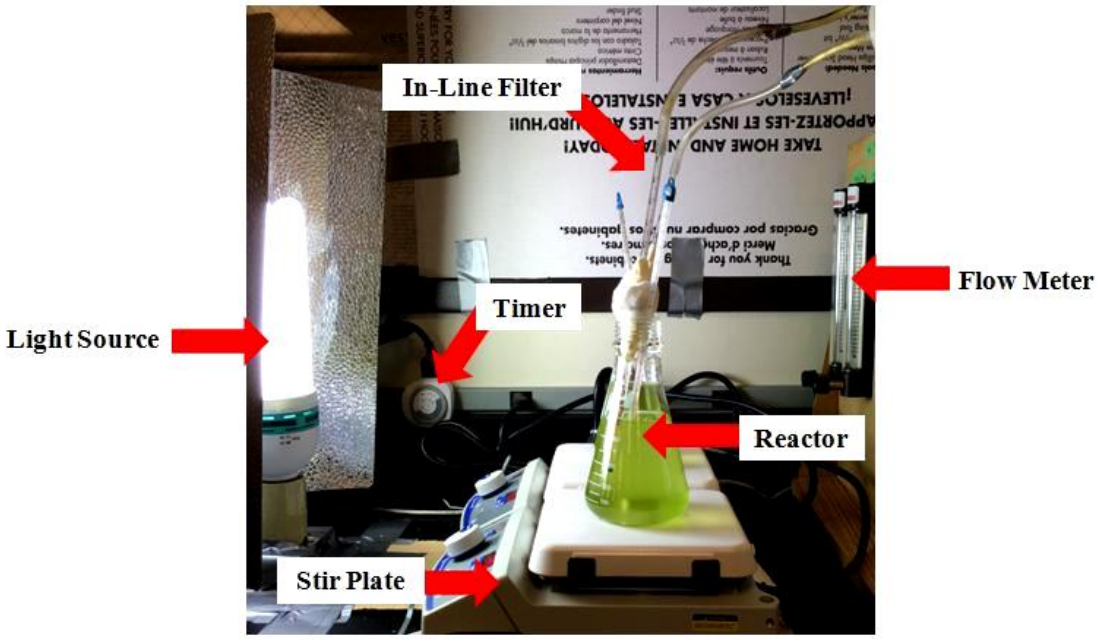

Figure 5: Laboratory cultivation of axenic *S. dimorphus* with infusion of filtered air or pure CO_2 into the system as the carbon source.

The same illumination schedule was used for both the air and pure CO_2 experiments; however, different sparging schedules were used for each. These schedules are presented visually in Figure 6. For the CO_2 experiment, represented by the image on the left in Figure 6, pure CO_2 was provided at a flow rate of 0.4 standard cubic feet per minute (scfm) over a 6-hour interval (8 am to 2 pm). At this time, the flow was stopped and the reactor was allowed to equilibrate with the ambient air for a period of 18 hours (2 pm to 8 am). During times when headspace samples were collected for gas analysis via gas chromatography (GC) (see Section 4.2.3.3), however, the reactor was closed to the atmosphere at 2 pm, marked by a star on Figure 6 (left), and pressurized for 18 hours (until 8 am) after which samples of 20 µL were collected and subsequently analyzed.

For the air experiment, represented by the image on the right in Figure 6, filtered air was provided at a flow rate of 0.4 scfm over a 12-hour interval from 8 am to 8 pm. At this time, the flow was stopped and the reactor was allowed to equilibrate with the ambient air for a period of 12 hours (8pm to 8 am). During times when headspace samples were collected for gas analysis via GC (see Section 4.2.3.3), however, the reactor was closed to the atmosphere at 8 pm, marked by a star on Figure 6 (right), and pressurized for 12 hours (until 8 am) after which samples of 500 µL were collected and subsequently analyzed.

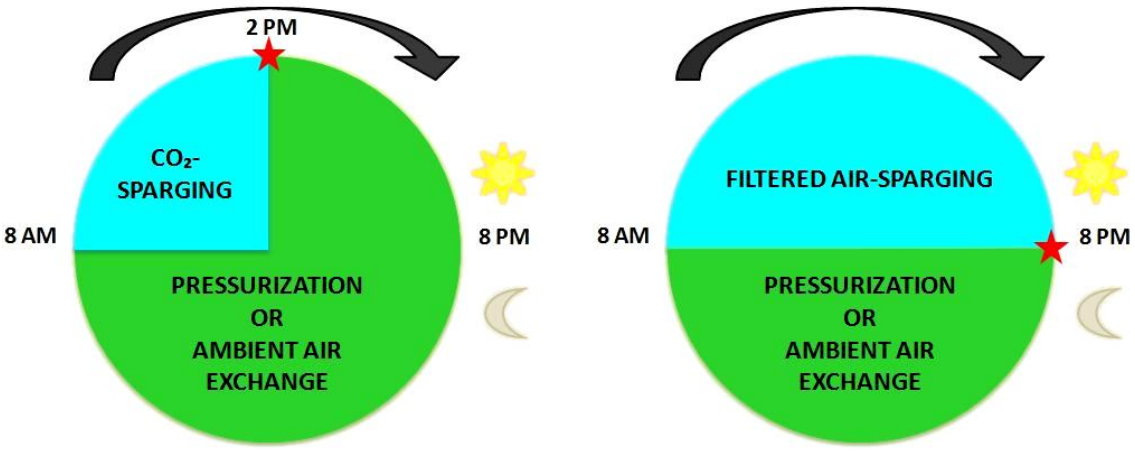

Figure 6: Schedule of sparging, pressurization, and sample collection for *S. dimorphus* cultures receiving either pure CO_2 (left) or ambient air (right). During pressurization, reactors were closed to the atmosphere. Stars mark the time at which samples were collected for analysis by GC.

4.2.2 Algae Cultivation Measurement Methods

The initial sources of nitrogen in all reactors included cellular nitrogen existing in the algae innocula and also aqueous nitrate (NO_3^-) in the MB3N medium. Over the course of the experiment, it was anticipated that aqueous nitrate would be converted into additional cellular nitrogen plus also perhaps gaseous nitrogen in the form of N_2O or other species. Thus, it was necessary to measure nitrogen in all three phases.

4.2.2.1 Optical Density & Total Suspended Solids Measurements

For each cultivation experiment, a growth curve for *S. dimorphus* was constructed by measurement of optical density (OD) with a Cary 100 Conc Spectrophotometer set to wavelength of 662 nm. This wavelength was chosen because it corresponds to maximum absorbance of chlorophyll A, which is abundant in the cells of microalgae. OD measurements were converted into algae mass using a calibration equation constructed from total suspended solids (TSS) measurements in a preliminary experiment (Appendix B, Figure 1). TSS measurements were collected according to APHA Standard Methods involving filtration through 0.7-µm pore size glass microfiber filters (Millipore, Billerica, MA) dried at 105 °C (APHA, 1998).

4.2.3 Experimental & Analytical Methods

Throughout each experiment, the efficiency of nitrogen conversion into biomass versus nitrogen gases was measured. In order to evaluate the differences in nitrogen content of algae before, during, and after a set cultivation period, several analytic

techniques were used. Using these different techniques, nitrogen content in the aqueous phase, the biomass phase, and the gaseous phase were all measured throughout each cultivation period.

4.2.3.1 Nitrogen in the Aqueous Phase

To measure nitrogen content in the aqueous phase, a total nitrogen (TN) high range commercial kit (Hach Co., Method #10072) was used. The TN method converts all forms of nitrogen into nitrate, which then reacts with chromotropic acid to form a yellow-colored complex that can be measured using a spectrophotometer. These measurements were performed on the medium, following separation of the biomass via centrifugation at 3000 rpm for 10 minutes using a Thermo Scientific, Sorvall Legend XTR centrifuge. A Hach DR 3900 Spectrophotometer set to a wavelength of 410 nm was used to measure OD of the samples following addition of the kit reagents. OD measurements were then converted into TN mass in units of mg/L based on a five-point calibration curve. Calibration information for this method can be found in Appendix B, Figure 2.

4.2.3.2 Nitrogen in the Solid (Biomass) Phase

Nitrogen content was also analyzed in the solid phase comprising algae cells. This was done in two ways: 1) estimation, via measurement of algae TSS and multiplication by an assumed nitrogen content based on theoretical stoichiometry; and 2) direct measurement, using a Thermo Scientific Flash 2000 NC Soil Analyzer, also referred to as a CHN (carbon, hydrogen, nitrogen) analyzer. A CHN analyzer combusts a sample and breaks it down into individual elements, which are then quantified by use of

infrared spectroscopy. This analyzer was used to measure the percentage of nitrogen in the algae cells at both the beginning and end of some but not all of the experiments.

4.2.3.3 Nitrogen in the Gaseous Phase

A Shimadzu QP2010 Ultra EI GC-MS, with a carrier gas of helium, was used to measure the concentration of N_2O in the headspace of each reactor over time via gas chromatography-mass spectrometry (GC-MS). Methodology was adapted from previously validated methods, such as Ferrón et al. (2012) and others. Chromatographic separation was achieved using an Rt-Q-Bond column of 30 m length, 8 µm thickness, and 0.25 mm diameter. Column oven temperature was 33°C (Restek #19765, USA). Mass spectrometry (MS) was used to identify the amount of N_2O present in the sample by measuring for a mass-to-charge (m/z) ratio of 44. The retention time for N_2O under these conditions was measured at 2.10 minutes. All samples were collected in a gas-tight 1750RN 500 µL syringe (Restek #24572, USA). Figure 7 summarizes the baseline chromatogram for N_2O flux in air using the described method. More examples of chromatographs can be found in Appendix B, Figures 3 and 4.

Figure 7: Representative chromatogram for the GC method used to analyze N_2O fluxes. The chromatogram shows the baseline peak response of N_2O in air. The N_2O peak has a retention time of 2.10 min under the conditions used for this study.

A commercial standard with original concentration of 10.1 ppm_v N_2O in air (Air Liquide America Specialty Gases, LLC, Plumsteadville, PA) was used to produce a calibration curve for GC measurements. Different injection volumes over the range of 20-500 µL were used to calibrate the instrument from 0 to 10 ppm_v N_2O. Calibration curves for each injection volume used can be found in Appendix B, Figures 5 and 6.

4.3 Integration into LCA Framework

A LCA-based modeling framework for algae-derived biofuels was adapted from previous work by Liu et al. (2013). Outputs from the original model include GWP of HTL production pathways, which were used as the baseline GWP for the LCA analysis conducted for this study. The baseline values did not previously account for N_2O as a contributor to GWP. Baseline values were revised to account for N_2O emissions taken

from published literature studies and experimental results in this thesis research, focusing on the GWP of petroleum-based gasoline at both pilot- and full-scales. GWP thresholds for RFS2 were taken from the GREET model (Argonne, 2013).

5.0 Results & Discussion

A three-pronged approach was used to satisfy the main objectives of this thesis research. First, an extensive literature review was conducted on previously published studies to track the N_2O emissions from eutrophic lakes exhibiting algal blooms; second, bench-scale experiments were conducted to measure the N_2O production from simulated algae cultivation; and third, literature and experimental results were analyzed for overall GWP impact using a previously published LCA-based model by Liu et al. (2013) for algae-derived biofuel production. Results and analyses for these three components are discussed below.

5.1 Literature Review Findings

As a first step towards understanding whether N_2O emissions may constitute a significant, previously undocumented contribution to the GWP footprint of algae-derived biofuels, an extensive literature search was performed on published studies that report N_2O flux emissions from eutrophic lakes. From these studies, measurements of N_2O fluxes from shallow lakes exhibiting algal blooms were collected. Results are summarized in Table 1. These studies suggest that the flux of N_2O from the shallow, irradiated portions of lakes containing dense algae cultures is on the range of 7-99 μg $N_2O/m^2/hr$. From this examination, it can be seen that heavily eutrophic lakes emit

significant quantities of N_2O into the atmosphere. This is potentially significant given (1) the estimated size of algae cultivation facilities (approximately 100-300 acres); (2) the estimated quantity of finished fuel produce produced by the facility each year (approximately one million gallons); and (3) the very strong GWP of N_2O (Sapphire Energy Inc., 2013).

Table 1: N_2O fluxes emitted from eutrophic lakes containing dense cultures of microalgae, as taken from previously published literature.

Lake	N_2O Flux ($\mu g\ N_2O/m^2/hr$)	Conclusion	Source
Lake Baldeggersee & Lake Sempachersee (Switzerland)	22	Maximum N_2O emissions in oxic epilimnia where algal blooms occurred; Denitrifying bacteria growing on the surface of algae can produce N_2O in oxic waters	Mengis, 1997
Lake Kasumigaura (Japan)	7	Highest N_2O emissions from nutrient-rich, shallow waters; N_2O emissions released to atmosphere during denitrification at anoxic sediment surface	Hashimoto et al., 1993
Lake Taihu (China)	26	Maximum N_2O emissions where algal blooms occurred; N_2O emissions proportional to NH_4^+ concentration	Wang et al., 2009
Meiliang Bay in Lake Taihu (China)	99	Highest N_2O emissions in the littoral zone where algal blooms occurred	Wang et al., 2006
Lake Postilampi & Lake Vehmasjarvi (Finland)	11	Maximum N_2O emissions from nutrient-rich, shallow waters; N_2O emission proportional to sequential aerobic nitrification & anaerobic denitrification	Huttunen et al., 2003

Table 1 focuses specifically on the N_2O fluxes emitted from the shallow portion of each pond, specifically the oxic epilimnion, which constitutes the illuminated portion of the water column. This portion of a lake typically exhibits oxic conditions, because active photosynthesis produces molecular oxygen (O_2) in this region. This part of the lake is thought to most closely match the conditions of commercial raceway cultivation ponds. From previous LCA studies of algae-derived biofuels, the optimal depth for cultivating algae on a large-scale basis is a shallow 0.5 m, because algae require good light penetration to conduct photosynthesis (Liu et al., 2012; Liu et al., 2013).

From all the studies reviewed, it was seen that the maximum N_2O emissions occurred in oxic zones with algal blooms, meaning algae species were present in the lakes at the time of N_2O emission. Mengis et al. (1997) suggested that denitrifying bacteria which can grow on the surface of algal cells are responsible for producing N_2O in oxic waters. Hashimoto et al. (1993) suggested N_2O emissions are released to the atmosphere during denitrification at anoxic sediment surfaces, but did not state what organisms are thought to be performing denitrification in this case. Huttunen et al. (2003) reported that N_2O emissions are proportional to sequential aerobic nitrification and anaerobic denitrification, suggesting that algae could be the organism undergoing these reactions. However, from the data summarized in Table 1, it is not possible to say specifically if algae in these lakes are producing N_2O or whether it is produced by other organisms that are present within the algal blooms.

It is of engineering importance to determine what organism or organisms could be producing N_2O in algae cultivation ponds and via what specific nitrogen transformation

pathway or pathways. Once this is known, it may be possible to design the cultivation system in a way that mitigates N_2O formation. Aquatic environments like the one depicted in Figure 4 can be similar to that of an algae cultivation pond, whereby several classes of microbes are known to produce N_2O as a by-product of coupled denitrification and nitrifier denitrification in such aquatic environments (Durand et al., 2011). Figure 8 depicts these specific pathways as they may relate to algae cultivation ponds.

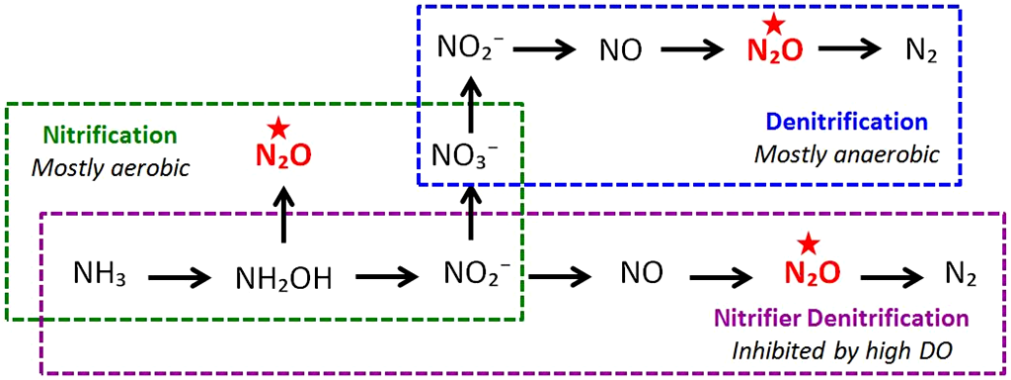

Figure 8: Microbial transformations of fertilizer nitrogen (NH_3 or NO_3) into gaseous N_2 or N_2O that may occur during algae cultivation, as mediated by bacteria and/or algae (Modified from Wrage et al., 2001; Robertson and Groffman, 2007).

Coupled denitrification comprises the combined transformations of nitrification and denitrification. N_2O can be produced as a by-product at two points during this combined pathway: during both nitrification and denitrification (Wrage et al., 2001). Nitrifier denitrification refers to the process by which N_2 is produced from NH_3, with possible creation of N_2O as a by-product. This process is less well understood than traditional coupled denitrification; however, there is evidence that it occurs under aerobic or anaerobic conditions, though it is inhibited by high O_2 (Wrage et al., 2001).

Reiterating, it is not clear from Table 1 which processed from Figure 8 are principally responsible for N_2O emissions from hypereutrophic lakes.

There is also emerging evidence that commercially relevant microalgae (e.g., *Chlorella, Scenedesmus,* and *Chlorococcus*) can produce N_2O through the series of transformations (Wrage et al., 2001). However, this is very controversial, because it is not known what portion of the N_2O is produced by algae versus bacteria in mixed communities (Wang et al., 2006; Guieysse et al., 2013). Figure 8 clearly shows that NH_3 and NO_3^- can be converted into gaseous N_2 or N_2O as by-products of different nitrogen transformations. This is noteworthy, because nitrogen fertilizer, in the form of NH_3 or NO_3^-, is used as a nutrient source for algae in typical algae cultivation ponds (Erisman et al., 2010).

Mixed communities containing both microalgae and bacteria are relevant for design of algae cultivation systems, because open ponds have been shown to be the most plausible configuration for algae cultivation for energy systems (Liu et al., 2013). In typical algae cultivation ponds, it is expected that both microalgae and bacteria will be present together, because the ponds are open to the atmosphere and to the surrounding environment. Therefore, all of the above nitrogen pathways in Figure 8 could be relevant to large-scale algaculture for energy production.

To further understand whether N_2O emissions may significantly contribute to the GWP of algae-derived biofuels, a literature review was conducted on published studies examining the release of N_2O from aquatic systems specifically simulating commercial

algae cultivation. For example, Fagerstone et al. (2011) measured N_2O emissions from *Nannochloropsis salina* grown under bench-scale conditions. This study reported headspace N_2O concentrations of up to 220 ppm_v under anoxic conditions during dark cultivation periods. Specialized controls with broad-spectrum antibiotics, combined with genomics testing, suggested that denitrifying bacteria, rather than the algae themselves, were the predominant N_2O producers in this system. Additionally, altering the composition of the initial headspace produced significant change in N_2O production, whereby 0.5-0.8 ppm_v N_2O was achieved during dark periods when the initial headspace was air under oxic conditions, as compared to 70-220 ppm_v N_2O during dark periods for reactors with N_2 as the initial headspace under anoxic conditions.

Ferrón et al. (2012) measured N_2O fluxes from a pilot-scale facility cultivating *Staurosira sp*. over a 24-hour period. Their results suggest that algae cultivation ponds can be a source or a sink for N_2O, depending primarily upon the availability of NO_3^-. A third study, focusing on energy-relevant *Chlorella vulgaris*, examined the pathways of algae-mediated N_2O release in detail using lab-grown pure cultures (Guieysse et al., 2013). This study confirmed the importance of dark conditions, and showed that NO_2^- availability strongly impacts N_2O release at lab-scale. These authors also asserted that N_2O could have "profound impacts" for algae biotechnologies" (Guieysse et al., 2013).

Taken together, the preliminary work of Fagerstone et al. (2011), Ferrón et al. (2012), and Guieysse et al. (2013) yield several compelling observations and additional questions about N_2O production in algae cultivation systems. First, it is still somewhat unclear whether it is algae or bacteria that mediate nitrogen transformation into N_2O.

Second, both studies stress the need for further examination of this phenomenon. For example, Ferrón et al. (2012) calls for research into better understanding temporal gas flux dynamics. Fagerstone et al. (2011) emphasizes that "future microalgae LCAs must consider the potential for direct N_2O emissions from the cultivation stage".

To achieve objective one of this thesis research, thorough examination of existing literature on eutrophic lakes emitting N_2O was conducted. It can be seen from Table 1 that eutrophic lakes similar to algae cultivation ponds are estimated to produce fluxes in the range of 7-99 μg $N_2O/m^2/hr$ from portions of the lakes containing dense algae cultures; confirming that eutrophic lakes are a significant contributor of N_2O into the atmosphere. This presumably reflects coupled denitrification and denitrification, as seen in Figure 8.

The research of Fagerstone et al. (2011) and Ferrón et al. (2012) underscore the need for further experimental research to determine if algae produce substantive quantities of N_2O during cultivation. Fagerstone et al. (2011) showed the importance of oxic versus anoxic state on the magnitude of N_2O fluxes from algae cultivation. Therefore, to achieve objective two of this thesis research, bench-scale experiments of simulated algae cultivation were conducted to determine the potential of N_2O production under growth conditions similar to that of previously published research, as well as in commercial algae cultivation ponds.

5.2 Analysis of Cultivation Experiments

The second portion of this thesis work sought to replicate experimental measurements in a commercially-relevant algae strain under simulated, anticipated large-scale cultivation conditions. From literature review findings, N_2O emissions from eutrophic lakes were principally associated with oxic rather than anoxic zones; however, Fagerstone et al. (2011) observed much higher quantities of N_2O emissions under anoxic conditions. This difference shows the importance of determining which conditions are most representative of commercial algae cultivation ponds. In this thesis, bench-scale experiments were conducted to simulate algae cultivation under different growth conditions subject to either ambient air- or CO_2-sparging; CO_2-sparging was anticipated to be commercially relevant for full-scale cultivation ponds. Because it is desirable to maximize the efficiency of nitrogen uptake during algae cultivation, and thereby minimize the environmental and economic costs associated with losses, a nitrogen mass balance was conducted to track this efficiency by which aqueous nitrate (NO_3^-) was converted into algae biomass. The nitrogen mass balance of each experiment is summarized below.

5.2.1 Nitrogen Mass Balance for Air-and CO_2-Sparging Experiments

Nitrogen mass balances were performed for two sets of experiments: one in which CO_2 for photosynthesis was supplied as ambient air and another in which pure CO_2 was provided from a pressurized tank. Figure 9 presents the distribution of nitrogen among the solid, aqueous and gaseous phases over the 7-day cultivation period for the air-

sparging experiment. As seen in Figure 9, nitrogen is redistributed throughout the three phases as the experiment progresses. Total nitrogen was measured at the beginning and end of the experiment. On day 7, the total nitrogen measured within the system was 8 ± 1% less than the total nitrogen measured at the start of the experiment. This difference means that there is some inefficiency in nitrogen uptake by the algae. This could reflect production of other gaseous nitrogen species, such as N_2, NO, or NO_2. These were not measured for this experiment, since these gaseous species do not contribute to GWP.

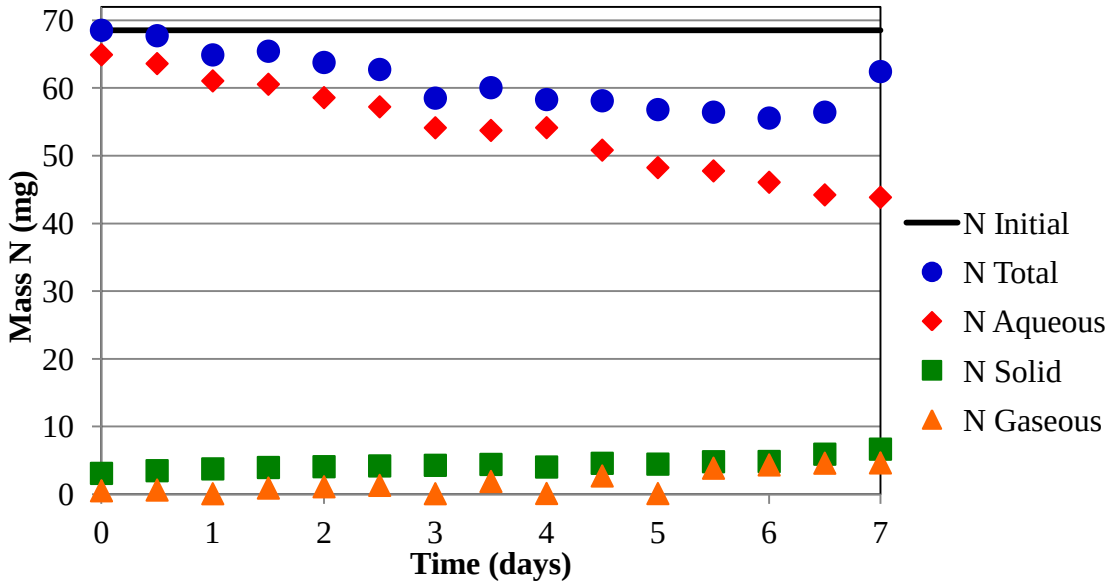

Figure 9: Distribution of nitrogen among the growth medium (aqueous phase), algae biomass (solid phase), and gaseous N_2O flux over a 7-day cultivation period of duplicate reactors sparged with filtered air.

Figure 10 presents the nitrogen distribution throughout the cultivation period for the CO_2-sparging experiment. Results are similar to those of the air-sparging experiment, whereby there is also some inefficiency in the nitrogen uptake. The difference in total

nitrogen from day 1 to day 7 is 3 ± 0.5%, which is less than that of the air-sparging experiment; however, there is still a portion of nitrogen that is unaccounted for.

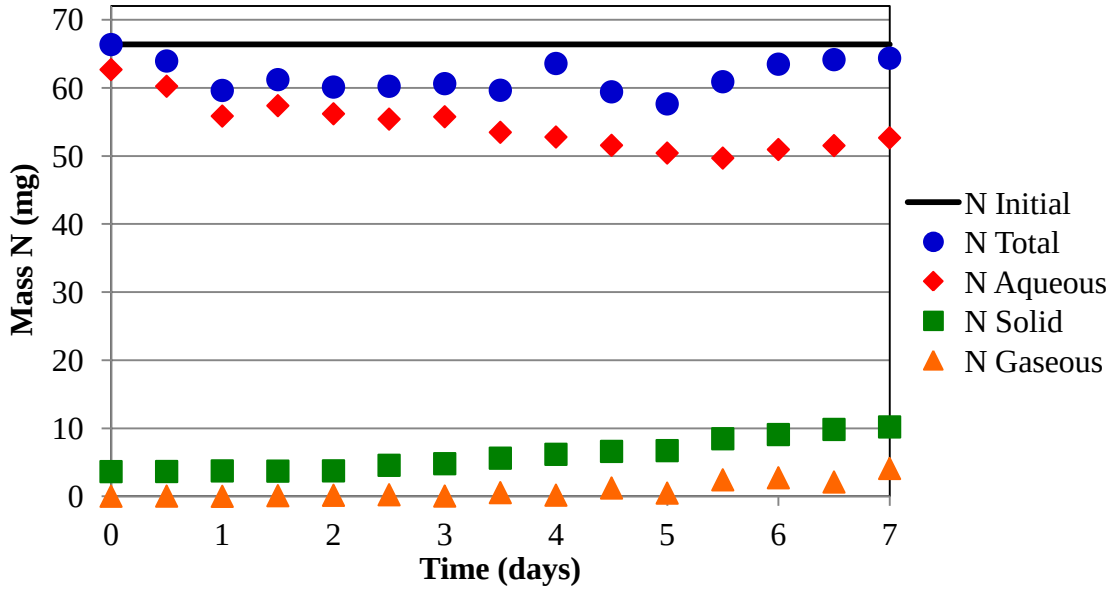

Figure 10: Distribution of nitrogen among the growth medium (aqueous phase), algae biomass (solid phase), and gaseous N_2O flux over a 7-day cultivation period of duplicate reactors sparged with pure CO_2.

When comparing the air- and CO_2-sparging experiments, algae growth behaved similarly. For the air-sparging experiment, stationary algae growth occurred at a density of approximately 200 mg/L and aqueous nitrogen decreased from 150 to 110 mg TN/L throughout the cultivation period. For the CO_2-sparging experiment, stationary phase algae biomass density was approximately 250 mg/L and aqueous nitrogen decreased from 150 to 130 mg/L. For both experiments, aqueous nitrogen was never depleted, allowing the algae to grow for the dull duration of both experiments.

5.2.2 Gaseous N_2O Flux Results

Headspace N_2O concentrations were measured periodically via GC-MS throughout the algae cultivation period for both the ambient air- and CO_2-sparging experiments. These results are presented in Figure 11. Original N_2O measurements were in units of ppm_v, which were then converted to mg/L using the Ideal Gas Law.

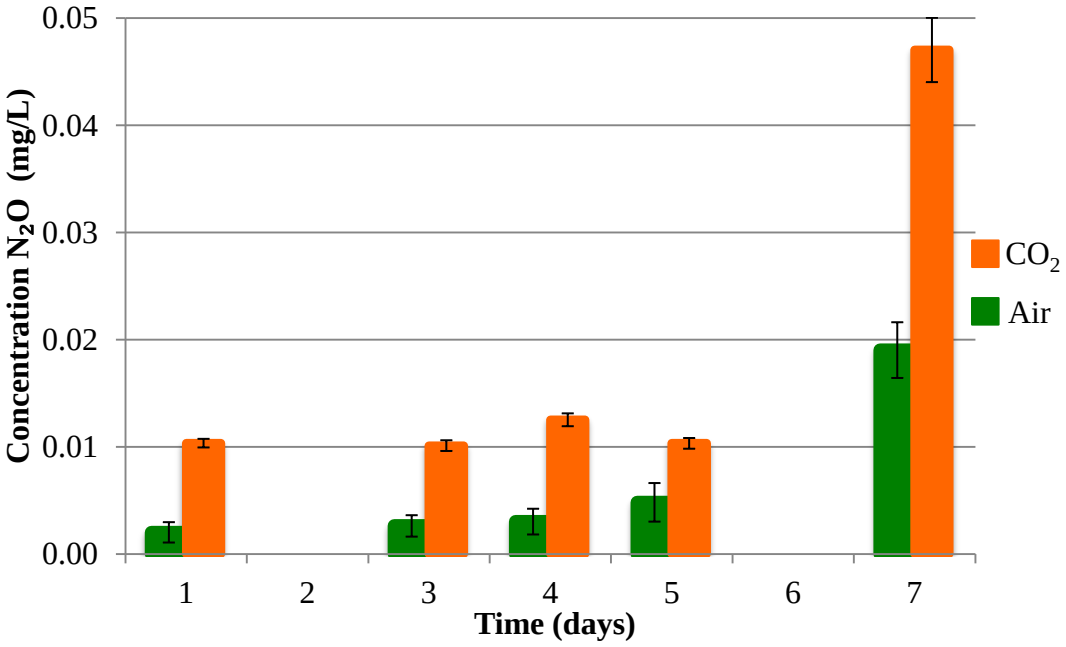

Figure 11: Measured headspace N_2O concentrations from *S. dimorphus* experiments subject to either ambient air- or CO_2-sparging over 7-day period.

The data presented in Figure 11 shows a significant difference in the amount of headspace N_2O produced under CO_2-sparging versus ambient air-sparging. About 2.5 times as much N_2O was produced during the CO_2-sparging experiment when compared to the air-sparging experiment, though the algae growth rates of the two experiments are comparable (Figures 9 and 10).

The headspace N_2O concentrations measured in this study can be compared to that of the bench-scale algae cultivation experiments conducted by Fagerstone et al. (2011). Figure 12 shows the average N_2O emissions, culture density, and nitrate concentrations in the growth media for both the air- and N_2-filled headspace experiments reported by Fagerstone et al. (2011). From this figure, maximum N_2O concentrations were measured during dark periods, rather than light periods, for both the air-headspace (top) and N_2-headspace (bottom). For the air-headspace experiment, N_2O concentrations ranged from 0.3 to 1.3 μg N_2O/L, with the highest concentration occurring during day 4 of cultivation. The highest N_2O concentration produced from the air-sparging experiment of this research was greater than that of Fagerstone et al.'s air-headspace experiment: 4.8 μg N_2O/L on the last day (day 7) of cultivation. Fagerstone et al. (2011) states that under an air-headspace, algae was cultivated under oxic conditions.

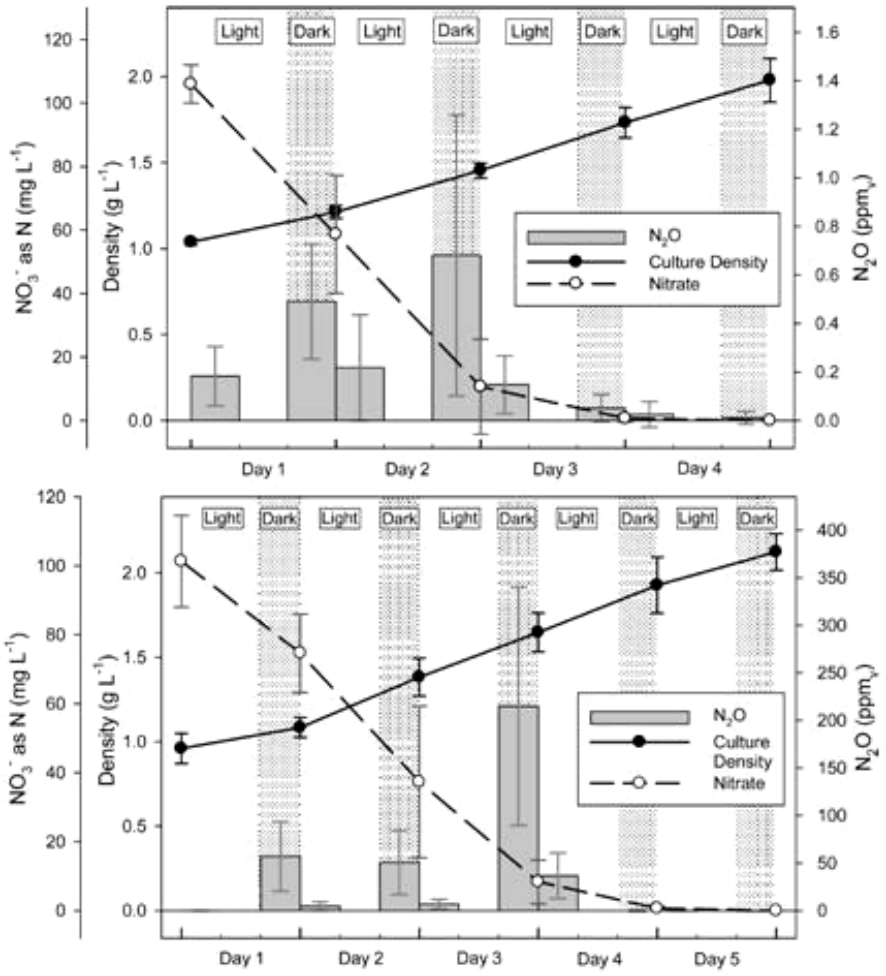

Figure 12: Average N_2O emissions, culture density, and nitrate in growth media for air-filled headspace (top) and N_2-filled headspace (bottom) experiment by Fagerstone et al. (2011) (Fagerstone et al., 2011).

For Fagerstone et al. (2011)'s N_2-headspace experiment, N_2O concentrations ranged from 0.3 to 399 μg N_2O/L, with the highest concentration occurring during day 3 of cultivation. On 7-day of cultivation of the CO_2-sparging experiment conducted for this thesis, a concentration of 19 μg N_2O/L was measured. Fagerstone et al. (2011) states that under a N_2-headspace, algae was cultivated under anoxic conditions. Both the CO_2-

sparging and N_2-headspace experiments resulted in higher N_2O production when compared to their respective air experiments. For both of the Fagerstone et al. (2011) experiments, N_2O production stopped when NO_3^- concentration levels fell below 10 mg/L. This could explain why maximum N_2O concentrations in both of their experiments were observed fairly early into their experimental duration. In contrast, NO_3^- levels for the experiments conducted in this thesis research remained above 100 mg/L for the entirety of both cultivation periods, such that maximum N_2O emissions were observed at the end of both experiments. It is also noteworthy that the algae density used by Fagerstone et al. (2011) is one order of magnitude greater that what was used for the experiment in this thesis research: 2-2.5 g/L versus 0.2-0.3 g/L.

The observation of greater N2O emissions under oxic rather than anoxic conditions contradicts some observations from the studies of eutrophic lakes presented in Table 1. However, the results from Fagerstone et al. (2011) confirm that oxic state is a potential contributor to possible N_2O production during algae cultivation. Because of this importance of oxic state on N_2O production, dissolved oxygen (DO) profiles were created for both the air- and CO_2-sparging experiments conducted for this thesis.

5.2.3 DO Profiles

In order to investigate the importance of DO on the production of N_2O during algae cultivation, as previously reported by Fagerstone et al. (2011), DO was measured throughout the 7-day cultivation period of both the air- and CO_2-sparging experiments. Figure 13 presents DO measurements during the cultivation experiments referenced

above. Both experiments show diurnal variation in DO concentrations, whereby DO is highest during daylight hours, when algae are producing O_2 as a product of photosynthesis. During dark hours, this reaction stops and the algae may consume O_2 for respiration. The difference between daytime and nighttime DO is subtle for the air-sparging experiment. DO concentrations for daytime and nighttime hours were 9-10 mg/L and 7-8 mg/L, respectively. Because the reactors were open to the atmosphere at night, DO never drops below 7 mg/L and conditions are always oxic. More dramatic variability in DO is observed for the CO_2-sparging experiment, whereby daytime and nighttime DO levels were roughly 6 mg/L and 1-4 mg/L, respectively. Thus, the CO_2-sparging reactors were subject to anoxic conditions for a significant portion of each day.

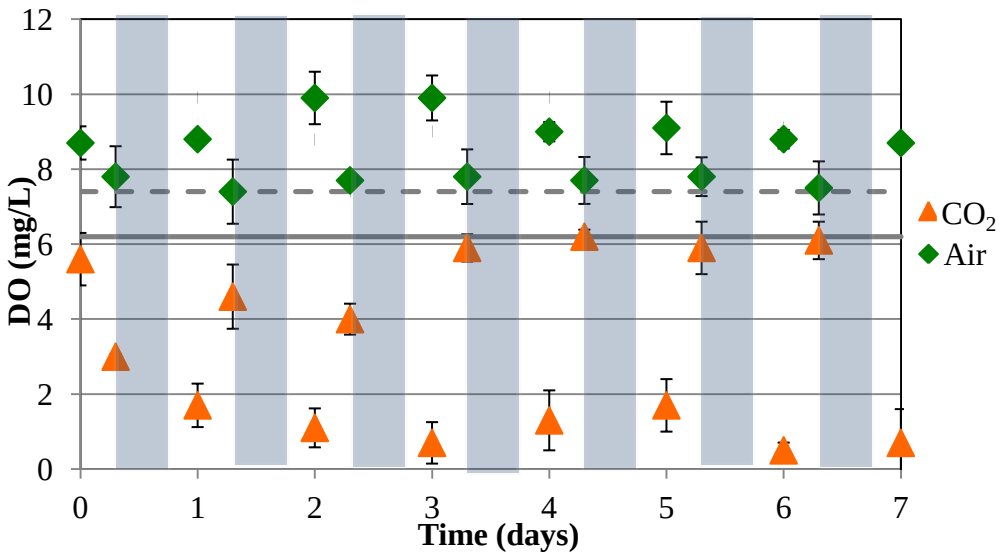

Figure 13: Time variant DO profile for air- and CO_2-sparging cultivation experiments. Shaded regions represent dark period. Dashed line represents the minimum DO level achieved throughout the air-sparging experiment; solid line presents the maximum DO level achieved throughout the CO_2-sparging experiment.

DO levels shown in Figure 13 are comparable to those recorded during the bench-scale algae cultivation experiments of Fagerstone et al. (2011). Figure 14 presents the 24-hour DO profile for both the air- and N_2-filled headspace experiments by Fagerstone et al. (2011). During light periods of the air-filled headspace experiment, DO levels ranged from 12-16 mg/L, while during dark periods when N_2O concentration measurements were taken, DO levels ranged from 4-8 mg/L. This is very comparable to the oxic DO levels that were achieved during the air-sparging experiment conducted for this research, with the lowest O_2 concentration of approximately 7 mg/L occurring at the end of the dark period.

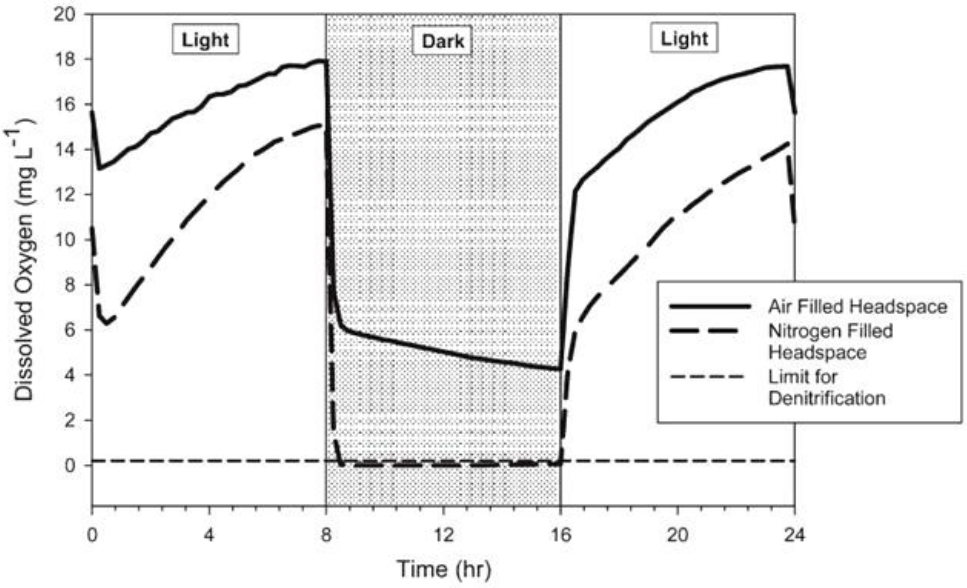

Figure 14: DO concentrations over 24-hrs for both air- and nitrogen-filled headspace experiments by Fagerstone et al. (2011). Dashed line represents the upper limit for anoxic denitrification (Fagerstone et al., 2011).

As seen from Figure 14, Fagerstone et al. (2011) also reported greater DO concentrations during the dark for their N_2-headspace experiment. Daytime DO levels were 8-14 mg/L; while dark DO levels were 0-4 mg/L. The authors characterized some portions of dark conditions as "anoxic", as defined by DO levels less than 0.2 mg/L, the detection limit for anoxic denitrification. By this metric, the N_2-headspace reactors were subject to anoxic conditions for the majority of the dark period. As seen in Figure 8, N_2O can be produced as a by-product of anoxic denitrification (Wrage et al., 2011).

The results from Figure 13 are particularly important, because the cultivation conditions of the CO_2-sparging experiment are anticipated to be commercially relevant. Many full-scale algae cultivation facilities use CO_2 as their carbon source, and do not provide gas flow during the night, as it is not economically feasible to supply energy for pumping, or to use CO_2 supplies when algae are not actively photosynthesizing (Liu et al., 2013; Ferrón et al., 2012). Thus, commercial algae cultivation ponds will likely be subject to anoxia for at least some portion of each day, potentially increasing the magnitude of possible N_2O emissions.

With respect to completion of objective two of this thesis, there is fairly good consistency between the N_2O measurements observed in this thesis and previously published studies, most notably Fagerstone et al. (2011). Also, we have confirmed that greater N_2O emissions are observed under significantly less oxic conditions, showing the importance of oxic state on N_2O production. If substantive fluxes of N_2O are being produced during full-scale algae cultivation, there is a need to quantify the GWP adjustment from these emissions, so it can be accounted for in the overall GWP of an

algae-derived biofuel production process. Objective three of this thesis research used an existing LCA-based framework for algae-derived biofuels for integration of literature and laboratory results from the analysis of N_2O emissions from algae cultivation ponds.

5.3 LCA Analysis

The third and final objective of this work was to assess whether the magnitude of N_2O emissions taken from previously published literature and arising from experiments performed in this study would substantively impact the estimated GWP of an algae-derived biofuel produced via HTL under RFS2. An existing LCA model constructed by Liu et al. (2013) was used as a source for baseline estimates of life-cycle GWP during algae cultivation. The baseline values are presented as filled rectangles in Figure 15. At pilot-scale, baseline GWP for algae-derived biofuels is 52.3 g CO_2 eq/MJ, while at full-scale, baseline GWP is 28.4 g CO_2 eq/MJ. Neither of these numbers accounts for the production of N_2O during algae cultivation.

In Figure 15, the y-axis represents Energy Return on Investment (EROI), which is the ratio of energy production to energy consumption over the life cycle of a product or process. EROI values greater than one are desirable for sustainable energy systems. The x-axis presents life-cycle GWP per MJ of fuel product. With these axes, values in the upper left are most desirable (i.e., high EROI, low GWP), whereas values in the lower right are least desirable (i.e., low EROI, high GWP). The RFS2 threshold for certification as an "advanced biofuel" based on 50% reduction of the GWP of 2005 petroleum gasoline (marked with the filled circle) from the GREET model is depicted

using a dashed vertical line (Han et al., 2011; Zhang et al., 2013). Fuels exhibiting GWP values less than this threshold (i.e., falling to the left of the dashed line) would qualify for certification as an "advanced biofuel" under RFS2.

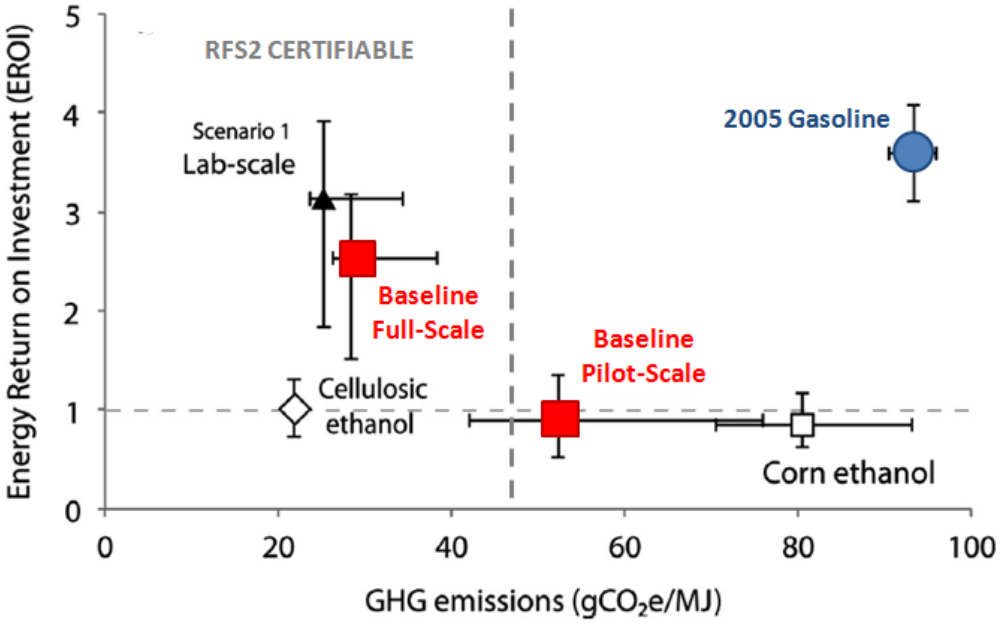

Figure 15: EROI and estimated life-cycle GWP for algae-derived gasoline produced using HTL at either full- or pilot-scale production. Baseline results are benchmarked against 2005 petroleum-based gasoline. The vertical dotted line represents RFS2 threshold for an "advanced biofuel". Values to the left of the dashed vertical line are eligible for certifiability under RFS2 (Modified from Liu et al., 2013).

From Figure 15, the baseline GWP estimates from Liu et al. (2013) suggest that algae-derived gasoline produced at full-scale, but not at pilot-scale, would likely be certifiable under RFS2. It was expected that expanding the model to also account for N_2O emissions would increase life-cycle GWP, reducing the likelihood of certifiability.

In order to quantify the increase in life-cycle GWP, N_2O GWP adjustments were first computed for results arising from the published literature studies presented in this thesis. Table 2 presents the conversion of N_2O flux measurements from Fagerstone et al. (2011) and Ferrón et al. (2012) into N_2O-based GWP adjustments, which can then be added to baseline pilot- and full-scale values shown in Figure 15. N_2O emissions from the various eutrophic lakes presented in Table 1 were not included in this analysis, because the magnitudes of the fluxes from these lakes were much lower than what was observed for simulated culture conditions (e.g., Fagerstone et al, 2011; this thesis). Also, the seeming divergence related to N_2O emissions increasing or decreasing during anoxia could be not resolved. A summary of GWP adjustment calculations appears in Appendix D.

Table 2: N_2O flux measurements from previously published studies and their corresponding N_2O-based GWP adjustments; for addition to baseline GWPs for algae-derived gasoline production at pilot- and full-scale, as taken from Liu et al. (2013).

Published Study		**N_2O Emission ($\mu g\ N_2O/m^2/hr$)**	**Baseline GWP (g CO_2 eq/MJ)**	**N_2O-Based GWP Adjustment (g CO_2 eq/MJ)**
Baseline (Liu et al., 2013)	*Pilot-Scale*	-	52.3	-
	Full-Scale	-	28.4	-
Air-Headspace (Fagerstone et al., 2011)	*Maximum*	78.0	-	6.24
	Average	40.0	-	3.18
N_2-Headspace (Fagerstone et al., 2011)	*Maximum*	24,500	-	1,760
	Average	8,300	-	600
CO_2-Headspace (Ferrón et al., 2012)	*Maximum*	60.0	-	0.69
	Average	30.0	-	0.35

The N_2O GWP adjustments were also computed for N_2O emissions arising from the experiments conducted for this thesis. This was done using the following procedure. First, the N_2O concentration measured in each headspace (as ppm_v) was converted to mg/L and then multiplied by the headspace volume to compute mass. This mass was then divided by the corresponding mass of algae biomass in the same reactor, to yield a normalized N_2O emission in units of g N_2O per g algae biomass. This emission was converted to flux dimensions by dividing by either 24 or 12 hours per day, since it was unknown whether algae make N_2O for the entire day or just during darkness or light. The normalized flux (in units of g N_2O/g algae/hour) was then multiplied by the total algae yield reported by Liu et al. (2013) (i.e., 96,562 and 128,750 lb algae/ha/yr for pilot- and full-scale, respectively) and the number of hours per year. The resulting quantity was then in units of g N_2O/ha-yr. This was converted to GWP in units of mass CO_2 equivalents, based on the 100-year impact factor for N_2O: 298 g CO_2 eq/g N_2O (IPCC, 2007). Finally, the N_2O-based GWP was divided by the energy output corresponding to total algae yield in the model by Liu et al (2013) (i.e., 287,221 and 957,405 MJ/ha-yr for pilot- and full-scale, respectively), so that it would be normalized to the 1-MJ basis referenced in Figure 15 and Table 2.

The calculation summarized above was repeated for each N_2O headspace concentration measured experimentally in this thesis. Results can be found in Appendix D. As seen in Tables D-3 and D-4 (Appendix D), there was significant variability among these measurements. To cover this variability, N_2O-based GWP adjustments were computed for the average and maximum N_2O headspace concentrations. Other

parameters referenced above were also evaluated using multiple values to address uncertainty and/or variability, including: average versus steady-state algae biomass concentrations; and partial-day (12-hours) versus full-day (24-hour) N_2O production time horizons. A wide range of N_2O-based GWP adjustments were computed for the combinations of these various permutations.

Table 3 presents the minimum and maximum N_2O-based GWP adjustments calculated from N_2O headspace concentrations measured in this study. Presentation of the extreme values highlights the dramatic variability arising from the LCA calculations. Parameter conditions that produced minimum GWP values for both the air- and CO_2-sparging experiments are as follows: average N_2O headspace concentration, average biomass concentration, and assuming measured N_2O concentration reflects 24 hours of N_2O production prior to GC analysis. Parameter conditions that produced maximum GWP values for both experiments are as follows: maximum N_2O headspace concentration, steady-state biomass concentration, and assuming measured N_2O concentration reflects 12 hours of production prior to GC analysis. Additional GWP adjustment calculations for other values can be found in Appendix D.

Table 3: Minimum and maximum N_2O-based GWP adjustments for addition to baseline values for petroleum-based gasoline from Liu et al. (2013).

GWP Adjustment for N_2O (g CO_2 eq/MJ)	Minimum		Maximum	
	CO_2-Sparging	Air-Sparging	CO_2-Sparging	Air-Sparging
Pilot Scale	20.8	9.21	781	368
Full Scale	8.33	3.68	312	147

The N_2O-based GWP adjustments from Table 3 are presented together with baseline values from Liu et al. (2013) for full-scale production of algae-derived gasoline in Figure 16. Use of the minimum estimate for an N_2O-based GWP adjustment does not change the outlook for certifiability of algae-derived gasoline produced at full-scale. The original estimate and the revised estimate are both less than the RFS2 cutoff, which is indicated visually using the gray dashed line. In contrast, use of the maximum estimate for N_2O-based GWP adjustment does indeed change the outlook for certifiability for full-scale algae-derived gasoline production. This maximum estimate increases the baseline full-scale GWP for petroleum gasoline by up to 630%, resulting in a value that greatly exceeds the RFS2 threshold for certification as an "advanced biofuel". Finally, because the baseline pilot-scale GWP was already greater than the RFS2 threshold, addition of any N_2O-based GWP adjustment does not change the certification outlook for algae-derived gasoline. As such, these data are not shown in Figure 16.

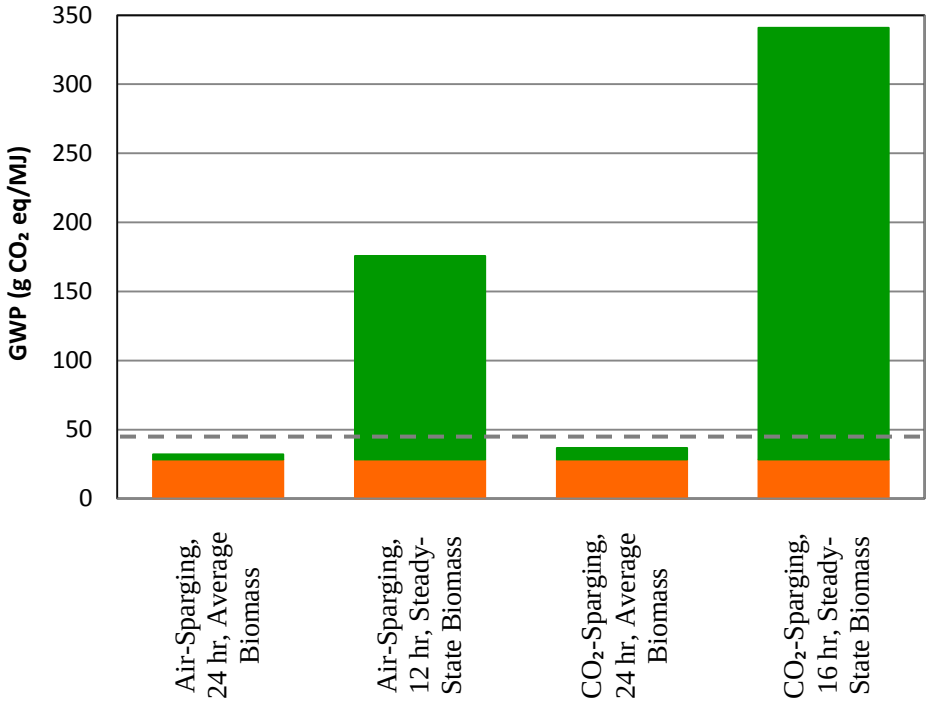

Figure 16: Minimum and maximum N_2O-based GWP adjustments from bench-scale experiments (top portion of bars) added to the baseline GWP of 28.4 g CO_2 eq/MJ for full-scale algae-derived biofuels (bottom portion of bars) from Liu et al. (2013). The horizontal dotted line represents the RFS2 threshold for an "advance biofuel". Values below the dashed horizontal line are eligible for certifiability under RFS2.

Figures 16 underscores the significant negative impacts N_2O emissions could have on the GWP performance of algae-derived biofuels. These impacts jeopardize the certification of algae-derived gasoline under RFS2, which in turn diminishes the likelihood of widespread commercialization of algae biofuels in the U.S. However, the wide variability of N_2O-based GWP adjustments that can be computed based on previously published data and the experiments conducted for this thesis research

necessitates future work in this vein to improve the accuracy of the N_2O-based GWP estimates and also guide the design of algae production facilities, so as to minimize N_2O emissions.

6.0 Conclusions

A three-pronged approach was used for this thesis research. To achieve objective one, a thorough literature review on eutrophic lakes emitting N_2O was conducted, confirming that eutrophic lakes, similar to algae cultivation ponds, are a significant contributor of N_2O into the atmosphere. Measured emissions are 7-99 μg $N_2O/m^2/hr$ from portions of the lakes containing dense algae cultures.

To achieve objective two, bench-scale experiments of simulated algae cultivation under two different growth conditions (i.e., air- and CO_2-sparging) were conducted to determine the potential of N_2O production under growth conditions similar to that of previously published research. Higher N_2O emissions were produced when cultivated under a headspace of CO_2, which is anticipated to be the preferred method for commercial algae cultivation. N_2O emissions produced under these conditions were up to 32 μg N_2O/g algae biomass. Headspace N_2O concentrations measured for this thesis research were comparable to those reported by Fagerstone et al. (2011), further validating this research. For both studies, greater N_2O emissions are observed under significantly less oxic conditions, where anoxic denitrification could be producing N_2O as a by-product, showing an importance of oxic state on N_2O production.

Finally, to receive approval as a renewable fuel, an "advanced biofuel" must show a 50% reduction in the average life-cycle GHG emissions for gasoline or diesel used as transportation fuel in 2005 (RFA, 2007). Therefore, objective three of this thesis research used an existing LCA-based framework by Liu et al. (2013) for algae-derived biofuels for integration of literature and laboratory results from the analysis of N_2O emissions from algae cultivation ponds, which revealed that accounting for N_2O could dramatically increase the life-cycle GWP of algae-derived gasoline by as much as a factor of 10, depending on system boundaries and parameter selection.

Prior to the inclusion of N_2O into the LCA by Liu et al. (2013), the process's GWP was within reach of that mark (Liu et al., 2013). However, even a small amount of N_2O production during cultivation could push an algae-derived biofuel production process out of compliance and possibly prevent approval under the EPA's RFS2. Thus, it is imperative to quantify the exact amount of N_2O released, along with any other previously undocumented GHG emissions that could contribute to the GWP of the production of algae-derived biofuels. Due to this substantive increase in GWP, future research is needed to ensure that algae-derived biofuel production processes are in compliance with the regulations set by RFS2.

7.0 Future Work

The engineering goal for algae-derived biofuels is to minimize the formation of nitrogen gases, especially N_2O, from algal cultivation facilities. From previously published data, it can be seen that N_2O formation is strongly impacted by environmental

conditions. Thus, the goal is to identify engineering approaches to optimize nitrogen use efficiency and mitigate N_2O-based GWP impacts in commercial algalculture. For forthcoming dissertation work, there are three main goals of focus.

First, bench-scale experiments will be conducted for longer cultivation periods, such as 14 and 21 days. These extended experiments will help us to better determine the appropriate N_2O flux for each experiment. N_2O flux curves will be created for each experiment to find the flux that is most commercially relevant to the cultivation of algae for biofuel production. Second, future bench-scale experiments will be conducted to determine if algae produce N_2O during or without illumination. Experiments will alter the daylight hours in order for N_2O measurements to be taken after a period of sparging during the dark hours, as seen in Figure 17. DO profiles for each growth condition experiment will also be analyzed.

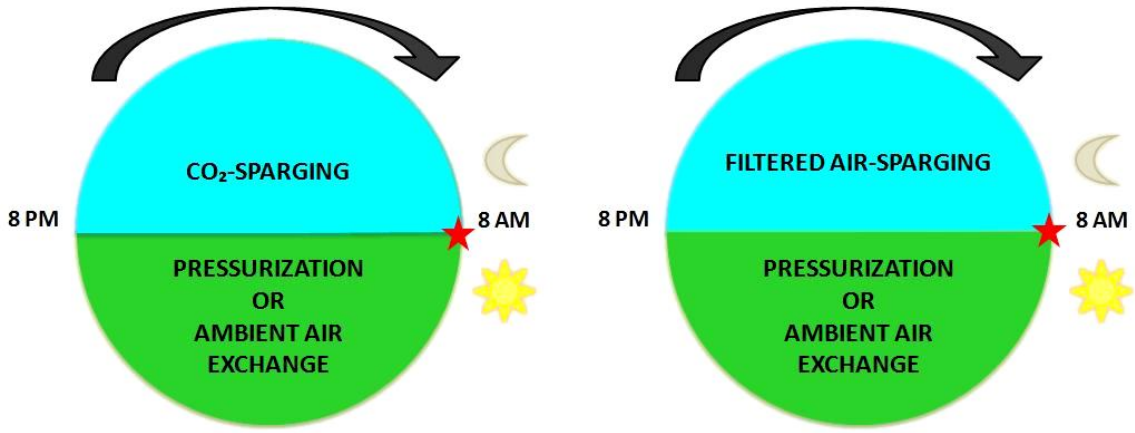

Figure 17: Future bench-scale experimental setup for cultivation of *S. dimorphus* under pure CO_2- (left) or ambient air-sparging (right) conditions to quantify N_2O production during dark conditions.

Lastly, future research will use Liu et al. (2013) LCA-based model as a framework to expand on the evaluation of the GWP of N_2O produced during algae cultivation to account for a multi-distillation system, not just for petroleum-based gasoline. In Liu et al.'s 2013 model, the model does not represented simultaneous distillation. For one barrel of algae-derived biofuels, the biofuel is either made into gasoline or diesel. In commercial systems, biofuels are typically divided into multiple distillates. Future evaluation will include research into the GWP of other distillates for diesel, jet-fuel, and co-distillation.

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Appendix A: Batch Cultivation of *S. dimorphus* in Freshwater Medium

1. Inoculate 14 x 10-mL screw-capped test tubes filled with 10 mL protease-peptone with 2 scoops of algae from solid slant using inoculating loop.

2. Incubate for 5 days in a rotating rotisserie subjected to 12 hours light and 12 hours dark (8 am – 8 pm) using a 125-W 6500-K fluorescent growth lamp in an aluminum reflector assemblage. Ensure the distance from the culture to the light to be between 6" – 12".

3. Passage to 4 x 250-mL flasks with sterile cover filled with 65 mL protease-peptone medium.

4. Incubate for 5 days in a crab shaker subjected to 12 hours light and 12 hours dark (8 am – 8 pm) using a 125-W 6500-K fluorescent growth lamp in an aluminum reflector assemblage. Ensure the distance from the culture to the light to be 6".

5. Passage to 6 x 500-mL flasks with sterile cover and magnetic stirring bar filled with ~233 mL MB3N medium. Add 1 mL of each of the three vitamins (filtered sterile) to each of the flask. Pump sterile air at Q = 7 scfh.

6. Flasks sit on top of a magnetic stirrer. Stir at minimum level (~200 – 300 rpm).

7. Stationary phase achieved at ~8 – 10 days.

Media Formulation:

Proteose-Peptone Medium - General purpose freshwater medium suitable for axenic

cultures.

Directions:

For 1 L total pH ~6.8.

1. **Add proteose-peptone to Bristol Medium**.

*For 1.5% agar medium, add 15 g of agar into the flask; do not mix.

2. Cover and autoclave medium.

#	Component	Amount	Stock Solution Concentration	Final Concentration
1	Bristol Medium	1 L		
2	Proteose Peptone (BD 211684)	1 g/L		

Bristol Medium - H.C. Bold's modification of Bristol's recipe (Bold 1949). General purpose freshwater medium and as Bristol's solution, an essential component of other media.

Directions:

For 1 L total.

1. To approximately 900 mL of **dH_2O** add each of the components in the order specified while stirring continuously.

2. Bring total volume to 1 L with dH_2O.

*For 1.5% agar medium, add 15 g of agar into the flask; do not mix.

3. Cover and autoclave medium.

4. Store at refrigerator temperature.

#	Component	Amount	Stock Solution Concentration	Final Concentration
1	$NaNO_3$ (Fisher BP360-500)	10 mL/L	10 g/400mL dH2O	2.94 mM
2	$CaCl_2 \cdot 2H_2O$ (Sigma C-3881)	10 mL/L	1 g/400mL dH2O	0.17 mM
3	$MgSO_4 \cdot 7H_2O$ (Sigma 230391)	10 mL/L	3 g/400mL dH2O	0.3 mM
4	K_2HPO_4 (Sigma P 3786)	10 mL/L	3 g/400mL dH2O	0.43 mM
5	KH_2PO_4 (Sigma P 0662)	10 mL/L	7 g/400mL dH2O	1.29 mM
6	NaCl (Fisher S271-500)	10 mL/L	1 g/400mL dH2O	0.43 mM

Modified Bold 3N Medium - Modification of Bold's recipe. General purpose freshwater medium used for axenic cultures, especially blue-greens and reds.

Directions:

For 1 L total pH 6.2.

1. To approximately 850 mL of dH_2O, add each of the components in the order specified (except vitamins) while stirring continuously.

2. Bring the total volume to 1 L with dH_2O.

*For 1.5% agar medium, add 15 g of agar into the flask; do not mix.

3. Cover and autoclave medium.

4. When cooled add vitamins.

*For agar medium, add vitamins, mix, and dispense before agar solidifies.

5. Store at refrigerator temperature.

#	Component	Amount	Stock Solution Concentration	Final Concentration
1	$NaNO_3$ (Fisher BP360-500)	30 mL/L	10 g/400mL dH2O	8.82 mM
2	$CaCl_2 \cdot 2H_2O$ (Sigma C-3881)	10 mL/L	1 g/400mL dH2O	0.17 mM
3	$MgSO_4 \cdot 7H_2O$ (Sigma 230391)	10 mL/L	3 g/400mL dH2O	0.3 mM
4	K_2HPO_4 (Sigma P 3786)	10 mL/L	3 g/400mL dH2O	0.43 mM
5	KH_2PO_4 (Sigma P 0662)	10 mL/L	7 g/400mL dH2O	1.29 mM
6	NaCl (Fisher S271-500)	10 mL/L	1 g/400mL dH2O	0.43 mM
7	P-IV Metal Solution	6 mL/L		
8	Soilwater: GR+ Medium	40 mL/L		
9	Vitamin B_{12}	1 mL/L		
10	Biotin Vitamin Solution	1 mL/L		
11	Thiamine Vitamin Solution	1 mL/L		

P-IV Metal Solution

Directions:

For 1 L total.

Note: Final concentration listed is for the stock solution.

1. To approximately 950 mL of dH_2O, add the nutrients in the order listed while stirring continuously.

   Note: The Na_2EDTA should be fully dissolved before adding other components.

2. Bring total volume to 1 L with dH_2O.

3. Store at refrigerator temperature.

#	Component	Amount	Stock Solution Concentration	Final Concentration
1	$Na_2EDTA{\cdot}2H_2O$ (Sigma ED255)	0.75 g/L		2 mM
2	$FeCl_3{\cdot}6H_2O$ (Sigma 1513)	0.097 g/L		0.36 mM
3	$MnCl_2{\cdot}4H_2O$ (Baker 2540)	0.041 g/L		0.21 mM
4	$ZnCl_2$ (Sigma Z-0152)	0.005 g/L		0.037 mM
5	$CoCl_2{\cdot}6H_2O$ (Sigma C-3169)	0.002 g/L		0.0084 mM
6	$Na_2MoO_4{\cdot}2H_2O$ (J.T. Baker 3764)	0.004 g/L		0.017 mM

Soilwater: GR+ Medium - The basic garden-type soilwater; includes a pinch of $CaCO_3$, which is added to the soil and water prior to steaming; suitable for most phototrophic freshwater algae. Optional ingredients: add vitamin B_{12} to cultures of *Volvox*; a pinch of NH_4MgPO_4. $6H_2O$ added to soilwater cultures of *Botryococcus*, *Synechococcus* and

some *Euglenoids* enhances growth and to LB 826 Gonium pectorale increases the numbers of 16-celled colonies formed.

Directions:

For 200 mL total.

1. Combine all components listed.

2. Cover the medium container and steam for 2 consecutive days, 3 hours on each day. Pasteurization is a gradual rising of temperature to approximately 95°C in 15 minutes. Then increased just over 98°C for the 3 hour duration. Cooling occurs gradually at room temperature.

3. Refrigerate 24 hours or more and bring to room temperature before using.

#	Component	Amount	Stock Solution Concentration	Final Concentration
1	Green House Soil	1 tsp/200 mL dH_20		
2	$CaCO_3$ (optional) (Fisher C 64)	1 mg/200 mL dH2O		0.05 mM

Green House Soil **-** Prior to its use in soil-water media, treat soil in batches by placing it in a heat-resistant pan lined with aluminum foil, fill the soil to a so depth of ¼ inch, and bake at 150°C for 2 hours. After it cools, cover the pan with aluminum foil and store in darkness at room temperature. Avoid excessive moisture during storage.

Considerations:

1. The soil should be a loam, with a mixture of particle sizes (sand, silt, clay).

2. It should contain a moderate amount (15 - 20%) of very-well-decomposed organic matter.

3. It must not contain pesticides, especially herbicides.

4. It should be soil that has been aged (preferably for 6 months or more) under moist conditions and not, for example, fresh potting soil, soil that contains fresh manure, or soil to which a commercial fertilizer was recently applied.

5. A slightly acidic soil derived from granite or other igneous rock is preferable to soil obtained from calcareous soils. Calcium carbonate can be added to the soilwater medium when it is prepared if a slightly alkaline medium is required.

6. Particulate matter in the soil such as gravel, Perlite, or vermiculite are not necessarily damaging but can be of considerable nuisance when wishing to quantitate the amount of soil used in the medium or when handling algae that are physically associated with the soil. Particulate organic matter, such as compost that is only partially degraded, should be avoided altogether.

Vitamin B_{12}

Directions:

For 200 mL total.

1. Prepare 200 mL of HEPES buffer (50 mM).
2. Adjust the pH to 7.8.
3. Add Vitamin B_{12} (0.1 mM) wait until fully dissolved.
4. Sterilize by 0.45 µm Millipore filter. Store in dark at freezer temperature.

** The amount of vitamins added can vary from medium to medium so the final concentration is not listed.

#	Component	Amount	Stock Solution Concentration	Final Concentration
1	HEPES buffer pH 7.8 (Sigma H-3375)	2.4 g/200 mL dH2O		
2	Vitamin B_{12} (cyanocobalamin, (Sigma V-6629)	0.027 g/200 mL dH2O		

Biotin Vitamin Solution

Directions:

For 200 mL total.

1. Prepare 200 mL of HEPES buffer (50 mM).
2. Adjust the pH to 7.8.
3. Add biotin (0.1 mM) wait until fully dissolved.

#	Component	Amount	Stock Solution Concentration	Final Concentration
1	HEPES buffer pH 7.8 (Sigma H-3375)	2.4 g/200 mL dH2O		
1	Biotin (Sigma B-4639)	0.005 g/200 mL dH2O		

Thiamine Vitamin Solution

Directions:

For 50 mL total.

1. Prepare 50 mL of HEPES buffer (50 mM).
2. Adjust the pH to 7.8.
3. Add Thiamine (6.5 mM) wait until fully dissolved.
4. Sterilize by 0.45 µm Millipore filter. Store in dark at freezer temperature.

** The amount of vitamins added can vary from medium to medium so the final concentration is not listed.

#	Component	Amount	Stock Solution Concentration	Final Concentration
1	HEPES buffer pH 7.8 (Sigma H-3375)	1.2 g/100 mL dH20		
2	Thiamine (Sigma T-1270)	0.11 g/100 mL dH20		

Appendix B: Calibration Curves for Experiment Analytical Methods

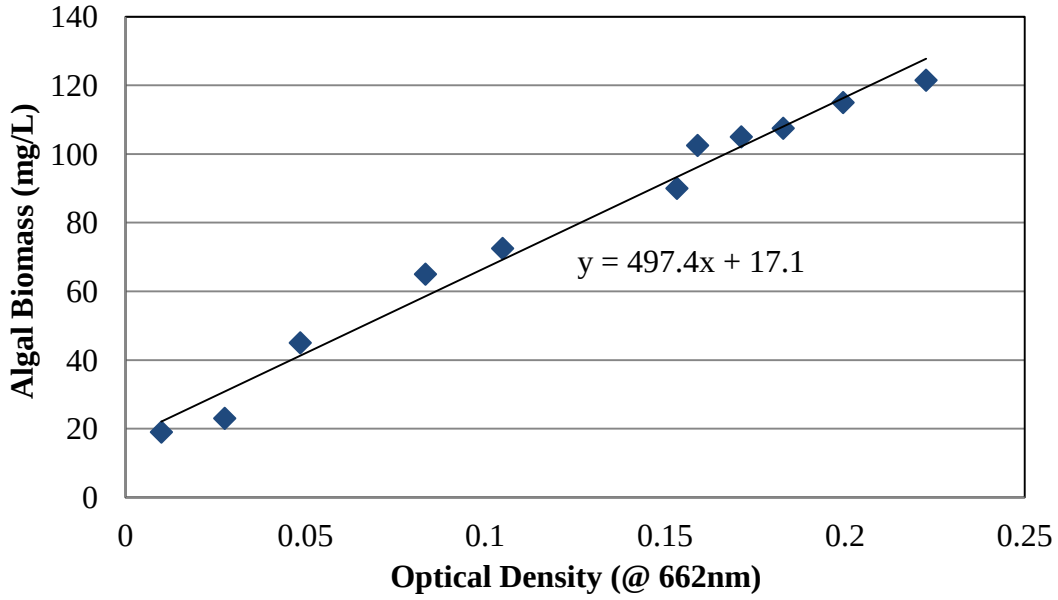

Figure B-1: Correlation Curve for Algae Biomass and Optical Density Measurements. Linear Fit Line R^2 Value 0.982. Calibration Range 0-0.25 abs.

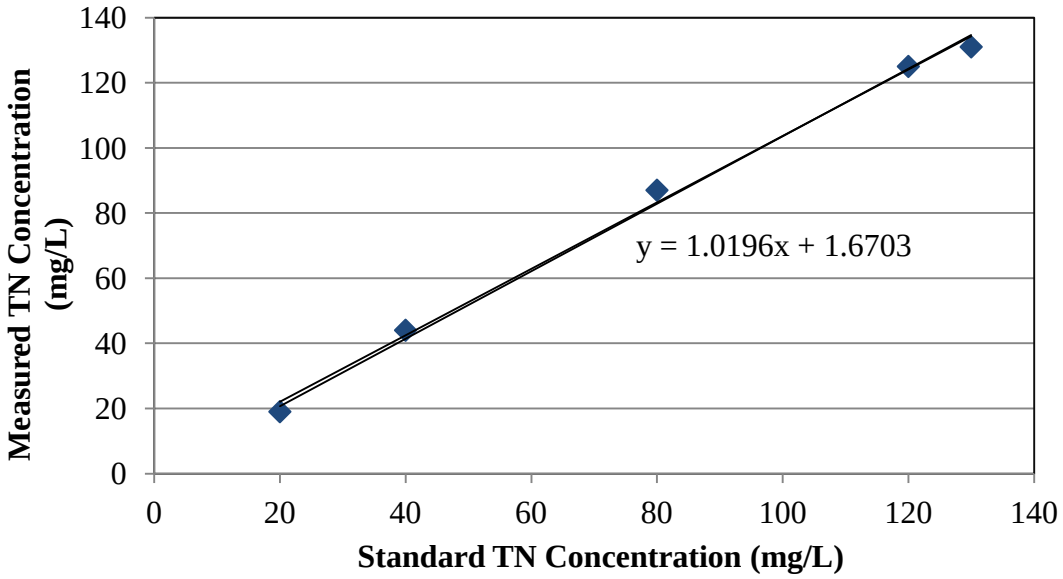

Figure B-2: Calibration Curve for Hach Total Nitrogen High Range Analytical Test Kit. Linear Fit Line R^2 Value 0.996. Calibration Range 0-150 mg/L.

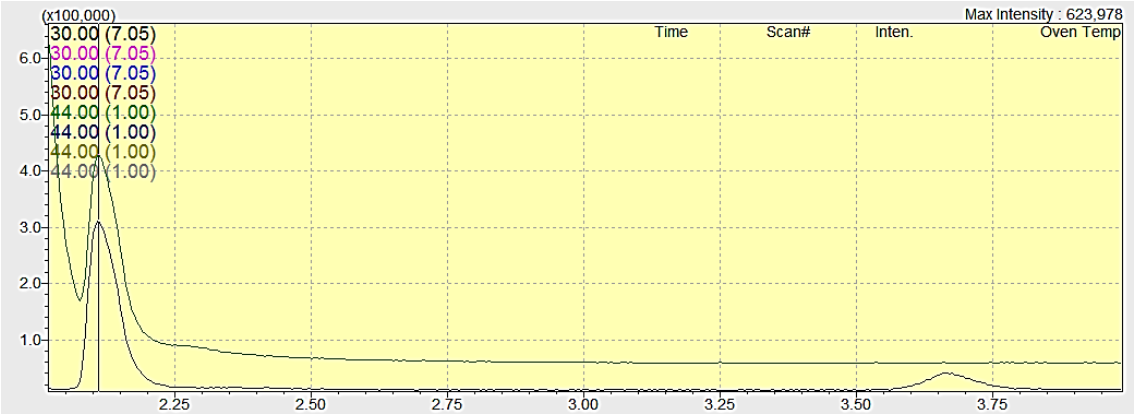

Figure B-3: Chromatograph profile from air-sparging cultivation experiment showing the peak response of N_2O flux from the algae reactors at the end of a 7 day cultivation period. N_2O output concentration shows distinct, clearly-resolved peaks that are suitable for analyzing fluxes.

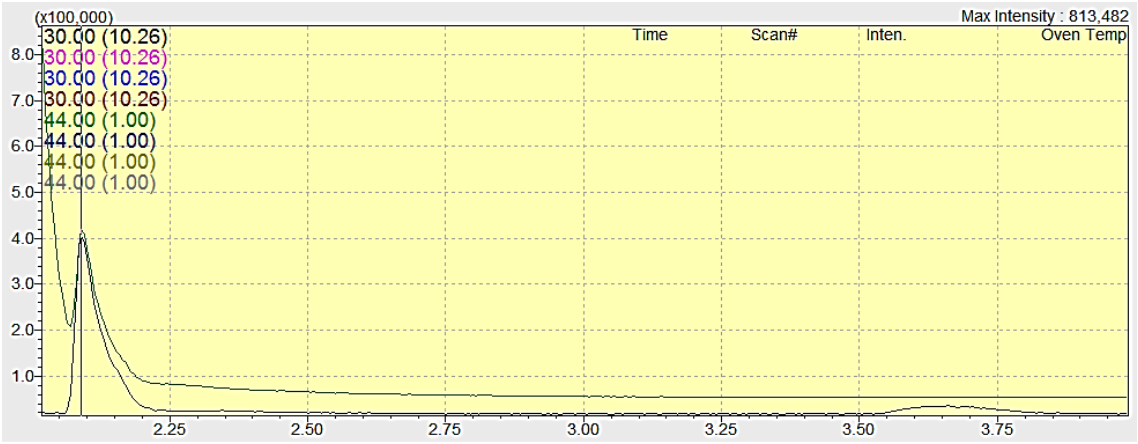

Figure B-4: Chromatograph profile from CO_2-sparging cultivation experiment showing the peak response of N_2O flux from the algae reactors at the end of a 7 day cultivation period. N_2O output concentration shows distinct, clearly-resolved peaks that are suitable for analyzing fluxes.

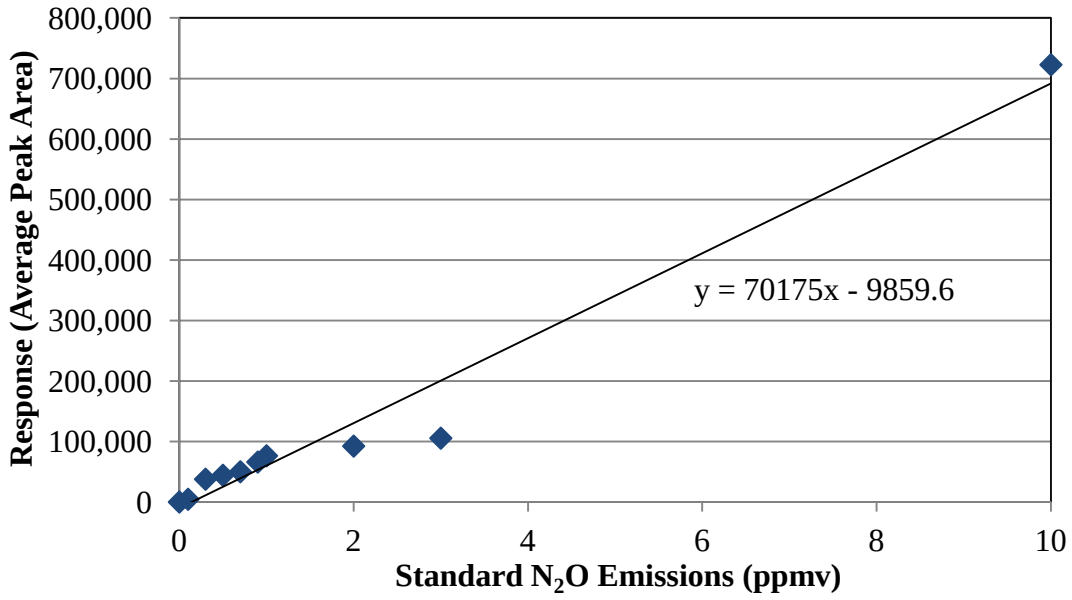

Figure B-5: Calibration Curve for N_2O by the Designed GC-MS Detection Method for 500 µL Injection Volume. Linear Fit Line R^2 Value 0.968. Calibration Range 0-10 ppmv.

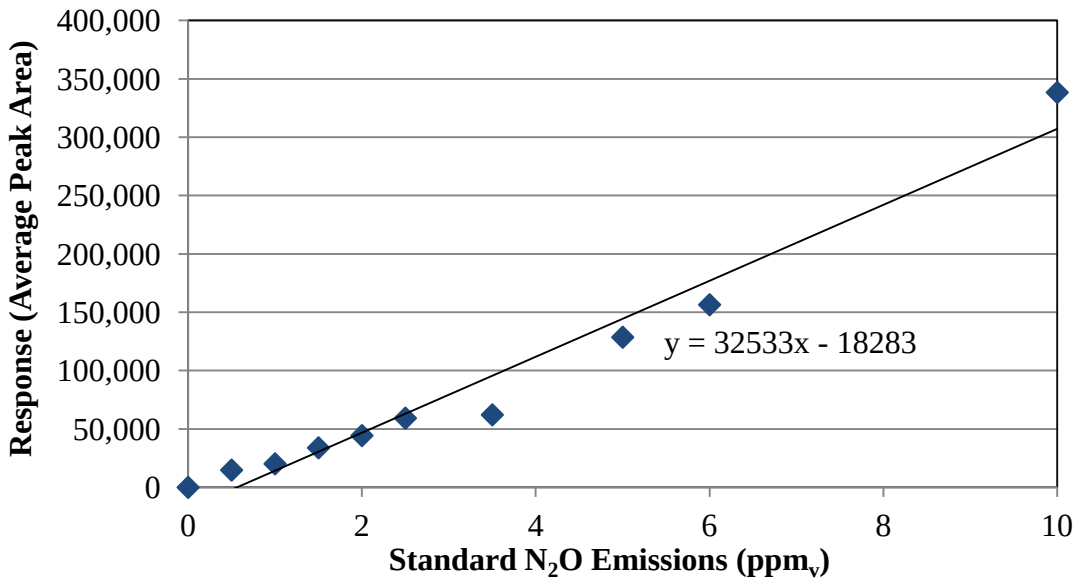

Figure B-6: Calibration Curve for N_2O by the Designed GC-MS Detection Method for 20 µL Injection Volume. Linear Fit Line R^2 Value 0.963. Calibration Range 0-10 ppmv.

Appendix C: Method Details for N_2O Flux Analysis using GC-MS

- GC-MS Real Time Analysis, Data Acquisition
    - Load Method
        - Download Initial Parameters
    - Sample Login
        - Download Initial Parameters
    - Start

Table C-1: Details for GC Tab of GC-MS Method for Analyzing N_2O Flux.

Parameter	Details	Parameter	Details
Inj. Port	SPL1	Total Flow (mL/min)	9.1
Inj. Heat Port	INJ1	Column Flow (mL/min)	1.43
Column Oven Temp. (°C)	33	Linear Velocity (cm/sec)	42.9
Injection Temp. (°C)	150	Purge Flow (mL/min)	0.5
Injection Mode	SPLIT	Split Ratio	5.0
Carrier Gas	He	Column Name	Rtx-Q-Bond
Prim. Press.	72.5-130.5	Length (m)	30
Flow Control Mode	Linear Velocity	Thickness (µm)	8.0
Pressure (psi)	14.5	Diameter (mm)	0.25
Final Temperature (°C)	33	Total Program Time (min)	4.0
Start Time (min)	2.02	Acq. Mode	SIM
End Time (min)	3.99	Event Time (sec)	0.3

Table C-2: Details for MS Tab of GC-MS Method for Analyzing N_2O Flux.

Parameter	Details	Parameter	Details
Ion Source Temp. (°C)	200	Micro Scan Width (µ)	0.0
Interface Temp. (°C)	150	Detector Voltage (V)	0.25
Solvent Cut Time (min)	2.0	Threshold	0.0

- GC-MS Post-Run Analysis, Qualitative & Quantitative Analysis
    - Data Explorer
    - Load Run
    - Qualitative
        - Peak Integrate for TIC (All Groups)
    - Quantitative
        - Peak Integrate for all IDS

Figure C-1: Sample chromatograph from algae cultivation experiments showing the peak response of N_2O flux during cultivation period. N_2O output concentration shows distinct, clearly-resolved peaks that are suitable for analyzing fluxes.

Appendix D: LCA-Based Model Framework Details

Table D-1: GWP for petroleum-based gasoline based on Liu et al. (2013) LCA-based model for algae-derived biofuels with RFS2 threshold for certification as renewable fuels.

GWP of Gasoline (g CO_2 eq/MJ)	**Baseline GWP from previous LCA (Liu et al., 2013)**	**GWP 2005 Transportation Fuel from GREET Model (Han et al., 2011)**	**RFS2 Threshold for GWP (Zhang et al., 2013)**
Pilot Scale	52.25	-	-
Full Scale	28.36	93.24	46.62

Table D-2: Algae yield and energy output for petroleum-based gasoline for pilot and full scale systems based on Liu et al. (2013) LCA-based model.

	Algae Yield (lb algae/ha-yr)	**Energy Output (MJ)**
Pilot Scale	96,562	287,221
Full Scale	128,750	957,405

Resulting GWP Adjustment for N_2O Production from LCA Analysis Using Different System Boundaries & Parameter Selection:

Accounting for Sparging Time Dimension & Algae Biomass Production

Table D-3 presents the GWP values calculated based on the different time dimensions of partial and full day production of N_2O for bench-scale experiments.

Minimum and maximum values of possible GWP impacts based on the different system boundaries and parameters selected are highlighted.

Table D-3: Algae producing N_2O for a portion of the day. 12 hours for air-sparging; 16 hours for CO_2-sparging. Minimum and maximum GWP values highlighted.

GWP N_2O Adjustment (g CO_2 eq/MJ)		**CO_2-Sparging Experiment**		**Air-Sparging Experiment**	
		Average Biomass	**Steady-State Biomass**	**Average Biomass**	**Steady-State Biomass**
Pilot-Scale	*Average*	31.2	219	18.4	129
	Maximum	112	781	52.6	368
Full-Scale	*Average*	12.5	87.5	7.37	51.6
	Maximum	44.6	313	21.1	147

Table D-4 presents the GWP values calculated based on the N_2O flux produced by average or steady-state biomass values. Minimum and maximum values of possible GWP impacts based on the different system boundaries and parameters selected are highlighted.

Table D-4: Algae producing N_2O throughout 24 hours of the day. Minimum and maximum GWP values are highlighted.

GWP N_2O Adjustment (g CO_2 eq/MJ)		**CO_2-Sparging Experiment**		**Air-Sparging Experiment**	
		Average Biomass	**Steady-State Biomass**	**Average Biomass**	**Steady-State Biomass**
Pilot Scale	*Average*	20.8	146	9.21	64.5
	Maximum	74.4	521	26.3	184
Full Scale	*Average*	8.33	58.3	3.68	25.8
	Maximum	29.8	208	10.5	73.7