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Growth of

Chlorella vulgaris and *Chlamydomonas reinhardtii* for biodiesel production and carbon dioxide capture

by

Mariana T. Farcas

**Thesis submitted to the
Davis College of Agriculture, Forestry, and Consumer Sciences
at West Virginia University
in partial fulfillment of the requirements
for the degree of**

**Master of Science
in
Applied and Environmental Microbiology**

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**Division of Plant and Soil Sciences
Morgantown, West Virginia**

2012

Keywords: growth, *Chlorella vulgaris*, *Chlamydomonas reinhardtii*, biodiesel, carbon dioxide capture, immobilized algae.

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ABSTRACT

Growth of *Chlorella vulgaris* and *Chlamydomonas reinhardtii* for biodiesel production and carbon dioxide capture

by Mariana T. Farcas

The growth of two strains of green microalgae, *Chlorella vulgaris* (UTEX 2714) and *Chlamydomonas reinhardtii* (UTEX 90) was tested in three types of media; Tris Acetate Phosphate (TAP), Bushnell Haas Broth (BHB), and Wright's Cryptophytes (WC buffered with either glycylglycine or Tris-base). Also, initial medium pH is ranging from 4 to 10, light intensity ranging from 100 to 600 µmol photons/m^{2}s, and CO_2 concentrations ranging from 0.038% (ambient) to 12%, were tested. WC medium at pH 8 buffered with glycylglycine sustained the highest yield and best buffering capacity for growth of both *C. vulgaris* and *C. reinhardtii.* A light intensity of 200 µmol photons m^{-2}s^{-1} provided for both good growth and electron transport rate (ETR). Both *C. vulgaris* and *C. reinhardtii* produced highest final yields when grown with 6% CO_2. Also, lipid content increased with increasing CO_2 concentration. Myristoleic acid (C14:1), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1n9), linoleic acid (C18:2), and docosahexaenoic acid (DHA) were found in higher content when *C. vulgaris* was grown on 12% CO_2, while the content of palmitoleic acid (C16:1), elaidic acid (C18:1t9), vaccenic acid (C18:1n7) were similar among all CO_2 concentration tested. CO_2 capture was explored using two approaches: consumption of known quantities of CO_2 in sealed serum bottles, and consumption of CO_2 flowing through immobilized algal beads. In both cases, fixation rate decreased with increasing CO_2 concentration. CO_2 consumption generally decreased over the five day experiment. The rate observed using immobilized algae was 20% of the maximum obtained in liquid culture, indicating the need to future optimize this novel method for CO_2 capture.

DEDICATION

I dedicate this thesis to my children and my husband. Without their love, encouragement, guidance, and support I never would have achieved this goal.

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GENERAL INTRODUCTION

As a result of the industrial revolution in the 20th century, an increased volume of CO_2 has been released into earth's atmosphere. The upper safety limit for atmospheric CO_2 is 350 parts per million (ppm) and since 1988 this limit has been exceeded (National Oceanic and Atmospheric Administration). CO_2 concentration in the atmosphere is still increasing and the outcome of this increase has already had a profound effect on the global environment. It is widely accepted that global warming is impacted by greenhouse gas emissions from anthropogenic activities (Intergovernmental Panel on Climate Change, 2005). Thus actions are being taken to mitigate greenhouse gas emissions from anthropogenic activities (Gough, 2008). There are three types of mitigations strategies: (1) chemical reaction-based approaches: washing with alkaline solutions, multiwalled carbon nanotubes, and amine coating activated carbon; (2) direct injection underground or to the ocean; and (3) biological CO_2 mitigation, with CO_2 being biologically converted to organic matter (Ho *et al.*, 2011). Biological CO_2 fixation is accomplished by all terrestrial plants and photosynthetic microorganisms through the process of photosynthesis. However, plants are expected to account for only a 3–6% reduction in global CO_2 emissions (Skjanes *et al.*, 2007). Costa *et al.* (2000) estimated that due to faster growth of microalgae and cyanobacteria, CO_2-fixation efficiency should be 10–50 times greater. Microalgal biomass also could be used as a feed stock for a variety of biofuels, medications, cosmetics, and nutritious foods, representing additional benefits from the microalgal CO_2 reduction process (de Morais and Costa, 2007).

In 2010, the U.S. Department of Energy funded twelve large projects (more than $107 M total) for "Innovative Concepts for Beneficial Reuse of Carbon Dioxide". Six of these projects are systems that use microalgae to capture CO_2 from power plant flue gas and convert it (via sunlight, water, and nutrients) into natural oils that are readily processed into liquid transportation fuels such as biodiesel. Photosynthetic green algae are good candidates to capture excessive amounts of atmospheric CO_2, since these organisms are capable of fixing CO_2 to produce energy and chemical compounds (fatty acids) upon exposure to sunlight.

Under optimal conditions of growth, fatty acids of microalgae constitute about 5–20%

of dry cell weight (DCW), but under unfavorable environmental or stress conditions for growth, algae alter their lipid biosynthetic pathways towards accumulation of neutral lipids (20–50% DCW), in the form of triacylglycerol (TAGs) (Guschina and Harwood, 2006; Hu *et al.*, 2008; Thompson, 1996). Based upon the photosynthetic efficiency and growth potential of algae, theoretical calculations indicate that annual oil production can be 100-fold greater than that of soybeans. However, few systems of algae-based biofuel production have progressed beyond the small laboratory or field testing stages (Hu *et al*., 2006).

The overall objective of the current project is to build an effective system to grow the green algae *Chlorella vulgaris* and *Chlamydomonas reinhardtii* for CO_2 capture and the production of biodiesel. Experiments were designed to compare photoautotrophic growth rates and biomass production by *Chlorella vulgaris* and *Chlamydomonas reinhardtii* with varying conditions of CO_2, light, and nutrient availability. This information was utilized to design two growth systems to investigate capture of CO_2 by *Chlorella vulgaris* and *Chlamydomonas reinhardtii* using: 1) fed-batch growth; and 2) a flow-through system based on immobilized algae in alginate beads.

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CHAPTER 1

Growth of *Chlorella vulgaris* and *Chlamydomonas reinhardtii* with varying growth media, pH, light, and CO_2 concentration

1.1. INTRODUCTION

Unicellular microalgae capture light energy for CO_2 fixation and biomass production. A portion of this biomass accumulates as triacylglycerols, which can be harvested to produce biodiesel transportation fuels (Francisco *et al.*, 2010). In laboratory experiments, growth conditions for production of *Chlorella vulgaris* and *Chlamydomonas reinhardtii* associated with fatty production were investigated.

Early attempts in the late 1800's to culture microalgae were reviewed by Allen and Nelson (1910) including basic media developed by Farmintzin, Beijerinck and Miquel. These works first described the significance of culture pH, iron, and vitamins for healthy algal growth and also the relationship to amounts of dissolved oxygen and carbonic acid, metal toxicity, and impurities in distilled water (Allen and Nelson, 1910). In the USA, the Stanford Research Institute was the first to attempt large-scale algal culture. They demonstrated the technical feasibility of *Chlorella* sp. production as an industrial raw material (Burlew, 1976). Spoehr and Milner (1949) first studied the effect of environmental conditions on chemical composition of *Chlorella* sp., and, in particular, the fatty acids profile of this alga. They found that nitrogen limitation increases lipid content.

Microalgae frequently are cultivated in batch culture. An algal inoculum is added to a specific growth medium containing nutrients necessary for growth until stationary phase is reached and the total culture is harvested (Richmond, 2004). Optimal batch growth conditions for microalgal cultures are strain specific, and final biomass production is influenced by many factors. The most important abiotic parameters regulating algal growth are: light cycle and intensity, temperature, nutrient quantity and quality, pH, carbon dioxide, salinity, and biotic factors like cell fragility and cell density. These factors affect photosynthesis, influence the pattern, pathway and activity of cellular metabolism and as a result dynamic cell composition (Anderson, 2005). Mechanical factors affecting growth include mixing, gas bubble size and distribution, and mass transfer (Schenk, 2008).

Growth of microalgae in culture media requires a suitable composition of essential macronutrients and micronutrient (Anderson, 2005). For autotrophic growth, supplemental CO_2/ HCO^{3-} often is necessary to supply the C-requirements of high yield autotrophic algal production. According to Redfield ratio, the stoichiometric ratio of the phytoplankton is C: N: P=106:16:1, thus, most media are nitrogen-rich relative to carbon, and carbon can become limiting (Riebesell *et al*., 1993).

The carbonate-bicarbonate system is the main buffer to control and uphold appropriate pH levels optimum for algal culture (Richmond, 2004). If culture pH increases quickly to 9 or higher, this may be a signal that carbon may be limiting. To prevent a reduced growth rate or cell yield, bubbling with CO_2 or adding more bicarbonate in late exponential phase often is used. Previous studies have shown that the CO_2 supply needs to be optimized, because higher levels can cause growth inhibition and low levels can limit growth (Rados *et al*., 1975). These maximum (inhibition) and minimum (limitation) concentrations vary among species. The most studied technique of supplying CO_2 is by injection of CO_2 mixed in the airflow (Chini *et al.,* 1996) or directly in the culture medium (Molina-Grima *et al.,* 1994).

Algal productivity requires the essential nutrients nitrogen and phosphorus (Schenk, 2008). The usual nitrogen sources in algal media are ammonium, nitrate, or urea. Special attention is required for ammonium as a sole source of nitrogen since the culture pH can drop sharply during active growth due to the release of H^+ ions, especially in dense cultures at high temperature. When nitrate is used as the sole nitrogen source, an increase in pH occurs (Richmond, 2004). Urea can also serve as a good nitrogen source once it is hydrolyzed, by the action of either the enzyme urease, or the enzyme urea amidolyase (Hodson, 1975). The major source of phosphorus in algal growth media is inorganic phosphates.

Other than C, N, and P, significant nutrients for algal nutrition are K, Ca, Mg, Cl, Mn, S, and Na in ionic form. They are constituents of chlorophyll (Mg) and are involved in photosynthesis (Cl, Mn), and cell metabolism (K, Na, Ca, S). Trace elements like Zn, Cu, Mo, and Mn are important nutrients involved in redox reactions (Taiz and Zeiger, 2002).

Some algae also require vitamins (Croft, 2006). The most common are vitamin B_{12} (cyanocobalamin), thiamine, and biotin. Usually, only few algae need all three vitamins (Provasoli and Carlucci, 1974).

Buffers are important components of algal media. The most common pH buffers used to prevent or reduce metal precipitation and to maintain constant pH values are: TRIS (2-amino-2-(hydroxymethyl)-1-3-propanediol) and glycylglycine (2-[(2-aminoacetyl) amino] acetic acid) (McLachlan, 1973). Comparing TRIS and glycylglycine with other buffers like MOPS (3-N-morpholino propanesulfonic acid), HEPES (N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulphonic acid]), and TAPS ([2-hydroxy-1, 1 bis (hydroxymethyl) ethyl] amino)-1-propanesulfonic acid]), Loeblich (1975) noted that TRIS and TAPS provided maximal growth with minimal pH change. TRIS also can serve as carbon source for contaminant bacteria, interfere with the analysis of dissolved organic nitrogen and ammonium, and toxic to some algal species (Fabregas *et al*., 1993). TRIS can have harmful effects on photosynthesis by inhibiting the mechanisms of HCO^{3-} transport across the plasma membrane (Axelsson *et al.* 2000; Hellblom *et al.,* 2001) or O_2 evolution in Photosystem II (Sofrova *et al*. 1978; Rickert *et al.* 1991).

Light energy is captured during photosynthesis for autotrophic CO_2 fixation. The photosynthetic process occurs in two phases, a photochemical phase that is light dependent and a biochemical dark phase that is light independent. ATP and NADPH produced during the light dependent phase are essential in the dark phase to synthesize molecules essential for growth (Laval and Mazliak, 1995). The optimal light intensity varies among different species, and the growth rate is lower at light intensities values below this value (Ojala, 1993). At high light intensities growth can be limited by the phenomenon of photoinhibition (Bouterfas *et al*., 2006). Therefore, the intensity, spectral quality and photoperiod of light must be considered because all can impact the circadian rhythm of photosynthesis, respiration (Piquemal, 1990), cellular division (Hobson *et al*., 1979), growth rate (Redalje and Laws, 1983), and enzymatic activities (Hobson *et al*., 1979). Light can be natural or provided by fluorescent tubes emitting either in the blue or the red light spectrum able to support photosynthesis. Care must be taken with artificial lights to prevent overheating of cultures. Algae exhibit a growth rate proportional to the duration of the effective light period (Foy and Gibson, 1993), and also a light/dark cycle is needed for a maximal growth.

Mechanical factors like mixing and aeration can influence optimal algal growth. In batch cultures, mixing is important for preventing sedimentation of algal cells and to ensure all algal cells are equally exposed to the light and nutrients (Molina-Grima *et al*., 1999). Air bubbles can

damage microalgae (Eriksen, 2008) due to shear sensitivity caused by eddies in the growth medium. To avoid cell adhesion to gas bubbles and reduce shear damage (cell death), cultures can be supplemented with non-ionic surfactants (Ma *et al*., 2004).

Biofuels are defined as solid, liquid, or gaseous fuels that are produced from biorenewable feedstock (Demirbas, 2009). As fossil hydrocarbons become limited and costly, conversion of algal biomass into liquid biofuels is becoming more cost-effective and attractive (Demirbas, 2011). The use of domestically produced biofuels can reduce political and economic vulnerability, reduce greenhouse gas emissions, and invigorate the economy by creating a new energy sector (Balat, 2010). For example, algae could use CO_2 from power plant emissions for conversion to biomass and into biofuels (Aksoy *et. al*, 2011). Studies showed the potential of microalgae to reduce CO_2 levels from industrial waste gas with an increase in biomass (Yun, 1997). There are advantages and disadvantages of biofuel production by microalgae. One advantage is the ability of algae to utilize less land resources and water than conventional agricultural crops. Another advantage is the high efficiency of CO_2 mitigation. The main problems that impede the commercial implementation of algae for biofuel production are the current low yield and high cost of biomass harvest, high capital costs to establish commercial facilities, and the increased care required by a microalgal farming facility compared to a conventional agricultural farm (Demirbas, 2011).

Previous studies (Converti *et al.*, 2009; Rodolfi *et al*., 2009) have shown that biochemical composition of microalgae and cell lipid content can vary as a result of changes in environmental manipulations (growth conditions, nutrient concentration, and nutrient availability). The purpose of the preliminary work was to study the growth of two strains of green microalgae, *Chlorella vulgaris* (UTEX 2714) and *Chlamydomonas reinhardtii* (UTEX 90). Three types of media Tris Acetate Phosphate (TAP), Bushnell Haas Broth (BHB), and Wright's Cryptophytes (WC), a pH range from 4 to 10, light intensity from 100 to 600 μmol photons $m^{-2}s^{-1}$, and CO_2 concentration of 0.038% (ambient), 3%, 6%, and 12%, were tested.

1.2.MATERIALS AND METHODS

1.2.1. Growth media and initial pH

Two strains of green microalgae *Chlorella vulgaris* (UTEX 2714) and *Chlamydomonas reinhardtii* (UTEX 90) were purchased from UTEX (The Culture Collection of Algae at the University of Texas at Austin). Both were maintained in under 12:12 light:dark cycle (200 µmol photons $m^{-2}s^{-1}$) at 25±2°C. Ambient air was bubbled through the vessels at~ 50 mL min^{-1}. The growth of both algae was compared in three media: Tris Acetate Phosphate (TAP), Bushnell Haas Broth (BHB), and Wright's Cryptophytes medium (WC).

Table 1 describes the chemical composition of Tris-Acetate-Phosphate (TAP) medium (Gorman and Levine, 1965). For the trace elements solution, EDTA was dissolved in boiling water, and the $FeSO_4$ was prepared last to avoid oxidation. All solutions were mixed, and EDTA was added last. Initially the mixture was green; however, after 1-2 weeks the solution eventually turned purple and could be used. Bushnell Haas Broth was purchased as a dehydrated medium (Difco, MD). The formula per liter is: $MgSO_4$ 0.2g, $CaCl_2$ 0.02g, K_2HPO_4 1g, KH_2PO_4 1g, NH_4NO_3 1g, and $FeCl_3$ 0.05g. The medium was prepared by dissolving 3.27g in 1L distilled water, followed by sterilization at 121-124˚C for 20 minutes. The third medium used was Wright's Cryptophytes (WC) medium (Guillard and Lorenzen, 1972). Table 2 describes the chemical composition of WC medium. Either glycylglycine (WC-Gly) or Tris base (WC-Tris) were used as buffers. Table 3 contains the formula for the trace metal solution used in WC medium. All components were added to 950 mL of dH_2O, the final volume was adjusted to 1 liter with dH_2O, and was sterilized at 121-124˚C for 20 minutes. To prepare the vitamin solution (Table 4) used in WC medium, the thiamine was dissolved in 950 mL of dH_2O, 1 mL of the primary stock of biotin (vit. H) and cyanocobalamin (vit. B_{12}) were added and the final volume was adjusted to 1 liter with dH_2O. The final solution was filter sterilized and stored at 4ºC. The initial pH of all three media (TAP, BHB, and WC) was adjusted to range from pH 4 to pH 10 using either 20% KOH or 1N HCl as needed.

Growth was compared using 24 vertical 200 ml glass test tubes attached to wood support. Six test tubes held 100 mL of TAP medium ranging from pH 4 to pH 10 (Fig. 1, Fig 2). Six more held BHB medium ranging from pH 4 to pH 10. Another twelve test tubes held WC medium ranging from pH 4 to pH 10, six of them using glycylglycine as buffer, and other six using Tris

base as buffer. The experiments were repeated twice. Two 460-watt metal GE grow lights (General Electric, Fairfield, CT) were suspended 1.5 m above the test tubes and supplied light (200 µmol photons $m^{-2}s^{-1}$) on a 12 hours light/12 hours dark schedule. Aluminum foil placed at a 45-degree angle at the base reflected light onto the test tubes. Un-inoculated controls were composed of a pH series of 4, 5, 6, 8, 9, and 10. The mouths of all tubes were closed using foam stoppers. Three mL of algal inocula (OD_{750}=0.250) (See Appendix 1) was injected into each cultures tubes using a syringe. Air (ambient CO_2 level = 0.038%) at 50 mL min^{-1} was bubbled through the testing tubes using a pipette to agitate the solution. Absorbance (OD_{750}) was measured every 24 hours during a 5-day period. The sampling was done by withdrawing three mL of algal suspension with a syringe, transfer to polymethacrilate cuvettes (Sigma-Aldrich, MO, USA), and determination the optical density (OD_{750}) using a Spectronic 20 Genesys spectophotometer (Spectronic Instruments, NY). The pH was measured every 24 hours using a Corning 320 pH Meter (Corning Incorporated, NY).

1.2.2. Light intensity

A second type of photobioreactor was constructed to investigate the effect of light intensity on growth rate and photosynthetic efficiency. Three mL inocula of *C. vulgaris* and *C. reihardtii* (OD_{750}=0.250) (See Appendix 1) were injected in 100 mL WC medium (pH=8) in the same test tubes used in the previous experiments but placed on their sides on two shelves illuminated with a variable light source (460-watt metal GE grow lights source, P.L Light Systems*)*. Light intensities were varied using a rheostat from 100-600 µmol photons $m^{-2}s^{-1}$ (12 hours light/ 12 hours dark) (Fig. 3) and air (CO_2=0.038%) was supplied at 50 mL min^{-1}. The experiments were repeated twice with three replicates for each algal species and light intensity. Control test tubes were run with air (ambient CO_2 level = 0.038%) at 50 mL/min in the same light condition but not inoculated. Absorbance was measured at 750 nm every 24 hours during a 5-day period. The sampling was done as described above.

Phytoplankton Analyzer (PHYTO-PAM, Heinz Walz GmbH, Germany) (Fig. 4) was used to measure the relative electron transport rate (ETR) as a function of PAR (Photosynthetically Active Radiation) on day two, three and four of the experiment. The measuring principle is based on selective amplification of a fluorescence signal which is

measured with the help of intense, but very short pulses of measuring light. Three mL of algal sample was withdrawn from the tests tubes and dark adapted, by keeping the polystyrene fluorometer cuvettes (Sigma-Aldrich, MO, USA) in the optical unit for five minutes. The minimal fluorescence yield (*F*o) was measured and immediately followed by the maximal fluorescence yield (*F*m). When the actinic light provided by the halogen lamp was switched on, the minimum fluorescence in actinic light (*F*t) was measured and followed by simultaneous measurement of the maximum fluorescence yield in actinic light (*F*m').

The maximum efficiency of dark-adapted PSII (*F*v/*F*m) was calculated as:

$Fv/Fm = Fm - Fo/Fm$

The maximum efficiency of PSII in actinic light (ΦPSII) was calculated as:

$\Phi PSII = (Fm' - Ft) / Fm'$

Photosynthetic ETR (μmol m^{-2} s^{-1}) was calculated as:

ETR (μmol electrons m^{-2} s^{-1}) = ΦPSII × incident PAR (μmol photons m^{-2} s^{-1}) × 0.5 × 0.84

where 0.5 is a correction factor based on the general assumption that half of the absorbed light energy was diverted to PSII, and 0.84 is absorptivity.

1.2.3. CO_2 concentration

To determine biomass production as a function of CO_2 concentration, a third type of photobioreactor was constructed. These photobioreactors consisted of six 160 mL glass serum bottles filled with 100 mL WC and sealed with solid stoppers (Fig. 5). Two needles were inserted through the stopper to bubble the gas through the medium and to provide an exit/sampling point. Laboratory air and CO_2 were mixed. CO_2 concentrations of ambient air (CO_2 level=0.038%), 3%, 6%, and 12% were established and verified by using a Carle AGC Series 100 gas chromatograph (Hach Co., Loveland, CO, USA) connected to a BD40 chart recorder (Kipp & Zonen, Inc., Bohemia, NY, USA). Light, held constant at 200 μmol m^{-2} s^{-1}, was provided by two GE grow lights source (12 hours light/12 hours dark) (General Electric, Fairfield, CT, USA). Three mL inocula of *C. vulgaris* and *C. reinhardtii* (OD_{750}=0.250) (See Appendix 1) were injected in 100 mL WC medium (pH=8). The experiments were repeated twice with three replicates for each algal species and CO_2 concentration. Two un-inoculated bottles were incubate under the same condition and used as controls.

Absorbance and ETR measurements were done as described above (See 1.2.2.).

1.2.4. Lipid extraction

Lipid and fatty acid composition were compared as a result of bubbling different CO_2 concentrations (mixed with air) through the medium as the carbon source for photosynthesis. Two systems were used for lipid extraction: 8000M Mixer/Mill® (SPEX SamplePrep LLC, Metuchen, NJ, USA) and Soxtec™ 2055 System (Foss Analytical, Denmark).

1.2.4.1. Mixer/Mill system

The SPEX SamplePrep 8000 Mixer/Mill is an efficient, compact laboratory mill capable of pulverizing samples in the 10-gram range by shaking the containers back and forth at approximately 1080 cycles per minute (Fig. 6). It has been used for pulverizing rocks, minerals, sand, cement, slag, ceramics, and many others hard samples (8000M Mixer/Mill, Spex SamplePrep, Operating Manual).

Extractions were performed using freeze dried algal biomass. One gram of dried cells was transferred to a 25 ml stainless steel vial of a mixer/mill shake container. Twenty mL chloroform/methanol (2/1) was added to the container and the sample was shaken for 10 minutes (1425 rpm). Then the entire mixture was transferred to 30 mL glass tube and centrifuged at 5000 rpm for 5 min to recover the liquid phase. After cell disruption, the mixture was washed with 4 ml 5% NaCl solution and vortexed for few seconds. After this step, the mixture was centrifuged at low speed (2000 rpm) for 10 min to separate the two phases. The upper phase was removed using a Pasteur pipette. The supernatant was collected and the solvent was vaporized under a nitrogen stream. The total lipid content was then measured gravimetrically using an analytical balance with e=0.0001g (Denver Instrument XE-100, NY, USA).

1.2.4.2. Soxtec system

The Soxtec™ 2055 System (Foss Analytical, Denmark) represents a new patented version of the Soxhlet extraction technique, consisting of an Extraction Unit and a Control Unit (Fig 7). The system is designed for maximum user convenience through batch handling of six samples at a time.

The extraction was performed using freeze dried algal biomass. One gram of freeze dried sample was weighed into a thimble and a defatted cotton plug was place on top of the sample.

Next, the samples were inserted into the extraction unit using the thimble support holder. A mixture of 45 ml chloroform/methanol (2/1) was added to the glass containers and 4-5 glass beads to relieve hot spots. After the 3-step extraction procedure consisting of boiling (20 min at 120ºC), rinsing (45 min), recovery (3 min), and drying (20 min), the extractable matter was collected in the cup. The total lipid content was measured gravimetrically as describe above.

1.2.5. Determinations of fat and fatty acids

The total fat and fatty acid content of the lipids were determined by the Agricultural Experimental Station, Chemical Laboratories, University of Missouri-Columbia. Samples were analyzed according to AOAC official method 996.06. Fat and fatty acids were extracted by the hydrolytic method and then methylated to fatty acid methyl esters (FAMEs) using BF3 in methanol, then quantified by capillary gas chromatography (GC).

1.3.RESULTS

1.3.1. Effect of growth medium and initial pH

Relative growth of *C. vulgaris* and *C. reinhardtii* was examined in four media: 1) Tris Acetate Phosphate (TAP); 2) Bushnell Haas Broth (BHB); 3) WC medium buffered with glycylglycine (WC-Gly); 4) WC medium buffered with Tris base (WC-Tris). The increase in OD_{750} of both algae in each medium over a five-day period is reported in Figure 8 and 10. Initial experiments focused on TAP and BHB media. TAP is widely accepted as a laboratory growth medium for microalgae (Anderson, 2005; Harris, 1989), while BHB is a pre-formulated simple salts solution used to examine carbon source utilization by aquatic bacteria (Roy *et al.*, 2002). At day five, all three factors of variability: algae type, medium type, and initial pH had a significant effect on final OD_{750} (See ANOVA table, Table 5). Overall, *C. vulgaris* displayed a greater growth response than *C. reinhardtii*. Between media types, TAP had a significantly higher final OD_{750} compared with BHB. The pH that supported the greatest final growth was pH 7, followed by pH 8. Lowest final growth was observed at pH 4 and pH 10.

There was a significant interaction between algae type and medium type (Table 5), i.e. the effect of algae type on growth is dependent on the medium type. *C. vulgaris* had a higher overall OD_{750} in TAP medium than BHB, while *C. reinhardtii* grew a better in BHB rather than TAP. The significant interaction between medium type and initial pH (Table 5) demonstrated

that TAP and BHB medium at pH 8 or 7 gave the highest growth. The lowest final OD_{750} was observed at pH 4. The effect of algae type on growth was dependent on the initial pH (Table 5). *C. vulgaris* at pH 8 and pH 7 showed the highest response and *C. reinhardtii* indicated a better growth at pH 7. The lowest response was observed at pH 4 for both algae.

The three-way interaction between algae type, medium type, and initial pH also was significant (Table 5). A simple comparison of the final OD_{750} obtained at day five for each alga in each medium is reported in Table 6. *C. vulgaris* grew optimally at pH 8 in TAP, while in BHB similar final growth was obtained at pH 6 and 7. In TAP *C. reinhardtii* grew equally well at pH 6 and 7, while in BHB had a similar growth response in the range of pH 6 to 8.

The growth responses described above in TAP and BHB media were complicated by the final pH measured in each culture vessel at day 5. Although un-inoculated controls maintained their initial pH readings throughout the incubation (Figure 12), inoculated samples generally exhibited a significant decrease of 0.5 to 1.0 pH units at the end of the growth experiment as a result of active algal growth (Figure 9 and 11, Table 7). In general, the observations of optimal growth at circumneutral pH in TAP and BHB are correct, but uncertainty of the actual medium pH at any given time compromises the interpretation of the statistical comparisons reported above. The data suggested the need for a more strongly buffered growth medium that would resist acidification resulting from algal growth. After an extensive literature review, a decision was made to test Wright's Cryptophyte Medium, buffered either with glyclglycine or Tris base. Superior growth was indeed observed in this medium (see below), however both WC-Gly and WC-Tris exhibited significant increases from initially acidic pH treatments, and significant decreases from initially basic conditions (range 0.5 to 3.1 pH units) over the 5 day incubation, again complicating an examination on the effect of pH on growth of these algae (Table 7 A, B).

Using WC medium, at day five all three factors of variability: algae type, medium type, and initial pH, had a significant effect on final OD_{750} (See ANOVA table, Table 8). Overall growth of *C. vulgaris* in both WC media types was significantly higher than that of *C. reinhardtii*. Furthermore, WC-Gly medium supported better overall growth of both algae than did WC-Tris. An initial pH of 8 resulted in a significantly higher final OD_{750} compared with all other pHs tested.

The effect of algae type on growth was dependent on the medium type (Table 8). Both algal species showed a significantly higher OD_{750} in WC-Gly medium compared with WC-Tris.

Also, there was no significant difference between *C. vulgaris* growth in WC-Tris and *C. reinhardtii* growth in WC-Gly. The interaction between algae type and initial pH (Table 8), demonstrated that *C. vulgaris* grew better at pH 8, while *C. reinhardtii* had the highest growth at pH 7. The interaction between medium type and initial pH (Table 8) demonstrated that WC-Gly at pH 8 gave the best growth response, and the lowest growth was observed with WC-Tris at pH 4.

The three-way interaction between algae type, medium type and initial pH was significant (Table 8). According to the Tukey HSD test, *C. vulgaris* grown in WC-Gly medium at pH 8 had the greatest growth response. The best response for *C. reinhardtii* was obtained in WC-Gly medium at pH 8 (Table 6).

Analysis of all growth data obtained using all four media demonstrated that the three-way interaction between algae type, medium type and initial pH was significant (Table 8). *C. vulgaris* grown in WC-Gly medium at pH 8 had the highest final growth. The best growth response for *C. reinhardtii* also was obtained in WC-Gly medium at pH 8 (Table 6). Therefore further experiments reported below were performed by growing both algae in WC-Gly medium adjusted to an initial pH of 8.

1.3.2. Effect of light intensity

Relative growth and the relative electron transport rate of *C. vulgaris* and *C. reinhardtii* were examined at light intensities ranging from 100 to 600 μmol photons $m^{-2}s^{-1}$. The absorbance at 750 nm is reported in Figure 13. The relative electron transport rate (ETR) as a function of PAR (Photosynthetically Active Radiation) during day two, three and four is reported in Figure 14, 15, and 16. The growth and ETR were analyzed by multivariate analysis of variance (MANOVA), followed by univariate ANOVA using a fully factorial three-way model and planned contrast analyses. MANOVA indicated a significant effect on growth of algae type ($F_{(1,24)}$=3.6844, p<0.0001) and light intensity ($F_{(5,24)}$=2.4308, p<0.0001). Also, the interaction between algae type and light intensity is significant ($F_{(5,24)}$=5.2018, p<0.0001).

On day two, neither algae species showed a significant difference in growth response (Table 9), but on day three *C. vulgaris* had significantly higher growth comparing with *C. reinhardtii* (Table 10). This result also was observed on day four (Table 11) (Figure 13).

The overall effect of light intensity over time among both algae species, indicated that on day two the highest growth was observed at higher light intensities, however on day three and four a light intensity of 200 or 300 µmol photons $m^{-2}s^{-1}$ offered a better growth.

The interaction between algae type and light intensity was significant (Table 9, 10 and 11) and suggested that *C. vulgaris* outgrew *C. reinhardtii* at all the light intensity tested. Also, *C. reinhardtii* growth was not significantly different among all light intensity studied. In the case of *C. vulgaris*, growth at light intensity of 300 µmol photons $m^{-2}s^{-1}$ was significantly higher comparing to all other tested.

MANOVA showed a significant effect on ETR of algal type ($F_{(1,11)}$=16.3654, p<0.0001), and light intensity ($F_{(5,11)}$=1.5951, p=0.0002). Also, the interaction between algae type and light intensity was significant ($F_{(5,11)}$=2.3229, p<0.0001).

Overall, *C. reinhardtii* showed a significant higher ETR over time compared to C. *vulgaris* (Table 12, 13, and 14) (Fig 14, 15, and 16).

On day four, light intensities from 200 to 500 µmol photons $m^{-2}s^{-1}$ resulted in significantly higher ETR than 100 and 600 µmol photons $m^{-2}s^{-1}$ (Table 14). ETR values obtained from *C. reinhardtii* at light intensities from 200 to 500 µmol photons $m^{-2}s^{-1}$ were significantly higher compared with results found at 100 and 600 µmol photons $m^{-2}s^{-1}$. ETR values for *C. vulgaris* were not significantly different among light intensities with the exception of 400 µmol photons $m^{-2}s^{-1}$.

1.3.3. Effect of CO_2 concentration

Relative growth and the relative electron transport rate of *C. vulgaris* and *C. reinhardtii* were examined at different CO_2 concentrations: 0.038% CO_2 (ambient), 3% CO_2, 6% CO_2 and 12% CO_2. The absorbance at 750 nm (Figure 17) and the relative electron transport rate (ETR) as a function of PAR (Photosynthetically Active Radiation) during day two, three and four is reported in Figure 18, 19, and 20. Growth and ETR were analyzed by multivariate analysis of variance (MANOVA), followed by univariate ANOVA using fully factorial three-way models and planned contrast analyses. MANOVA indicated a significant effect on growth of algae type ($F_{(1,16)}$=10.7660, p<0.0001), and %CO_2 tested ($F_{(3,16)}$=19.5426, p<0.0001). However, there is not

significant interaction between algae type and % CO_2 over time ($F_{(3,16)}$=0.5061, p=0.0805, α=0.050).

Overall, *C. vulgaris* showed significantly higher growth during entire experiment compared to C. *reinhardtii* (Table 15, 16, 17, and Fig. 17). Furthermore, the highest OD_{750} was obtained when 6% CO_2 was bubbled into the bottles, followed by 3% CO_2, 12% CO_2, and 0.038% CO_2. On day four, the growth of *C. vulgaris* at 6% CO_2 was not significantly different from the response at 12% CO_2, and *C. reinhardtii* growth at 6% CO_2 was not significantly different from the response of *C. reinhardtii* at 3% CO_2. The lowest OD_{750} was obtained during growth on ambient CO_2 for both algae (Table 17).

MANOVA indicated a significant effect on ETR of algae type ($F_{(1,16)}$=19.1880, p<0.0001), and different % CO_2 tested ($F_{(3,16)}$=3.7597, p<0.0001), and also a significant interaction between algae type and % CO_2 over time ($F_{(3,16)}$=3.4448, p<0.0001, α=0.050). Overall, ETR among both algae at CO_2 concentration tested decreases from day two (ETR=69), day three (ETR=54) and to day four (ETR=35).

During the entire experiment, *C. reinhardtii* showed a significant higher ETR compared to C. *vulgaris* (consistent with the results determined in the light experiment) (Fig. 18, 19, 20 and Table 18, 19, and 20). The highest ETR was obtained when 0.038% CO_2 was bubbled into the bottles and then decreases with increasing CO_2 concentration (exception day four, when ETR was higher at 6% CO_2 but not significantly different from ambient and 3% CO_2. Determinations on day three and four indicated that 12% CO_2 negatively affected the ETR.

1.3.4. Total lipid content of *C. vulgaris* and *C. reinhardtii* grown at ambient, 6% and 12% CO_2

The total lipid contents for the microalgae cultured in this study ranged from 8.57% to 17.79% of the dry weight (Table 23). ANOVA (Table 21) showed that the algal type and CO_2 concentration had a significant effect on lipid content, but the extraction type was not significant. The total lipid content of *C. vulgaris* (14.9121 g lipid/ 100g algae biomass) was significantly higher compared to *C. reinhardtii* (12.5746 g lipid/ 100g algae biomass). Furthermore, a higher CO_2 concentration resulted in significantly higher lipid content. The total lipid content at 12% CO_2

was 16.0475 g lipid/ 100g algae biomass, at 6% CO_2 the lipid content was 13.8056 g lipid/ 100g algae biomass, and at ambient CO_2 the lipid content was 11.3769 g lipid/ 100g algae biomass.

1.3.5. Total fat content of *C. vulgaris* and *C. reinhardtii* grown at ambient, 6% and 12% CO_2

Analyzing the percentage of total fatty acids per 100mg biomass, ANOVA indicated that algal type and CO_2 concentration had a significant effect on the total fat content, but the extraction type was not significant (Table 22). The total fat content of *C. vulgaris* (10.61 g fat/ 100g algae biomass) was significantly higher compared to *C. reinhardtii* (8.67 g fat/ 100g algae biomass). Furthermore, the data indicate that higher CO_2 concentrations resulted in significantly higher fat content. The total fats at 12% CO_2 were 11.3839 g/ 100g algae biomass, at 6% CO_2 the total fatty acids was 9.8777 g/ 100g algae biomass, and at ambient CO_2 total fats were 7.6600 g fat/ 100g algae biomass. There was no effect of algal type on the fat content depending on the level of extraction type, and no effect of algae type on the fat content depending on the level of %CO_2.

1.3.6. Fatty acids profiles of *C. vulgaris* and *C. reinhardtii* grown at ambient, 6% and 12% CO_2

Generally, linoleic acid (C18:2) and palmitic acid (C16:0) were found in a significantly higher concentration, followed by linolenic acid (ω18:3), elaidic acid (C18:1t9) and oleic acid (C18:1n9) ($F_{(9,29)}$=386.6372, p<0.0001). Fig. 21 presents the major fatty acid composition of *C. vulgaris* at different CO_2 concentrations.

The higher CO_2 concentrations had a significant effect on each fatty acids of *C. vulgaris* ($F_{(2,29)}$=11.1242, p<0.0001) (Fig. 21). Myristoleic acid (C14:1), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1n9), linoleic acid (C18:2, and DHA were found in higher content when *C. vulgaris* was grown on 12% CO_2, while the content of palmitoleic acid (C16:1), elaidic acid (C18:1t9), vaccenic acid (C18:1n7) were similar among all CO_2 concentration tested. On the contrary, linolenic acid (ω18:3) was greater when *C. vulgaris* was grown at ambient CO_2 rather than higher CO_2 concentrations.

For *C. reinhardtii,* linoleic acid (C18:2) and palmitic acid (C16:0) are found in a significant higher concentration, next oleic acid (C18:1n9) ($F_{(9,29)}$=65.1665, p<0.0001) Fig. 22 presents the

major fatty acid composition of *C. reinhardtii* at different CO_2 concentrations. Similar to *C. vulgaris,* the CO_2 concentrations have a significant effect on each fatty acids of *C. reinhardtii* ($F_{(2,29)}$=24.0857, p<0.0001) (Fig. 22). Cells grown at 12% CO_2 and 6% CO_2 will results with a higher content of fatty acids compared with cells grown at ambient CO_2. Palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1n9), linoleic acid (C18:2), and DHA is found in higher content when *C. reinhrdtii* is grown at 12% CO_2. The CO_2 concentration did not effect the content of these fatty acids: myristoleic acid (C14:1), palmitoleic acid (C16:1), elaidic acid (C18:1t9), linoleic acid (C18:2), and vaccenic acid (C18:1n7).

1.4. DISCUSSION

1.4.1. Growth media and initial pH

TAP and WC media previously have been used by several researchers to examine such factors as growth rate, stress response, photosynthetic rate, oil accumulation, and heavy metal removal by microalgae (Harris, 1989; Anderson, 2005; Fischer *et al*., 2006; Jo *et al*., 2006; Laurinavichene *et al*., 2002; Ribeiro *et al*., 2010; Siaut *et al*., 2011, Vandamme *et al*., 2011). In the present study the highest growth yield of both *Chlorella vulgaris* and *Chlamydomonas reinhardtii* was obtained using WC medium supplemented with glycylglycine. This WC medium, buffered with glyclylglycine rather than Tris, uses nitrate rather than ammonium as a nitrogen source, is supplemented with vitamins and Na_2SiO_3, and contains lower concentrations of $CaCl_2$ and $MgSO_4$ than the other media tested. TAP medium originally was developed for growth of photoheterotrophs using acetate as a carbon source, and may not be optimimal for photoautotrophic growth. Commonly, TAP is used for those algae which use ammonium rather than nitrate as a nitrogen source (Awasthi, 2005). BHB medium, a simple phosphate buffered salts solution formulated without a carbon source, normally is used to study hydrocarbon-degrading bacteria (Roy *et al.,* 2002) and has not previously been used for photoautotrophic growth of microalgae.

The buffering capacity of both WC medium with glycylglycine and WC medium supplemented with Tris buffer was strong as evidenced by changes in initial pH to more circumneutral values. Consistent with the known ecophysiology of these algae (Taiz, 2002), greatest growth yields were obtained under neutral to slightly alkaline conditions. Lowest growth yields occurred at the lowest (pH 4) and highest (pH 10) initial medium pH. Decreasing pH in

TAP and BHB media generally was observed with time. Fabregas (1993) noticed pronounced bacterial populations in non-axenic cultures which inhibited algal growth; however, once TRIS buffer was omitted from the medium normal microalgal growth was recovered. Ursi *et al.* (2008) found that TRIS had a deleterious effect on growth rate and pigment content of *Gracilaria birdiae*. Sofrova *et al.* (1978) demonstrated that TRIS and substances with similar structure (tricine, pentaerythritol, mannitol and sucrose) affected photochemical activity and decreased oxygen evolution and photoreduction of an artificial electron acceptor (DCPIP). Hellblom *et al.* (2001) reported a 70% reduction in the net photosynthetic rate of *Zostera marina* when TRIS buffer was used. In contrast, comparing TRIS with eight other buffers, Loeblich (1975) reported the maximal growth rate and minimal pH fluctuations when TRIS was added in a seawater medium containing vitamins and sodium nitrate as nitrogen source for growth of *Cachonina niei*. In the present study, TRIS buffer worked well for growth with WC medium but not TAP. Most likely this was due to the use of nitrate rather than ammonium as the nitrogen source in WC medium. Ammonium utilization can cause production of excess acidity. Since WC medium with glycylglycine sustained the highest yield and best buffering capacity for growth of both *Chlorella vulgaris* and *Chlamydomonas reinhardtii,* this medium was chose to conduct all subsequent experiments.

1.4.2. Growth variation at different light intensity

Photosynthetic microalgae use light as their source of energy to assimilate inorganic carbon for conversion into organic matter. In an optimal system where no other factors are limiting, light availability controls the rate of photosynthesis and cell yield (Molina-Grima *et al.*, 1999). Light requirements vary greatly among different algal species (Ojala, 1993). Photoautotrophic growth systems must be designed to optimize light availability with increasing algal density and culture depth. Growth with light intensities ranging from 125 to 1,250 µmol photons $m^{-2}s^{-1}$ have been reported (Lavens and Sorgeloos, 1996). In the present study, growth yield of *C. vulgaris* increased with increasing light intensity from 100 µmol photons $m^{-2}s^{-1}$ to 300 µmol photons $m^{-2}s^{-1}$. There was no statistical difference in the observed growth yields of *Chlamydomonas reinhardtii* with light intensity. Bhola *et al.* (2011) reported that *C. vulgaris* tolerated light intensities ranging from 150 to 350 µmol photons m^{-2} s^{-1}, but that intensities

beyond 370 μmol photons m^{-2} s^{-1} resulted in decreased biomass production. Sorokin and Krauss (1958) reported decreased growth of *C. vulgaris* at light intensities above 90 μmol photons $m^{-2}s^{-1}$. Hartig *et al.* (1998) demonstrated a linear decrease of the photochemical efficiency of PSII of unspecified microphytobenthic algae with increasing irradiances. Fischer *et al.* (2006), observed a strong negative influence on the efficiency of photosynthesis and PSII photodamage in *C. reinhardtii* at high light (2,500 μmol photons $m^{-2}s^{-1}$) compared with low light (120 μmol photons $m^{-2}s^{-1}$) growth conditions. They suggested that the high charge separation at PSII exceeds the availability of the electron acceptor $NADP^+$ at PSI resulting in the degradation of the PSII core D1 protein. Sorokin and Krauss (1958) reported decreased growth of *Chlorella pyrenoidosa* and *C. reinhardtii* at light intensities above 300 μmol photons $m^{-2}s^{-1}$. Fans were used in the current study to decrease the temperature at the surface of the glass growth vessels for high light intensities. Temperatures measured at 400 to 600 μmol photons $m^{-2}s^{-1}$ ranged from 30 to 35ºC, compared with 25 to 27ºC for the experiments run at 100 to 300 μmol photons $m^{-2}s^{-1}$, which may somewhat confound comparison of these data.

1.4.3. Growth variation at different CO_2 concentration

In the present study, both *C. vulgaris* and *C. reinhardtii* produced highest final yields when grown with 6% CO_2 and were capable of growth in a 12% CO_2 atmosphere. de Morais and Costa (2007) observed no significant difference in growth rates in *Chlorella kessleri* and *Scenedesmus obliquus* cultivated with 6% and 12% CO_2. However, the growth rate value and biomass productivity of *C. kessleri* decreased when the CO_2 concentration reached 18%. Other studies have demonstrated that *C. vulgaris* can grow in elevated CO_2 atmospheres ranging from 10 to 40 % (Hirata *et. al*, 1996; Hanagata *et. al*, 1992). Maeda *et al*. (1995) found a strain of *Chlorella* sp. T-1 which could grow under 100% CO_2, even though the maximum growth rate occurred at 10%. Sung *et al*. (1999) compared the growth of a new species *Chlorella* KR-1 with concentrations of CO_2 ranging from 10 to 70% (v/v) in air. They reported optimum growth at 10% CO_2, while cultures at higher concentrations exhibited a longer lag period before growth started. Chinnasamy *et al.* (2009) reported increased chlorophyll content and biomass of *Chlorella vulgaris* ARC 1 grow with 6% and 14% CO_2.

The maximum CO_2 concentration reported for growth of *Chlamydomonas* sp. is 15% (Salih, 2011). Fischer *et al*. (2006) reported higher photosynthetic activity of *C. reinhardtii* cells

grown in a medium containing 5% CO_2 compared ambient air. Chinnasamy *et al.* (2009) reported that the carbonic anhydrase (an enzyme that catalyzes the rapid interconversion of CO_2 and water to bicarbonate and protons) of *Chlamydomonas reinhardtii* had much lower tolerance for high CO_2 levels compared with *C. vulgaris*. This mechanism may explain the inferior growth of *C. reinhardtii* at 12% compared with 3% and 6% CO_2 observed in the present study.

1.4.4. Variation of ETR at different light intensity and CO_2 concentration

The ETR measurements at different light intensities exhibited a decrease at higher light intensity (Fig. 14, 15, 16). Similar results were reported by Bhola et *al.* (2011) who noticed that for *C. vulgaris,* light intensity beyond 370 µmol photons $m^{-2}s^{-1}$ resulted in decreased ETR values. Also, Hartig *et al.* (1998) demonstrated a linear decrease of the actual photochemical efficiency of PSII of motile microphytobenthic algae with increasing irradiances. Fischer *et al.* (2006) showed that at high light intensity the photosynthetic electron transport chain is reduced because a high charge separation rate at PSII exceeds the availability of the electron acceptor $NADP^+$ at PSI. Also, they believe that this process stimulated the degradation of the PSII core D1 protein followed by inactivation of photosynthesis and photoinhibition.

Furthermore, ETR values decreased over time. A similar observation was described by Hofstraat *et al.* (1994) who noticed higher overall ETR for *Dunaliella tertiolecta* in the first five days of the experiment compared with the observation after 17 days. Masojídek *et al.* (2001) measured the light-response curves of photosynthetic oxygen evolution and ETR of a phytoplankton mixture from a fish pond, at various times of the day. Interestingly, they observed that the highest efficiency of light utilization was at 8:30 am and the highest inhibition of photosynthesis during the day was at 10:30 am. However, at 14:00 pm the ETR increased almost to the morning value.

The overall ETR results were considerably higher for *C. reinhardtii* than *C. vulgaris* during the light or CO_2 experiments (Fig. 14, 15, 16, 18, 19, and 20). The ETR results obtained from the light experiment did not demonstrate the same rapid decrease for *C. reinhardtii* compared to *C. vulgaris*; which might indicate that *C. reinhardtii* is able to easily adapt to different light intensities. This hypothesis is supported by the presence of an “eyespot” (the simplest visual system found in nature) in flagellates like *Chlamydomonas* sp. which assist

guidance of the algae in places where light conditions are optimal for photosynthetic growth (Hegemann, 1997).

1.4.5. Total lipid content of *C. vulgaris* and *C. reinhardtii* grown at ambient, 6% and 12% CO_2

Lipid content increased with increasing CO_2 concentration. Similar findings were reported by Widjaja *et al.* (2009) who demonstrated that increasing CO_2 concentrations increased lipid productivity of *C. vulgaris.* When the CO_2 flow was increased to 50 ml/min, the lipid productivity increased by more than 50% over standard controls conditions. Fulke *et al.* (2010) found maximum lipid productivity of *Chlorella* sp. at 3% CO_2, when a range from ambient to 15% was compared. However, Chiu *et al.* (2008) found that the lipid content of *Chlorella* sp. cultured at 2%, 5%, 10%, and 15% CO_2 was very similar in their single cell photobioreactor. Production of biomass and lipid content significantly increased (6x) in a six-cell parallel photobioreactor compared with the single-cell photobioreactor, implying that detention time and light exposure were the most important factors.

The highest lipid content of total algal dry biomass was almost 18% for *C. vulgaris* and for 14% *C. reinhardtii.* These numbers are lower than have been reported by others (Demirbas 2011; Liang, 2009; Mata, 2010). In his review, Demirbas (2011) reported the average lipid content on a dry matter basis of *C. vulgaris* was between 14-22 % and for *C. reinhardtii* was 21 %. Mata (2010) reported 5-58 % lipid content for *C. vulgaris*. In our experiments, the carbon source was provided via bubbling of CO_2-enriched air into the culture medium. The reason for these low amounts of lipid content might be due to low absorption of CO_2 since the bubbles formed were large and tended to rise to surface, leading to considerable waste of gas to the atmosphere. Carvalho and Malcata (2001), studying the transfer of CO_2 into microalgal cultures, demonstrated that the use of microporous hollow fibers, rather than plain bubbling, offers technological enhancements in the effectiveness of mass transfer. In addition, this type of system offers the opportunity to recirculate the gas and to use lower gas pressures, thus reducing operating costs.

1.4.6. Fatty acids profiles of *C. vulgaris* and *C. reinhardtii* grown at ambient, 6% and 12% CO_2

The quality of biodiesel is considerably affected by the fatty acids used in its production. In the present study, GC analysis showed that the main fatty acid components of the two examined microalgae ranged from C_{16} to C_{18} in chain length, and generally were unsaturated. As a result, biodiesel produced from these two microalgae would have low viscosity and desirable low-temperature properties (Knothe, 2008).

The fatty acids composition of *C. vulgaris* and *C. reinhardtii* varied with different CO_2 concentrations used during growth. Palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1n9), linoleic acid (C18:2) were higher in content when the algae were grown at 12% CO_2. These findings are comparable to observations made by Tsuzuki *et al.* (1990). Similarly, Yoo *et al.* (2010) reported the presence palmitic acid, oleic acid, and linoleic acid as the main fatty acids in *C. vulgaris*, whereas palmitoleic acid and stearic acid were minor components. They also reported that oleic acid content was higher in *Botryococcus braunii* compared to *C. vulgaris*. Decreased content of linolenic acid (ω18:3) in *C. vulgaris* exposed to higher CO_2 concentrations also was observed by Tsuzuki *et al.* (1990). In contrast, Moreno (2011) found a significantly higher content of linolenic acid when *C. vulgaris* UTEX 259 was grown at 5% CO_2. He also observed an increase in fatty acids grown at higher CO_2 concentration compared with ambient.

1.5. CONCLUSIONS

The choice of microalgae for CO_2 mitigation and production of biodiesel requires selection according to their growth rate, lipid content, and tolerance of high levels of CO_2. Results showed that *C. vulgaris* had a faster growth rate, higher lipid content, and better tolerance for high CO_2 levels compared to *C. reinhardtii* (Table 6, Table 11, Fig. 15). Furthermore, data indicate that CO_2 concentrations result in higher lipid content.

Wright's Cryptophytes medium supplemented with glycylglycine (WC-Gly) had the best buffering capacity and the highest growth rate among the media tested: Tris Acetate Phosphate (TAP), Bushnell Haas Broth (BHB), and Wright's Cryptophytes medium supplemented with TRIS (WC-Tris). A light intensity of 200 μmol photons $m^{-2}s^{-1}$ provided the best growth yield and ETR.

In future studies, it would be valuable to examine the effect of different temperatureon the growth yield and ETR. After nutrient quantity and quality, environmental temperature is the key parameter that controls the basic rates of all biochemical reactions in the algal cell. By optimizing the temperature, it may be possible to increase the biomass productivity and lipid content can be increased as has been suggested by Sandnes *et al.*, 2005, Converti *et al.*, 2009.

Examination on possible nitrogen limitation effects on the lipid content and biomass of the microalgae could provide a better perspective on the best method for enhancing algae biofuel. Deng *et al.* (2011) noticed that the external carbon source has little impact on lipid accumulation whereas N or S deficiency in a high carbon medium achieved significantly higher lipid accumulation.

1.6. REFERENCES

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Table 1. Chemical composition of TAP medium (Gorman and Levine, 1965).

Stock Solution (SL)	Quantity	Component	Concentration in SL	Conc. in final Medium
Tris base	2.42 g	$H_2NC(CH_2OH)_3$ Tris(hydroxymethyl)-aminomethan		2.00×10^{-2} M
TAP-salts (Beijerinck salts)	25 mL	NH_4Cl	15 g L^{-1}	7.00×10^{-3} M
		$MgSO_4 \cdot 7H_2O$	4 g L^{-1}	8.30×10^{-4} M
		$CaCl_2 \cdot 2H_2O$	2 g L^{-1}	4.50×10^{-4} M
Phosphate solution	0.375 mL	K_2HPO_4	28.8 g 100 mL^{-1}	1.65×10^{-3} M
		KH_2PO_4	14.4 g 100 mL^{-1}	1.05×10^{-3} M
Trace elements solution (Hutner trace elements)	1 mL	$Na_2EDTA \cdot 2H_2O$	5.00 g 100 mL^{-1}	1.34×10^{-4} M
		$ZnSO_4 \cdot 7H_2O$	2.20 g 100 mL^{-1}	1.36×10^{-4} M
		H_3BO_3	1.14 g 100 mL^{-1}	1.84×10^{-4} M
		$MnCl_2 \cdot 4H_2O$	0.50 g 100 mL^{-1}	4.00×10^{-5} M
		$FeSO_4 \cdot 7H_2O$	0.50 g 100 mL^{-1}	3.29×10^{-5} M
		$CoCl_2 \cdot 6H_2O$	0.16 g 100 mL^{-1}	1.23×10^{-5} M
		$CuSO_4 \cdot 5H_2O$	0.16 g 100 mL^{-1}	1.00×10^{-5} M
		$(NH_4)_6MoO_{24} \cdot 4H_2O$	0.11 g 100 mL^{-1}	4.44×10^{-6} M

Table 2. Chemical composition of WC medium (Guillard and Lorenzen, 1972).

Component	Quantity	Stock Solution	Molar Concentration in Final Medium
Buffer: Glycylglycine Tris base	500 mg	---	3.78×10^{-3} M
$NaNO_3$	1 mL	85.01 g L^{-1} dH_2O	1.00×10^{-3} M
$CaCl_2 \cdot 2H_2O$	1 mL	36.76 g L^{-1} dH_2O	2.50×10^{-4} M
$MgSO_4 \cdot 7H_2O$	1 mL	36.97 g L^{-1} dH_2O	1.50×10^{-4} M
$NaHCO_3$	1 mL	12.60 g L^{-1} dH_2O	1.50×10^{-4} M
$Na_2SiO_3 \cdot 9H_2O$	1 mL	28.42 g L^{-1} dH_2O	1.00×10^{-4} M
K_2HPO_4	1 mL	8.71 g L^{-1} dH_2O	5.00×10^{-5} M
Trace metal solution	1 mL	(see below)	---
Vitamin solution	1 mL	(see below)	---

Table 3. Trace metal solution for WC medium.

Component	Primary Stock Solution	Quantity	Molar Concentration in Final Medium
$FeCl_3 \cdot 6H_2O$	---	3.15 g	1.17×10^{-5} M
$Na_2EDTA \cdot 2H_2O$	---	4.36 g	1.17×10^{-5} M
$CuSO_4 \cdot 5H_2O$	10.0 g L^{-1} dH_2O	1 mL	4.01×10^{-8} M
$Na_2MoO_4 \cdot 2H_2O$	6.0 g L^{-1} dH_2O	1 mL	2.48×10^{-8} M
ZnSO4 • $7H_2O$	22.0 g L^{-1} dH_2O	1 mL	7.65×10^{-8} M
$CoCl_2 \cdot 6H_2O$	10.0 g L^{-1} dH_2O	1 mL	4.20×10^{-8} M
$MnCl_2 \cdot 4H_2O$	180.0 g L^{-1} dH_2O	1 mL	9.10×10^{-7} M
H_3BO_3	---	1.00 g	1.62×10^{-5} M

Table 4. Vitamin solution for WC medium.

Component	Primary Stock Solution	Quantity	Molar Concentration in Final Medium
Thiamine · HCl (vit. B_1)	---	100 mg	2.96×10^{-7} M
Biotin (vit. H)	0.5 g/L dH_2O	1 mL	2.05×10^{-9} M
Cyanocobalamin (vit. B_{12})	0.5 g/L dH_2O	1 mL	3.69×10^{-10} M

Figure 1. The experimental set-up for evaluation of growth medium and pH (before incoculation). The mouths of the tubes were covered with foam stoppers. Air at 50 mL/min was bubbled through the testing tubes using a pipette to agitate the solution. A syringe provides an exit/sampling point.

Figure 2. The experimental set-up for evaluation of growth medium and pH (day 5). Visible growth at pH 7 and pH 8. Controls show no growth.

Figure 3. The experimental set-up for optimization of light intensity. Test tubes were placed on their sides on two shelves illuminated with 460-watt metal GE grow lights source. Light was varied by rheostat from 100-600 µmol photons $m^{-2}s^{-1}$ (12 hours light/ 12 hours dark).

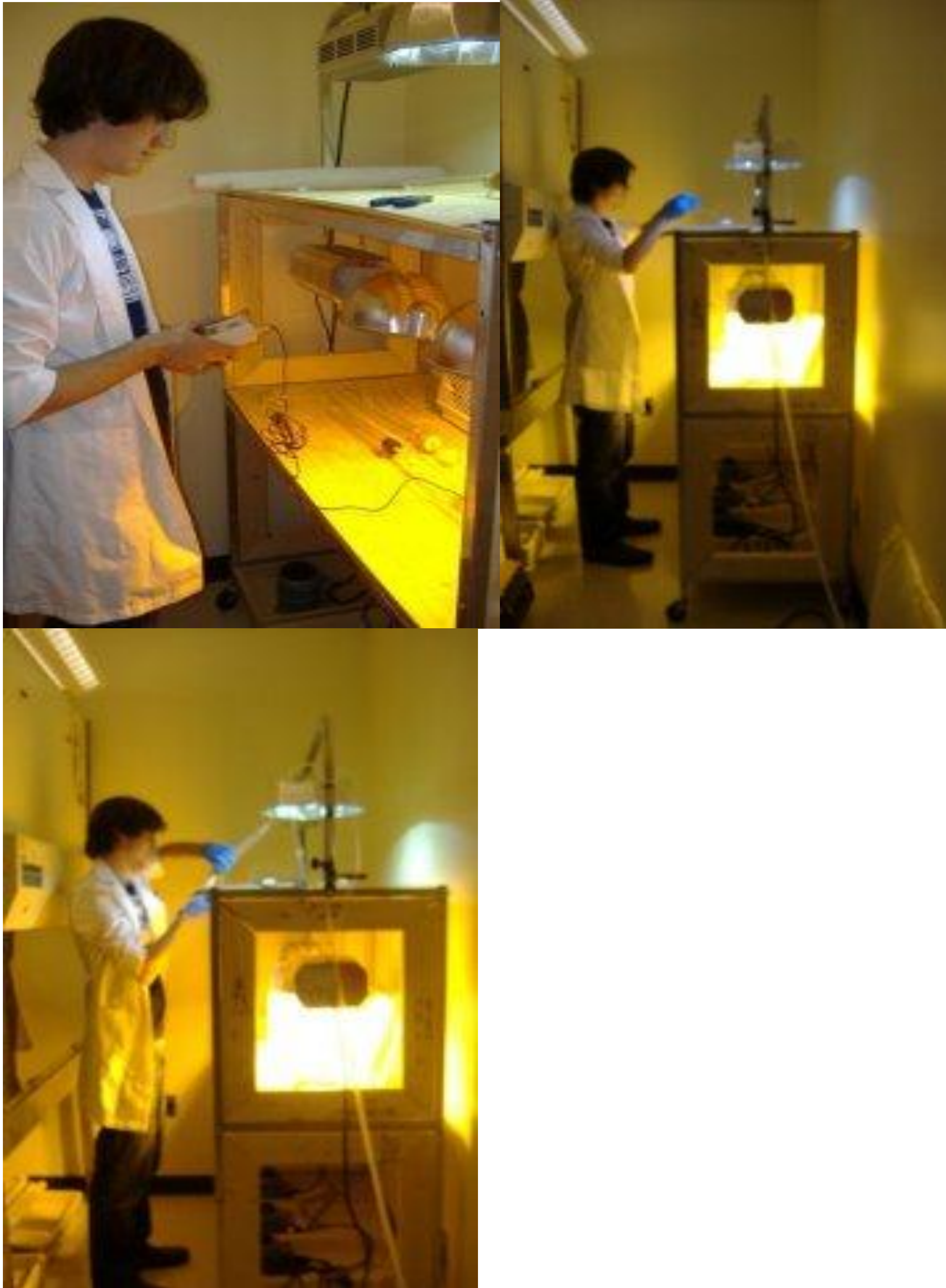


Figure 4. Phytoplankton Analyzer PHYTO-PAM (Heinz Walz GmbH, Germany) was used to measure the relative electron transport rate (ETR) as a function of PAR (Photosynthetically Active Radiation) on day two, three and four.

Figure 5. The experimental set-up for evaluation of CO_2 concentration**.** Glass serum bottles filled with WC medium and sealed with solid stoppers. Two needles were inserted in the stopper to bubble the gas (CO_2 level=0.038%, 3%, 6%, and 12%) through the medium and to provide an exit/sampling point. Light was held constant at 200 µmol $m^{-2}s^{-1}$. The medium was stirred continuously using magnetic stirrers.

Fig 6. 8000M Mixer/Mill® system (Spex SamplePrep,USA).

Fig.7.Soxtec™ 2055 system (Foss Analytical, Danemark).

Figure 8. *Chlorella vulgaris* growth curve using Tris Acetate Phosphate (TAP), Bushnell Haas Broth (BHB), and Wright's Cryptophytes (WC) medium over a 5-day period. The initial pH of the media ranged from pH 4 to pH 10.

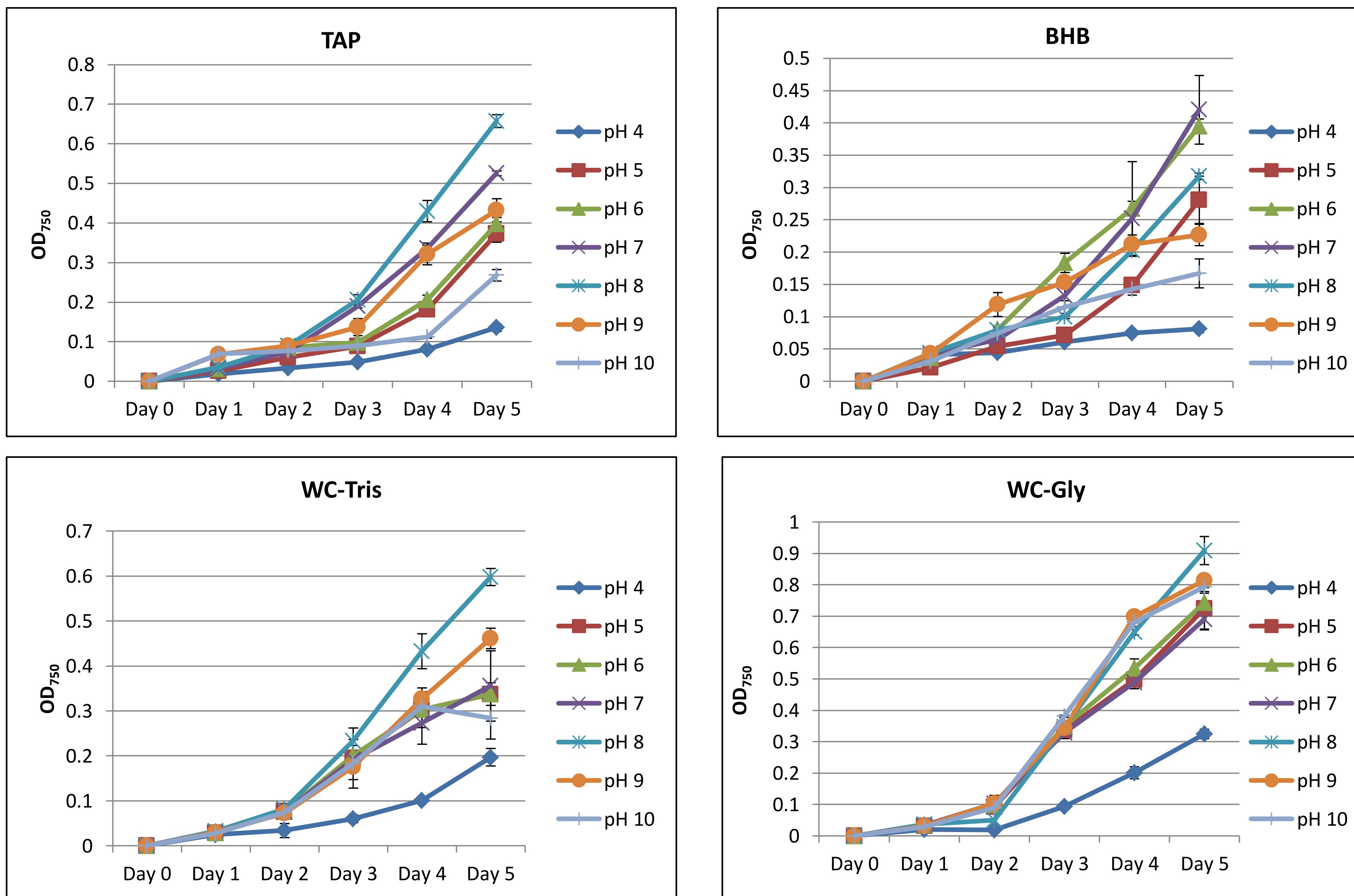


Figure 9. pH variation during growth of *Chlorella vulgaris* growth using Tris Acetate Phosphate (TAP), Bushnell Haas Broth (BHB), and Wright's Cryptophytes (WC) medium over a 5-day period.

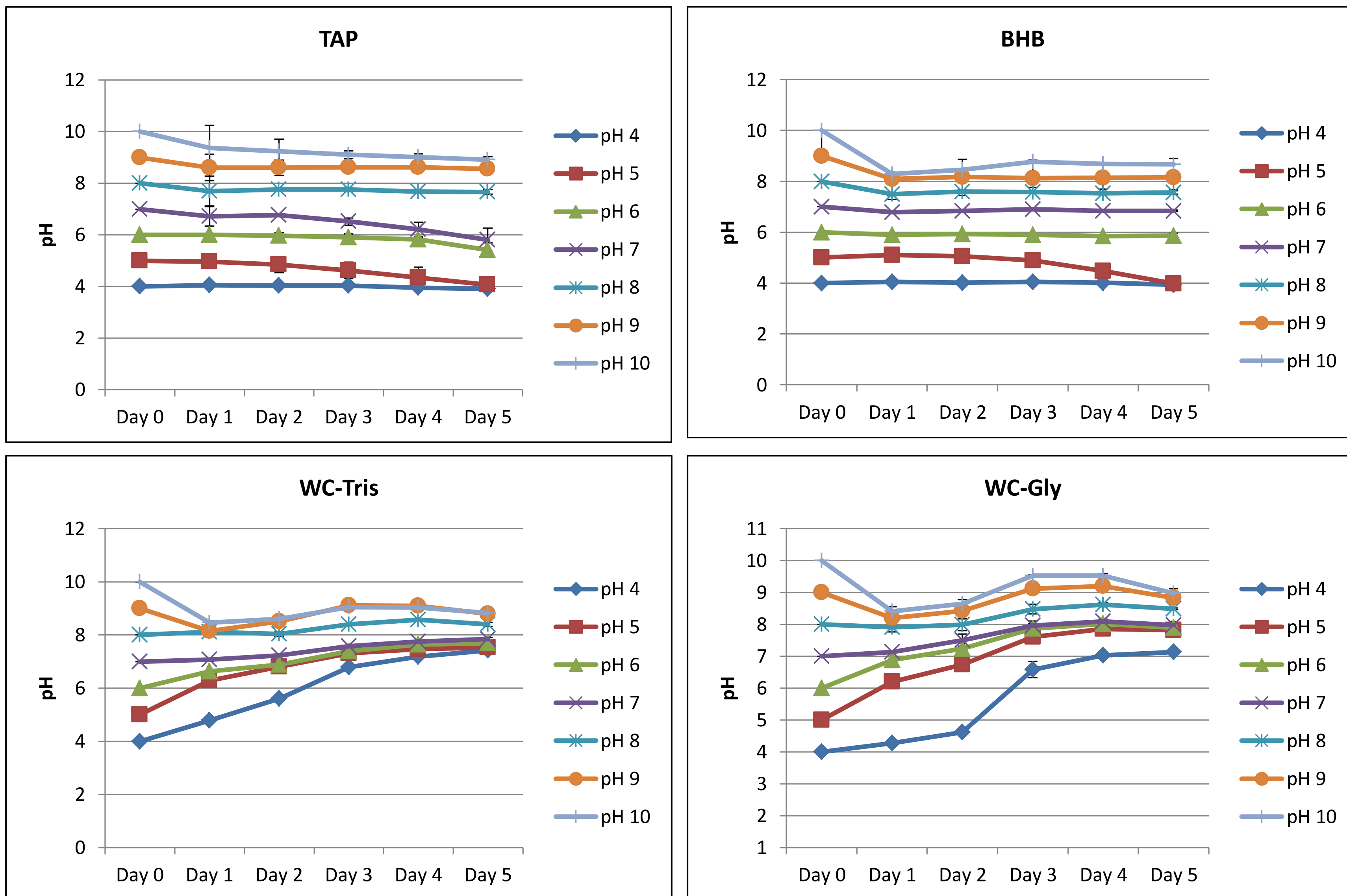


Figure 10. Growth of *Chlamydomonas reinhardtii* growth curve using Tris Acetate Phosphate (TAP), Bushnell Haas Broth (BHB), and Wright's Cryptophytes (WC) medium over a 5-day period. The initial pH of the media ranged from pH 4 to pH 10.

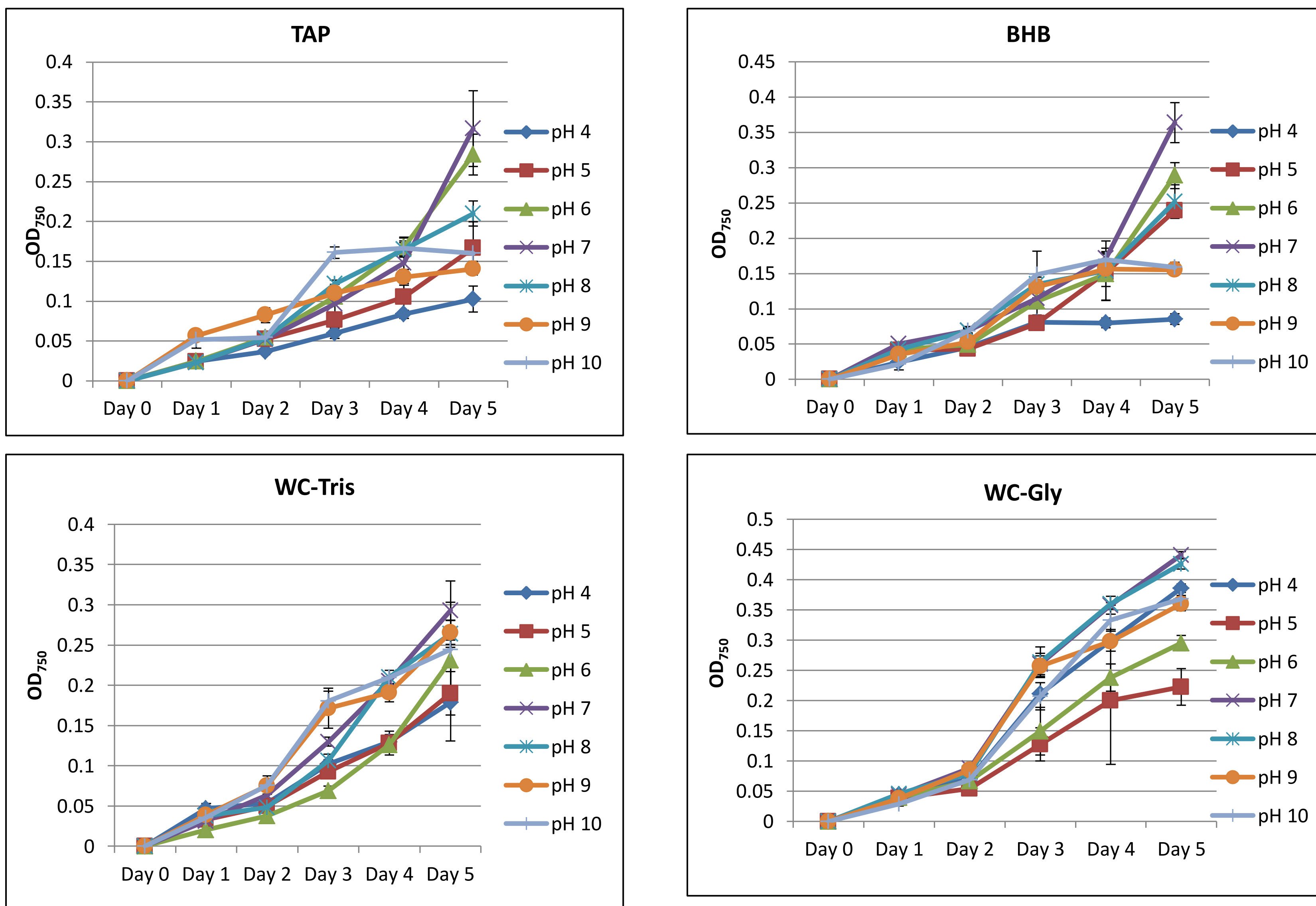
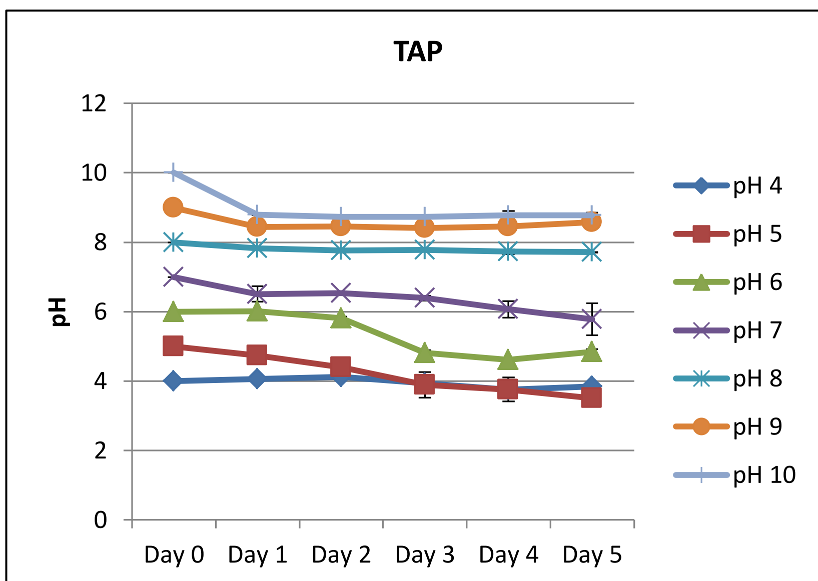
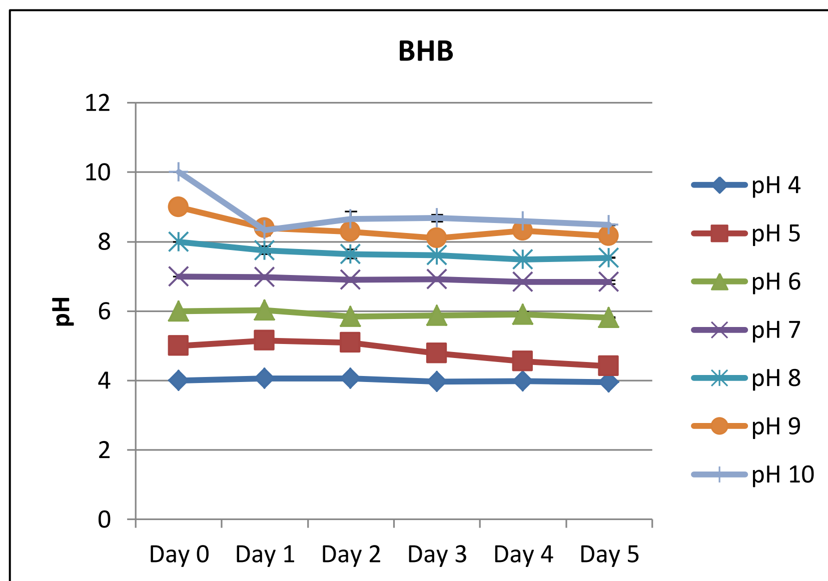
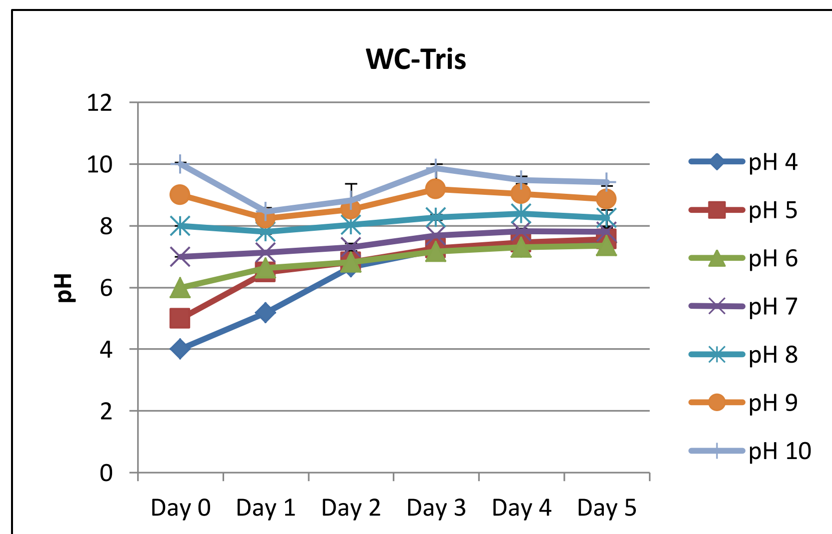


Figure 11. pH variation during *Chlamydomonas reihardtii* growth using Tris Acetate Phosphate (TAP), Bushnell Haas Broth (BHB), and Wright's Cryptophytes (WC) medium over a 5-day period.







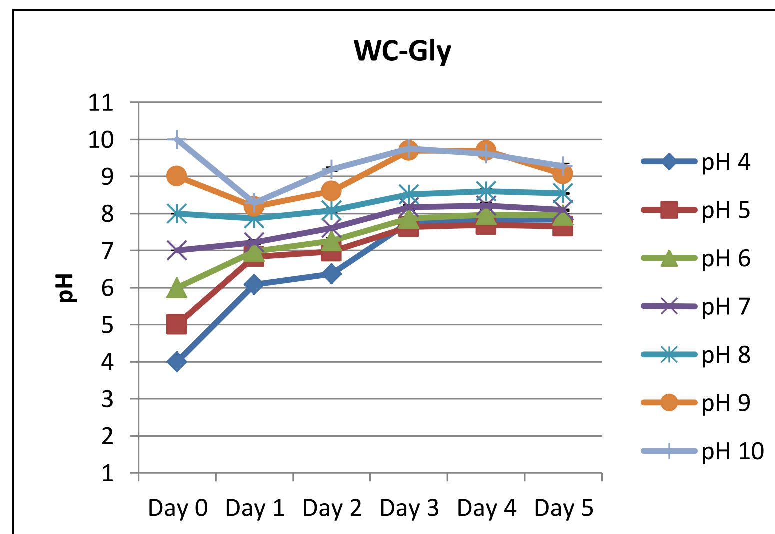
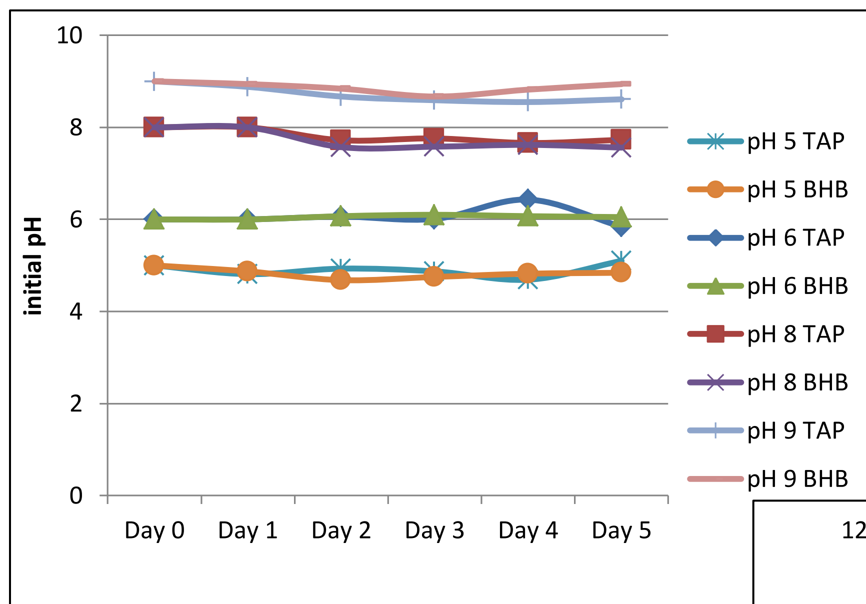


Fig. 12. Variation of pH in un-inoculated vessels.



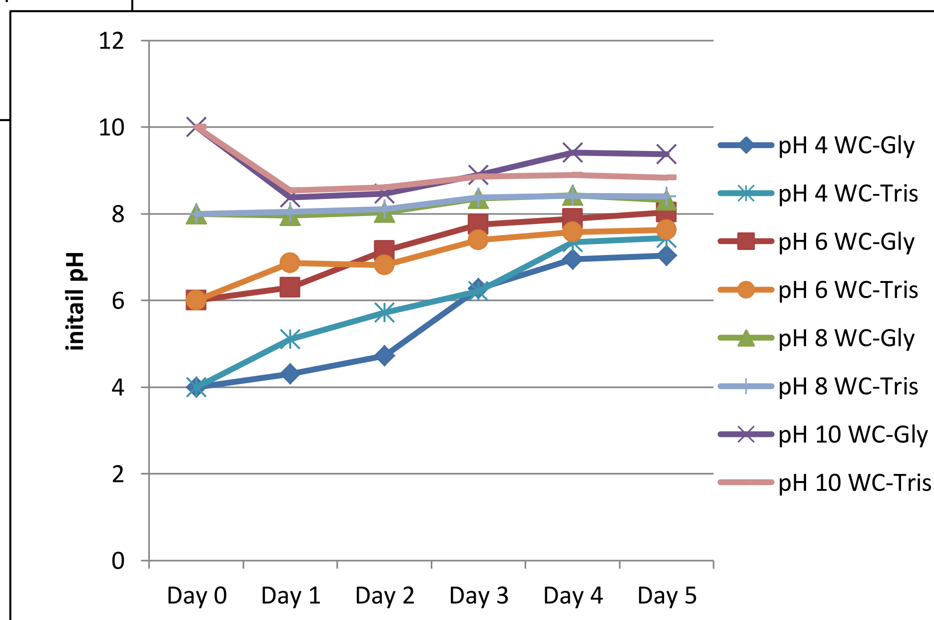


Table. 5. Analysis of variance (ANOVA) table at day five for TAP and BHB medium.

Source of Variation	dF	Sum of Squares	F Ratio	Prob > F
Model	27	2.0287	150.2228	<.0001
Algae type	1	0.3962	792.2854	<.0001
Medium type	1	0.0626	125.1723	<.0001
Algae type* Medium type	1	0.1725	344.9751	<.0001
Initial pH	6	1.1010	366.8946	<.0001
Algae type* Initial pH	6	0.1258	41.9232	<.0001
Medium type * Initial pH	6	0.0498	16.5848	<.0001
Algae type* Medium type* Initial pH	6	0.1206	40.1945	<.0001
Error	84	0.0420		
Total	111	2.0708		

Table 6. Comparison of OD_{750} at day five between the algae species (*=significant) and among all pH tested (Levels not connected by same letter are significantly different).

Medium type	TAP		BHB		WC-Gly		WC-Tris	
Initial pH	*C. vulgaris*	*C. reinhardtii*	*C. vulgaris*	*C. reinhardtii*	*C. vulgaris*	*C. reinhardtii*	*C. vulgaris*	*C. reinhardtii*
4	0.135^{f}	0.102^{d}	0.080^{e}	0.087^{d}	0.324^{e}	0.386*^{b}	0.197^{d}	0.223^{bc}
5	0.371*^{d}	0.170^{c}	0.269^{c}	0.237^{bc}	0.707*^{d}	0.229^{d}	0.332*^{bc}	0.190^{c}
6	0.390*^{d}	0.283^{a}	0.395*^{a}	0.289^{ab}	0.746*^{cd}	0.298^{c}	0.341*^{bc}	0.233^{bc}
7	0.512*^{b}	0.318^{a}	0.423^{a}	0.367^{a}	0.688*^{d}	0.439^{a}	0.382*^{b}	0.286^{a}
8	0.652*^{a}	0.207^{b}	0.314^{b}	0.299^{ab}	0.910*^{a}	0.442^{a}	0.582*^{a}	0.265^{ab}
9	0.431*^{c}	0.137^{cd}	0.227*^{c}	0.155^{d}	0.832*^{b}	0.361^{b}	0.505*^{a}	0.263^{ab}
10	0.268*^{e}	0.160^{c}	0.169^{d}	0.161^{cd}	0.796*^{bc}	0.365^{b}	0.282^{cd}	0.258^{ab}

Table 7. Comparison between initial pH and final pH (*=significant different) (α = 0.05).

A) C. vulgaris

Medium type	**Final pH**			
Initial pH	TAP	BHB	WC-Gly	WC-Tris
4	3.90	3.90	7.14*	7.41*
5	4.00*	3.97*	7.81*	7.52*
6	5.47*	5.79*	7.89*	7.67*
7	5.90*	6.82*	7.95*	7.84*
8	7.67*	7.53*	8.47*	8.38*
9	8.56*	8.14*	8.84*	8.76*
10	8.92*	8.66*	8.95*	8.76*

B) C. reinhardtii

Medium type	**Final pH**			
Initial pH	TAP	BHB	WC-Gly	WC-Gly
4	3.85*	3.93	7.83*	7.48*
5	3.53*	4.44*	7.67*	7.55*
6	4.84*	5.82*	7.91*	7.40*
7	5.79*	6.81*	8.09*	7.81*
8	7.74*	7.54*	8.53*	8.29*
9	8.57*	8.13*	8.99	8.87*
10	8.78*	8.47*	9.25*	9.43*

Table 8. Analysis of variance (ANOVA) table at day five for WC-Gly and WC-Tris medium.

Source of Variation	dF	Sum of Squares	F Ratio	Prob > F
Model	27	4.8698	248.7527	<.0001
Algae type	1	1.6391	2260.5490	<.0001
Medium type	1	1.4476	1996.4610	<.0001
Algae type* Medium type	1	0.3563	491.3842	<.0001
Initial pH	6	0.7150	164.3561	<.0001
Algae type* Initial pH	6	0.5144	118.2521	<.0001
Medium type * Initial pH	6	0.0605	13.9157	<.0001
Algae type* Medium type* Initial pH	6	0.1369	31.4642	<.0001
Error	84	0.0609		
Total	111	4.9307		

Table 9. Analysis of variance (ANOVA) table of OD_{750} with different light intensities on day two.

Source of Variation	dF	Sum of Squares	F Ratio	Prob > F
Model	11	0.0802	10.1755	<.0001
Algae type	1	0.0006	0.7928	0.3821
Light intensity	5	0.0547	15.2699	<.0001
Algae type* Light intensity	5	0.0249	6.9577	0.0004
Error	24	0.0609		
Total	35	0.0974		

Table 10. Analysis of variance (ANOVA) table of OD_{750} with different light intensities on day three.

Source of Variation	dF	Sum of Squares	F Ratio	Prob > F
Model	11	0.3558	11.5330	<.0001
Algae type	1	0.1126	40.1300	<.0001
Light intensity	5	0.0712	5.0756	0.0026
Algae type* Light intensity	5	0.1721	12.2711	<.0001
Error	24	0.0673		
Total	35	0.4232		

Table 11. Analysis of variance (ANOVA) table of OD_{750} with different light intensities on day four.

Source of Variation	dF	Sum of Squares	F Ratio	Prob > F
Model	11	1.1396	48.5503	<.0001
Algae type	1	0.4306	201.7673	<.0001
Light intensity	5	0.2966	27.7953	<.0001
Algae type* Light intensity	5	0.4125	38.6618	<.0001
Error	24	0.0512		
Total	35	1.1908		

Table 12. Analysis of variance (ANOVA) table of ETR with different light intensities on day two.

Source of Variation	dF	Sum of Squares	F Ratio	Prob > F
Model	11	3836.0716	15.3984	<.0001
Algae type	1	2437.3574	107.6218	<.0001
Light intensity	5	240.0179	2.1196	0.0978
Algae type* Light intensity	5	1158.6963	10.2325	<.0001
Error	24	543.5385		
Total	35	4379.6102		

Table 13. Analysis of variance (ANOVA) table of ETR with different light intensities on day three.

Source of Variation	dF	Sum of Squares	F Ratio	Prob > F
Model	11	4636.9867	14.6571	<.0001
Algae type	1	3741.3937	130.0882	<.0001
Light intensity	5	362.7087	2.5223	0.0570
Algae type* Light intensity	5	532.8843	3.7057	0.0126
Error	24	690.2505		
Total	35	5327.2372		

Table 14. Analysis of variance (ANOVA) table of ETR with different light intensities on day four.

Source of Variation	dF	Sum of Squares	F Ratio	Prob > F
Model	11	10203.237	68.6915	<.0001
Algae type	1	7199.7261	533.1801	<.0001
Light intensity	5	1339.7153	19.8427	<.0001
Algae type* Light intensity	5	1663.7960	24.6427	<.0001
Error	24	324.081		
Total	35	10527.318		

Figure 13. Growth of *Chlorella vulgaris* and *Chlamydomonas reinhardtii* at different light intensities (from 100-600 μmol photons $m^{-2}s^{-1}$).

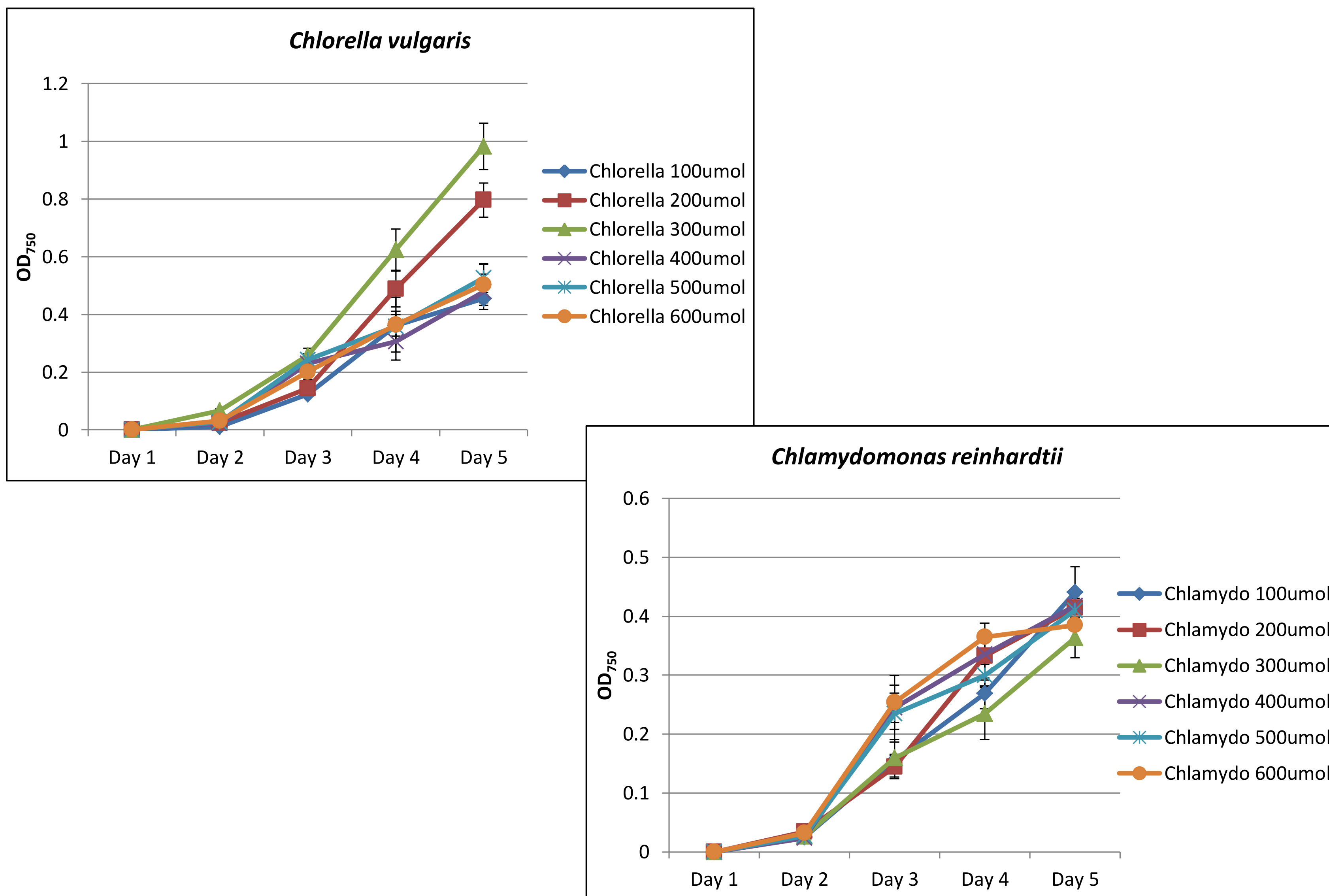
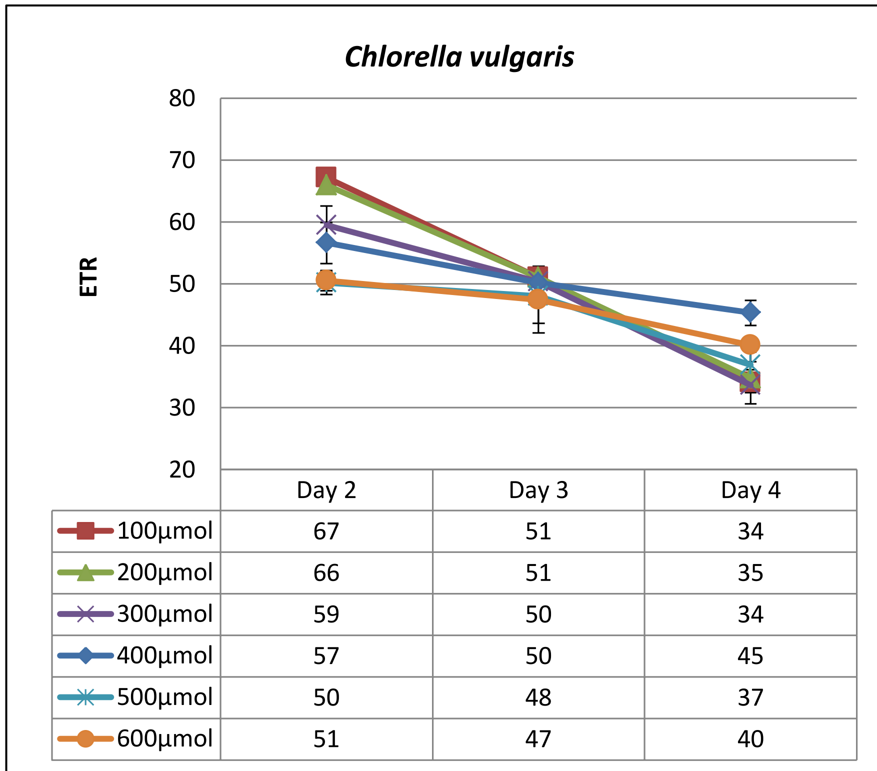
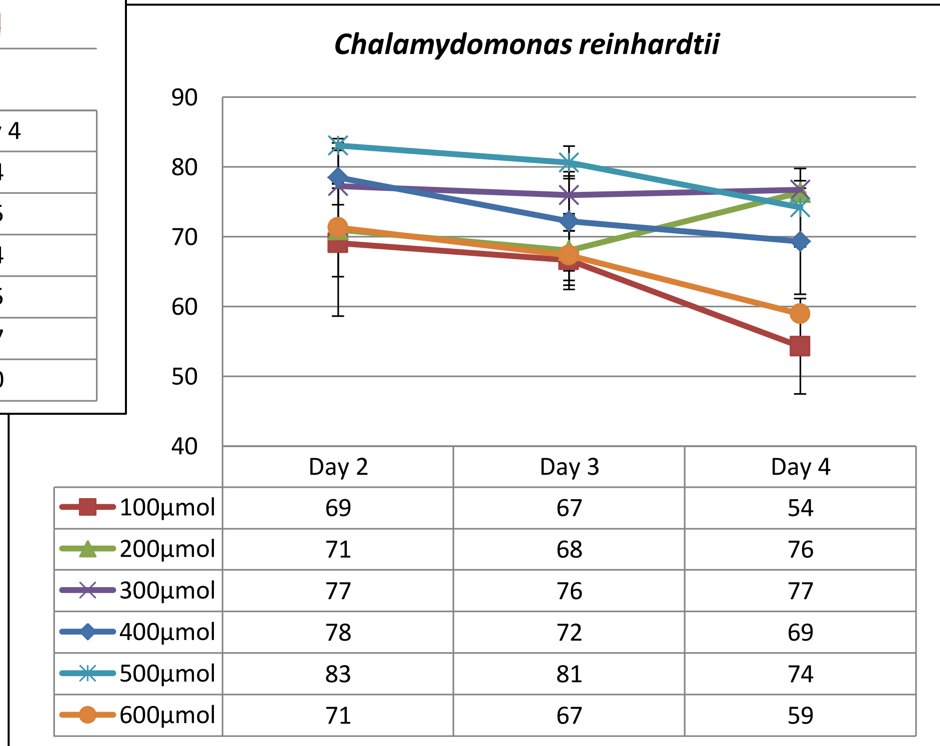


Figure 14. *Chlorella vulgaris* and *Chlamydomonas reinhardtii* relative electron transport rates (ETR) at different light intensities (from 100-600 µmol photons $m^{-2}s^{-1}$) measured on day two, three and four.



	Day 2	Day 3	Day 4
100µmol	67	51	34
200µmol	66	51	35
300µmol	59	50	34
400µmol	57	50	45
500µmol	50	48	37
600µmol	51	47	40



	Day 2	Day 3	Day 4
100µmol	69	67	54
200µmol	71	68	76
300µmol	77	76	77
400µmol	78	72	69
500µmol	83	81	74
600µmol	71	67	59

Figure 15. *Chlorella vulgaris* and *Chlamydomonas reinhardtii* relative ETR (Electron Transport Rate) as a function of PAR (Photosynthetically Active Radiation) at light intensities of 100-300 μmol photons $m^{-2}s^{-1}$ measured on day two, three and four.

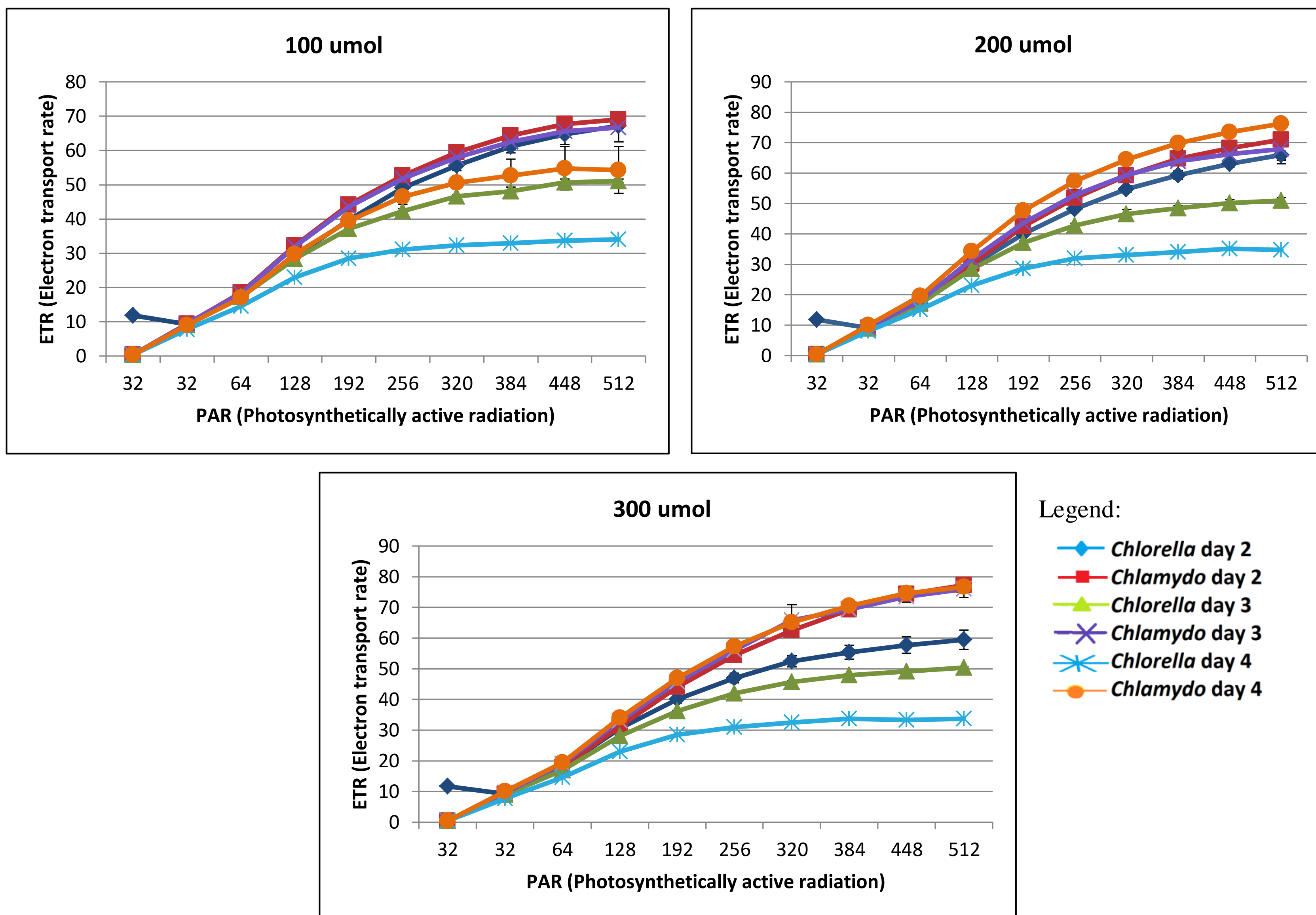


Figure 16. *Chlorella vulgaris* and *Chlamydomonas reinhardtii* relative ETR (Electron Transport Rate) as a function of PAR (Photosynthetically Active Radiation) at light intensities of 400-600 µmol photons $m^{-2}s^{-1}$ measured on day two, three and four.

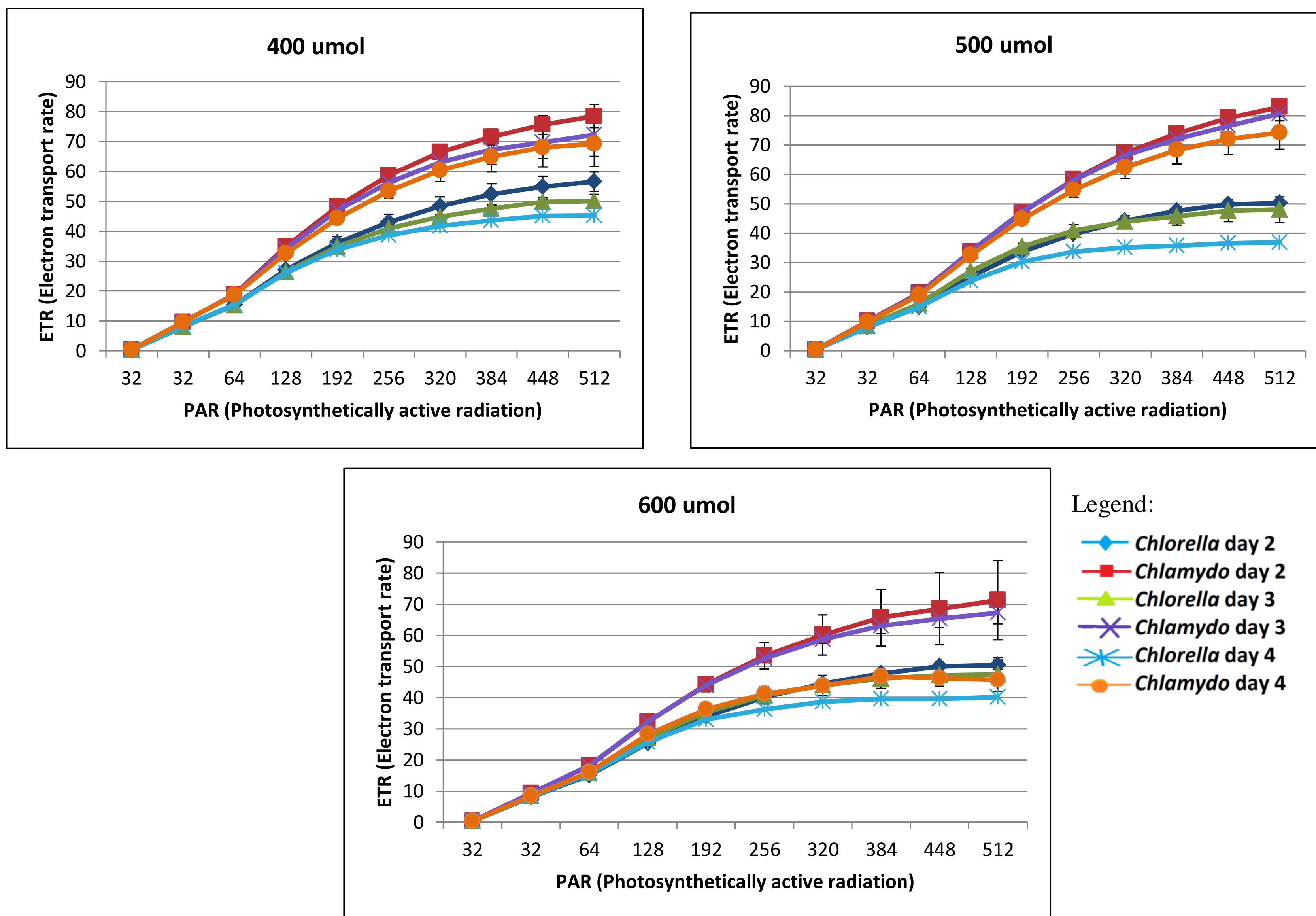


Figure 17. Growth of *Chlorella vulgaris* and *Chlamydomonas reinhardtii* in ambient air (0.038%), 3% CO_2, 6% CO_2, and 12% CO_2.

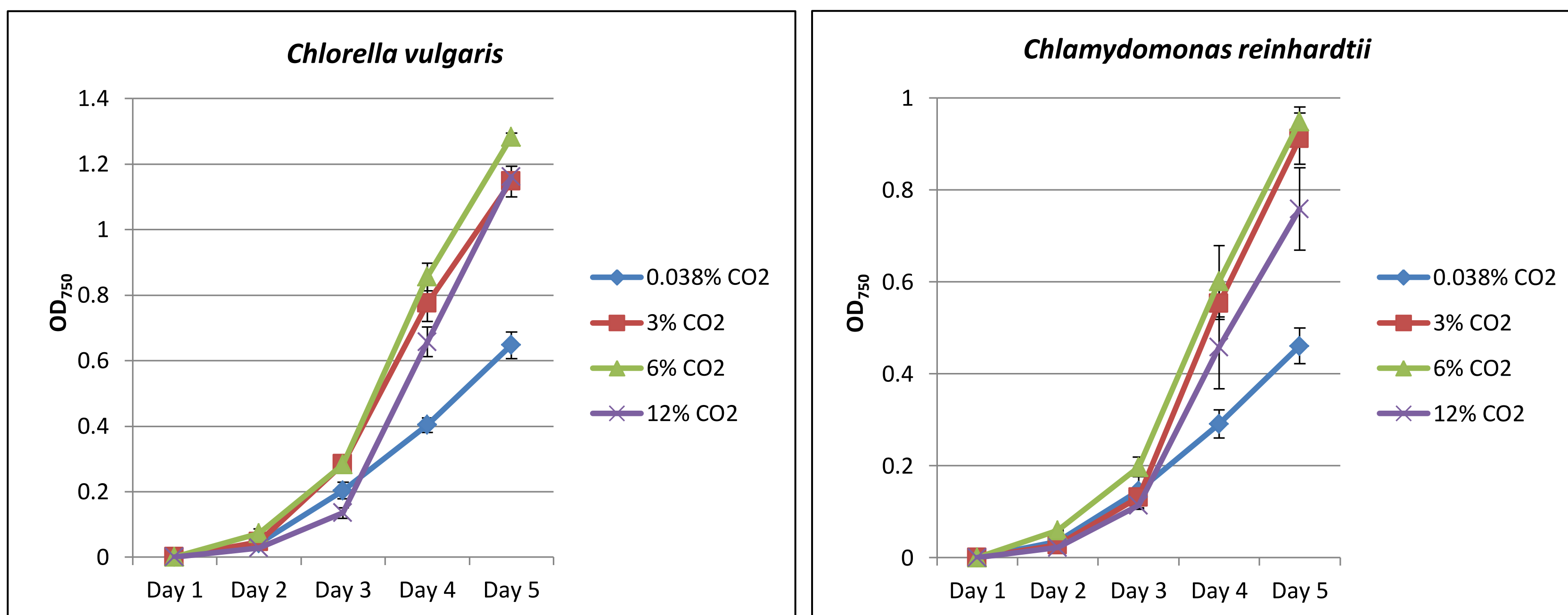


Table 15. Analysis of variance (ANOVA) table of OD_{750} with different CO_2 concentrations on day two.

Source of Variation	dF	Sum of Squares	F Ratio	Prob > F
Model	7	0.0955	27.1731	<.0001
Algae type	1	0.0376	72.9149	<.0001
% CO_2	3	0.0441	29.2650	<.0001
Algae type* % CO_2	3	0.0138	9.1673	0.0009
Error	16	0.0080		
Total	23	0.1035		

Table 16. Analysis of variance (ANOVA) table of OD_{750} with different CO_2 concentrations on day three.

Source of Variation	dF	Sum of Squares	F Ratio	Prob > F
Model	7	0.7515	36.0975	<.0001
Algae type	1	0.2322	79.0914	<.0001
% CO_2	3	0.5027	56.3368	<.0001
Algae type* % CO_2	3	0.0166	1.8604	0.1770
Error	16	0.0476		
Total	23	0.7991		

Table 17. Analysis of variance (ANOVA) table of OD_{750} with different CO_2 concentrations on day four.

Source of Variation	dF	Sum of Squares	F Ratio	Prob > F
Model	7	1.6581	107.5106	<.0001
Algae type	1	0.5017	227.7067	<.0001
% CO_2	3	1.1142	168.5734	<.0001
Algae type* % CO_2	3	0.0421	6.3826	0.0047
Error	16	0.0353		
Total	23	1.6934		

Table 18. Analysis of variance (ANOVA) table of ETR with different CO_2 concentrations on day two.

Source of Variation	dF	Sum of Squares	F Ratio	Prob > F
Model	7	5889.1250	70.7829	<.0001
Algae type	1	3981.4656	334.9797	<.0001
% CO_2	3	894.2447	25.0790	<.0001
Algae type* % CO_2	3	1013.4147	28.4211	<.0001
Error	16	190.1711		
Total	23	6097.2961		

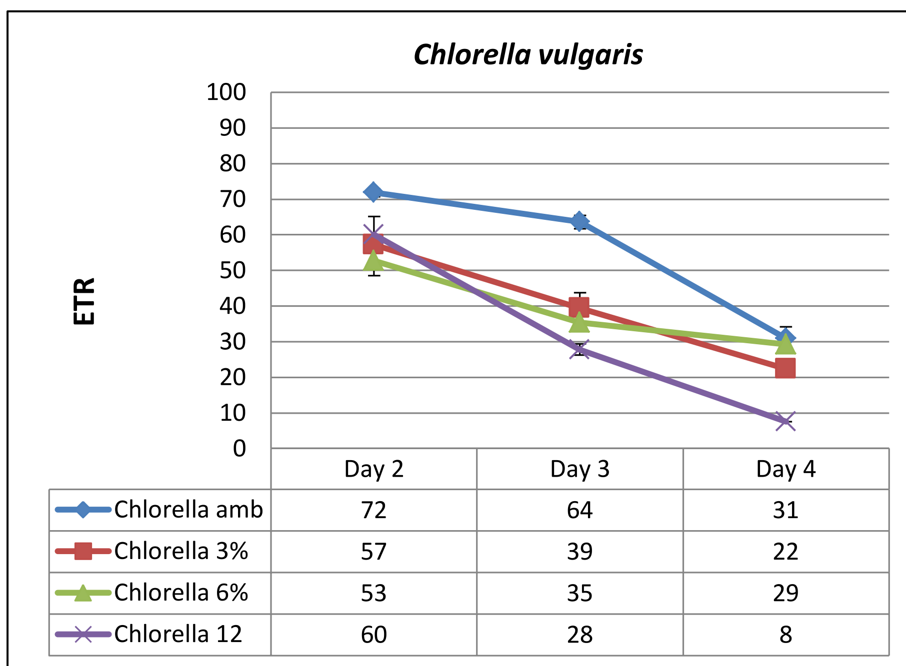
Table 19. Analysis of variance (ANOVA) table of ETR with different CO_2 concentrations on day three.

Source of Variation	dF	Sum of Squares	F Ratio	Prob > F
Model	7	6974.2753	17.7621	<.0001
Algae type	1	4291.9627	76.5155	<.0001
% CO_2	3	1495.4897	8.8870	0.0011
Algae type* % CO_2	3	1186.8230	7.0527	0.0031
Error	16	897.4841		
Total	23	7871.7594		

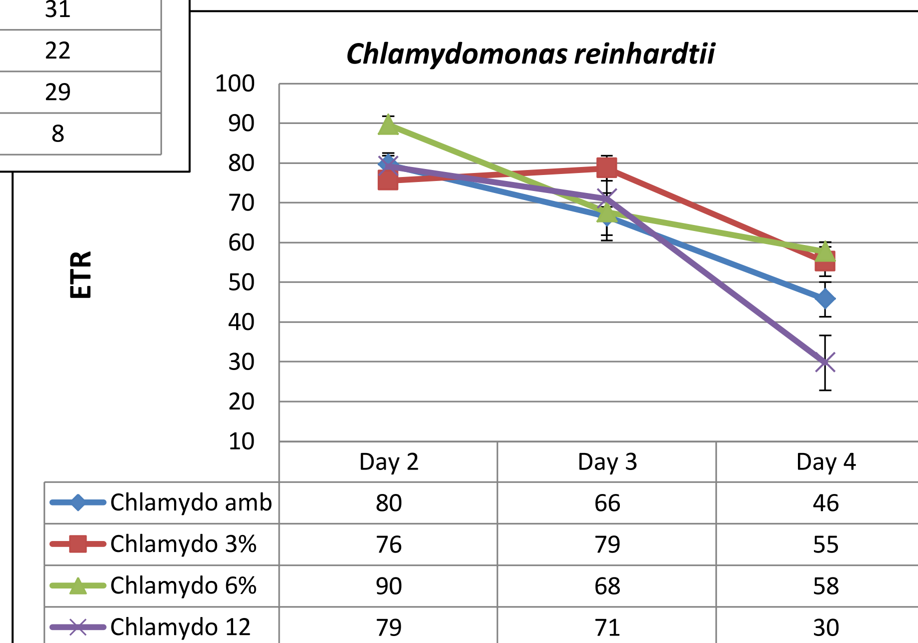
Table 20. Analysis of variance (ANOVA) table of ETR with different CO_2 concentrations on day four.

Source of Variation	dF	Sum of Squares	F Ratio	Prob > F
Model	7	6088.5057	869.7870	<.0001
Algae type	1	3629.7430	291.2247	<.0001
% CO_2	3	2184.9535	58.4350	<.0001
Algae type* % CO_2	3	273.8092	7.3228	0.0026
Error	16	199.4195		
Total	23	6287.9252		

Figure 18. *Chlorella vulgaris* and *Chlamydomonas reinhardtii* relative electron transport rates (ETR) in ambient air (0.038%), 3% CO_2, 6% CO_2, and 12% CO_2 measured on day two, three and four.

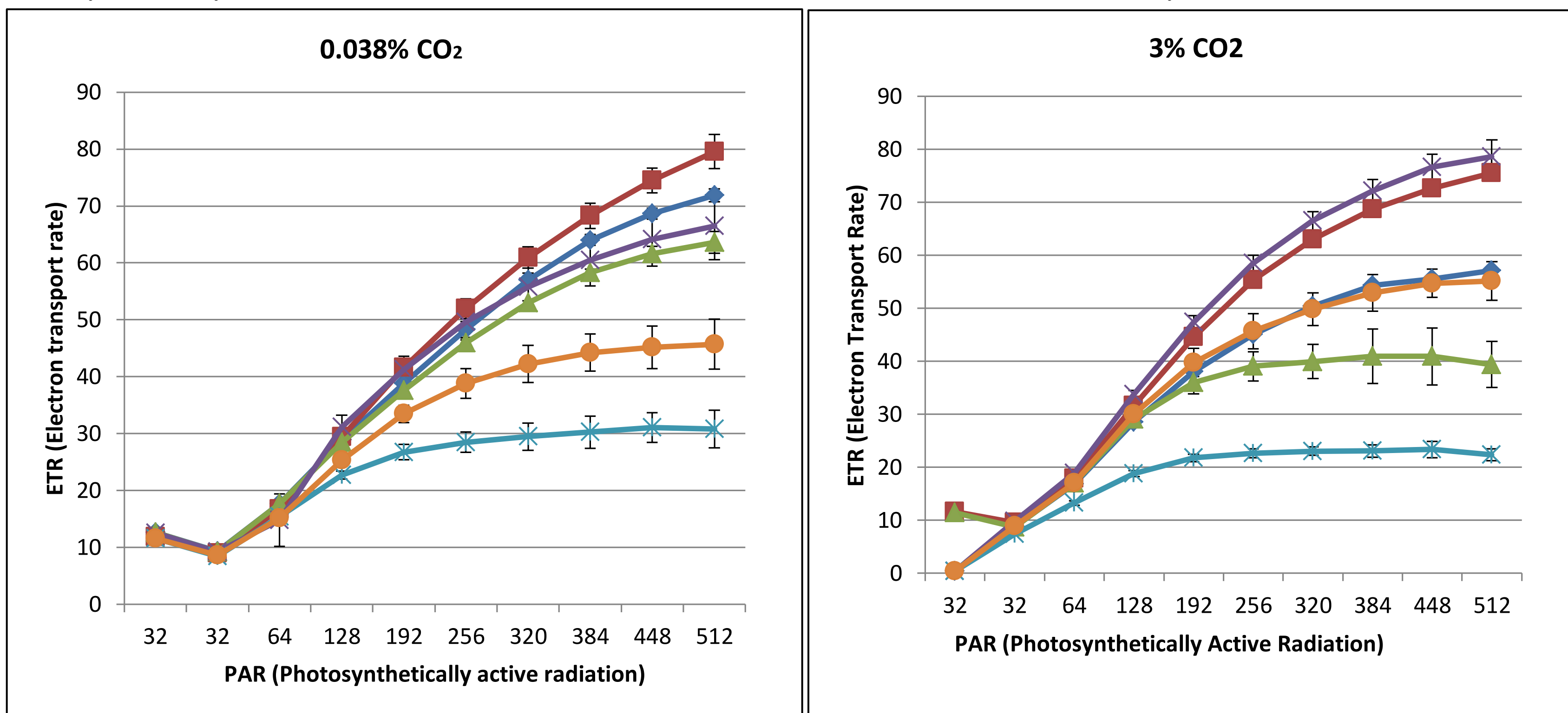


	Day 2	Day 3	Day 4
Chlorella amb	72	64	31
Chlorella 3%	57	39	22
Chlorella 6%	53	35	29
Chlorella 12	60	28	8



	Day 2	Day 3	Day 4
Chlamydo amb	80	66	46
Chlamydo 3%	76	79	55
Chlamydo 6%	90	68	58
Chlamydo 12	79	71	30

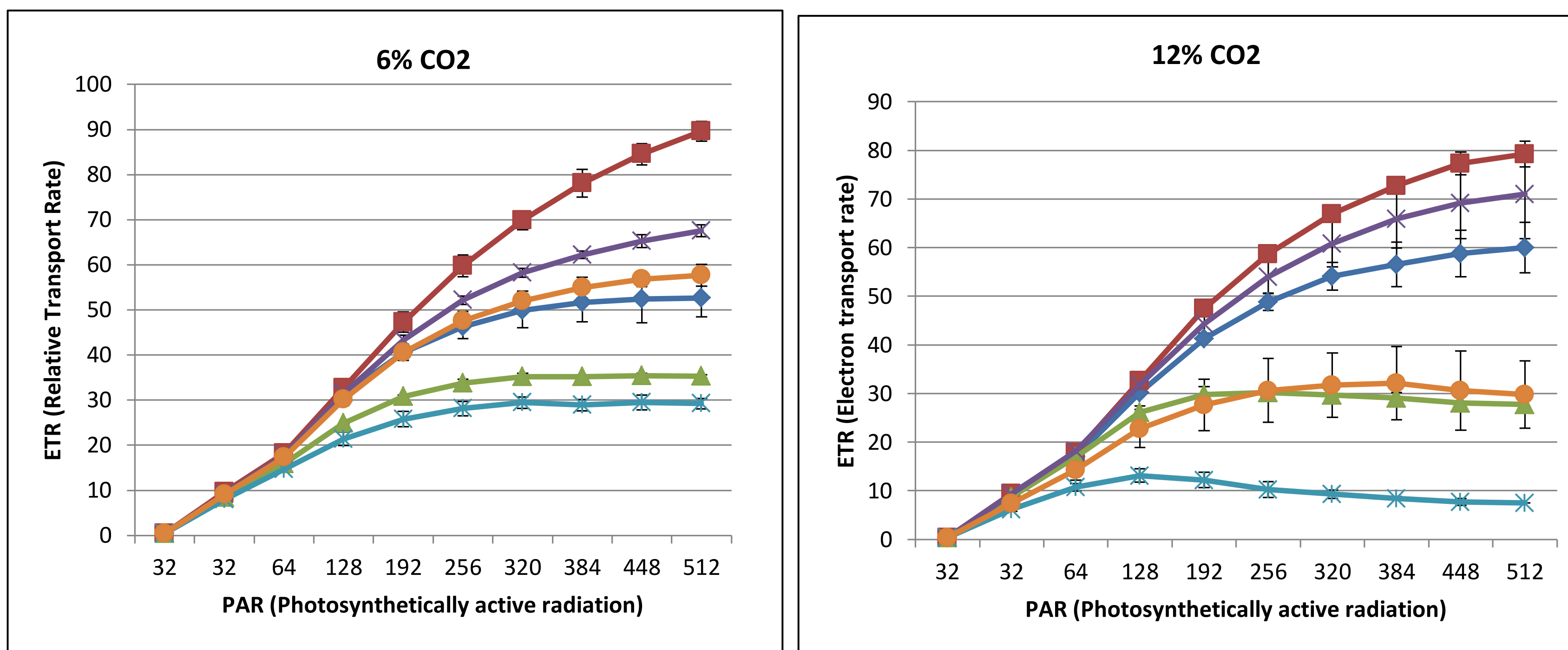
Figure 19. *Chlorella vulgaris* and *Chlamydomonas reinhardtii* relative electron transport rates (ETR) as a function of PAR (Photosynthetically Active Radiation) at ambient CO_2 (0.038% CO_2) and 3% CO_2 measured on day two, three and four.



Legend:

- ***Chlorella*** **day 2**
- ***Chlamydo*** **day 2**
- ***Chlorella*** **day 3**
- ***Chlamydo*** **day 3**
- ***Chlorella*** **day 4**
- ***Chlamydo*** **day 4**

Figure 20. *Chlorella vulgaris* and *Chlamydomonas reinhardtii* relative electron transport rates (ETR) as a function of PAR (Photosynthetically Active Radiation) at 6% CO_2 and 12% CO_2 measured on day two, three and four.



Legend:

- ***Chlorella*** **day 2**
- ***Chlamydo*** **day 2**
- ***Chlorella*** **day 3**
- ***Chlamydo*** **day 3**
- ***Chlorella*** **day 4**
- ***Chlamydo*** **day 4**

Table 21. Analysis of variance (ANOVA table) of the total lipid content of *Chlorella vulgaris* and *Chlamydomonas reinhardtii* grown at different CO_2 concentrations and using two types of extractions.

Source of Variation	dF	Sum of Squares	F Ratio	Prob > F
Model	11	137.3847	8.7883	0.0004
Algae type	1	32.7834	23.0683	0.0004
Extraction type	1	1.3680	0.9626	0.3459
Algae type*Extraction type	1	0.0294	0.0207	0.8880
% CO_2	2	87.3055	30.7165	<.0001
Algae type*% CO_2	2	10.2033	3.5898	0.0600
Extraction type*% CO_2	2	2.7361	0.9626	0.4095
Algae type*Extraction type*% CO_2	2	2.9589	1.0410	0.3829
Error	12	17.0538		
Total	23	154.4385		

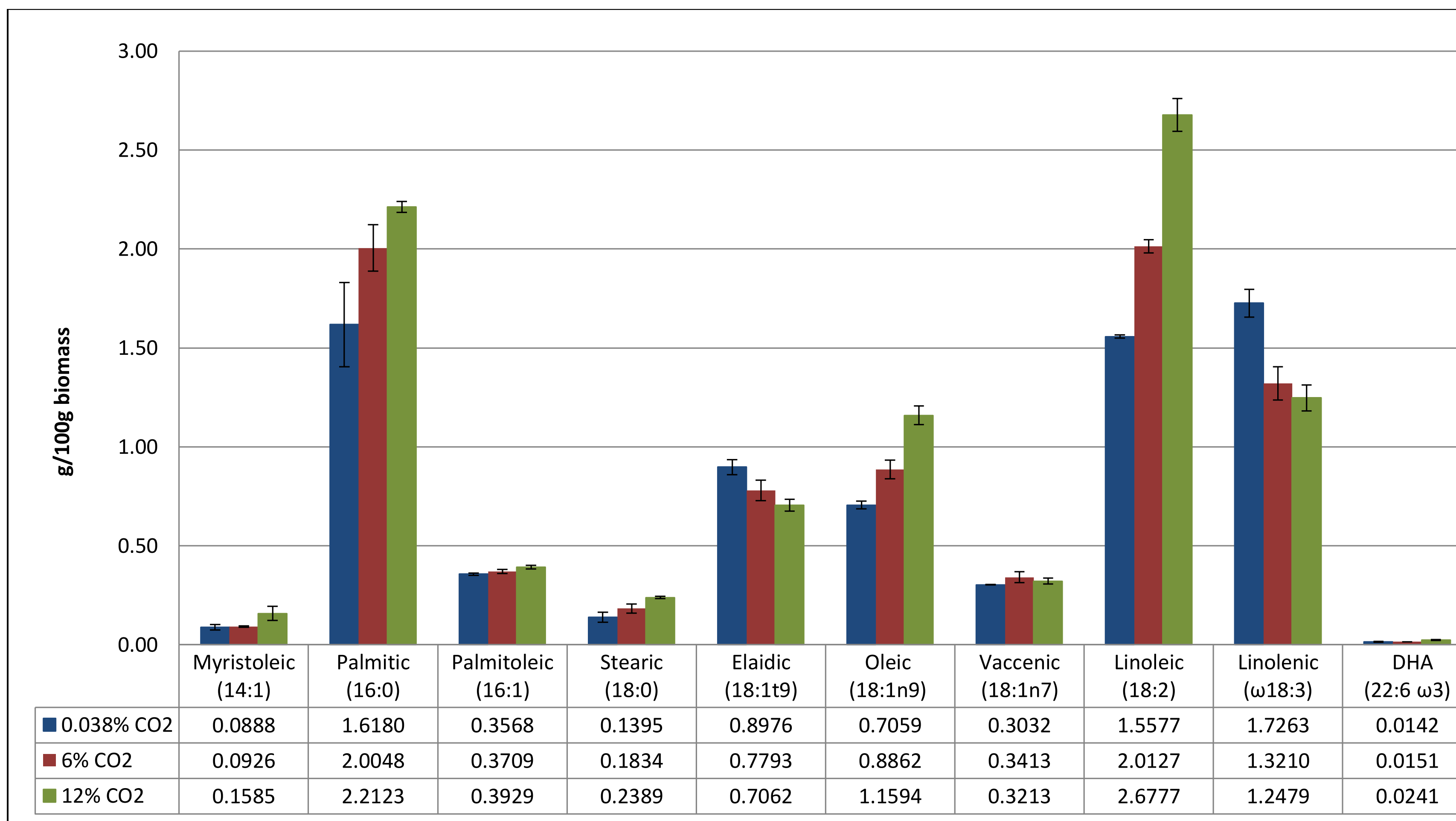
Table 22. Analysis of variance (ANOVA table) of the total fatty acid content of *Chlorella vulgaris* and *Chlamydomonas reinhardtii* grown at different CO_2 concentrations and using two types of extractions.

Source of Variation	dF	Sum of Squares	F Ratio	Prob > F
Model	11	89.3821	6.4777	0.0016
Algae type	1	22.6095	18.0240	0.0011
Extraction type	1	0.0077	0.0062	0.9388
Algae type*Extraction type	1	0.2952	0.2353	0.6363
% CO_2	2	56.1538	22.3826	<.0001
Algae type*% CO_2	2	7.3585	2.9330	0.0918
Extraction type*% CO_2	2	1.3754	0.5482	0.5918
Algae type*Extraction type*% CO_2	2	1.5821	0.6306	0.5490
Error	12	15.0529		
Total	23	104.4350		

Table 23. Lipid content, total fat content and fatty acid content of *C. vulgaris* and *C. reinhardtii* (g/ 100g biomass) grown at ambient CO_2 concentration, 6 % CO_2 and 12% CO_2 using 8000M Mixer/Mill and Soxtec extraction system.

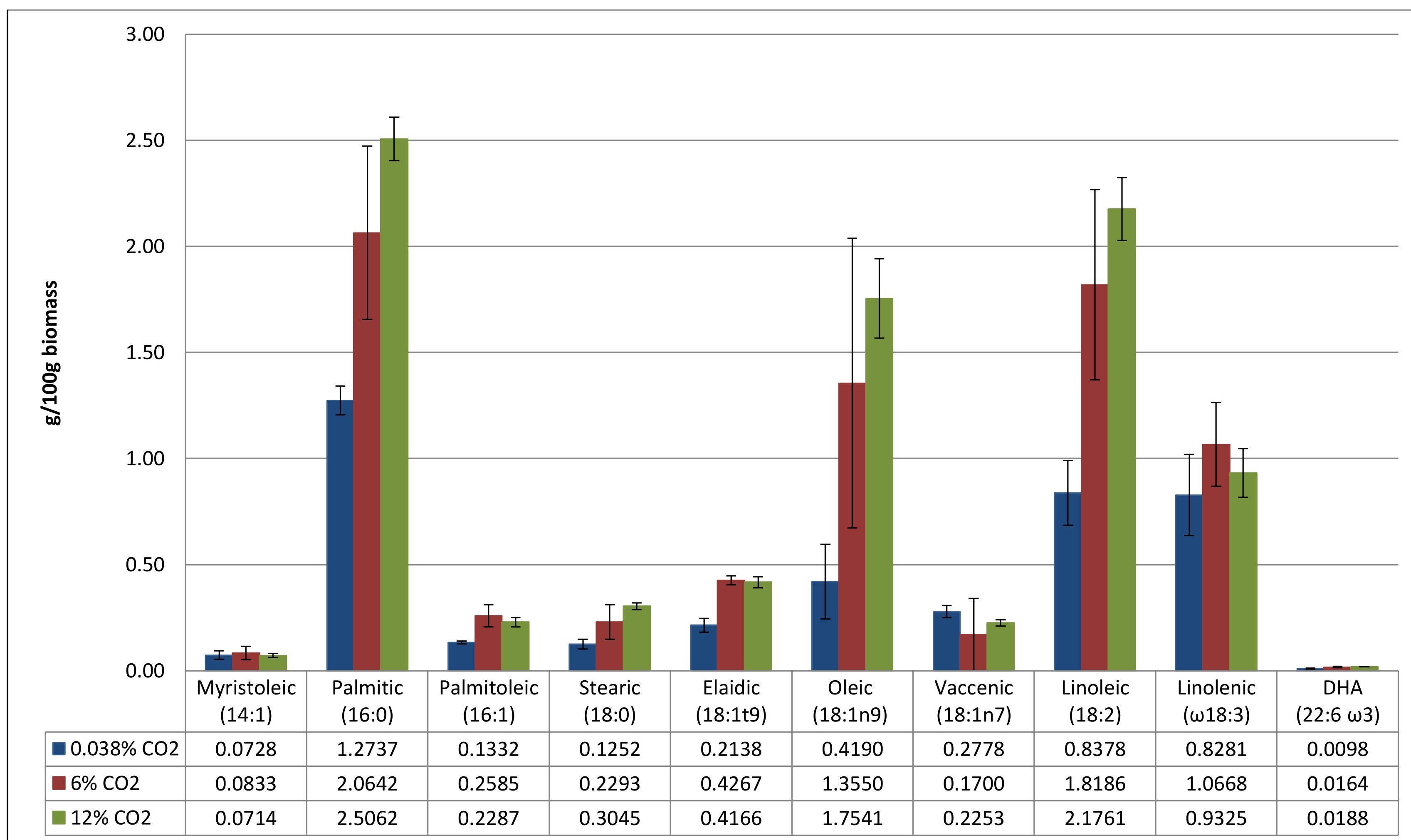
Algae type	Extraction type	% CO_2	Lipid content	Total fat content	14:1	16:0	16:1	18:0	18:1t9	18:1n9	18:1n7	18:2	ω18:3	DHA
Chlorella	Mixer/Mill	0.038	13.1250^{abc}	9.0739^{abc}	0.1033^{b}	1.4058^{bcd}	0.3511^{ab}	0.1146^{c}	0.8606^{a}	0.7249^{ab}	0.3049	1.5657^{abcd}	1.6561^{ab}	0.0120^{cd}
Chlorella	Mixer/Mill	6	14.4350^{ab}	10.2462^{ab}	0.0960^{b}	1.8871^{abcd}	0.3603^{ab}	0.1609^{abc}	0.8307^{a}	0.8391^{ab}	0.3686	2.0463^{abcd}	1.4047^{abc}	0.0150^{bcd}
Chlorella	Mixer/Mill	12	17.7875^{a}	12.8995^{a}	0.1951^{a}	2.1841^{abcd}	0.4020^{a}	0.2335^{abc}	0.7359^{ab}	1.2059^{ab}	0.3364	2.7603^{a}	1.3134^{abc}	0.0258^{a}
Chlorella	Soxtec	0.038	12.8525^{bc}	9.5864^{abc}	0.0743^{b}	1.8301^{abcd}	0.3624^{ab}	0.1644^{abc}	0.9346^{a}	0.6869^{ab}	0.3015	1.5496^{abcd}	1.7965^{a}	0.0163^{abcd}
Chlorella	Soxtec	6	13.6700^{ab}	10.1419^{abc}	0.0892^{b}	2.1224^{abcd}	0.3814^{ab}	0.2058^{abc}	0.7279^{ab}	0.9333^{ab}	0.3139	1.9791^{abcd}	1.2373^{abcd}	0.0152^{bcd}
Chlorella	Soxtec	12	17.6025^{a}	11.7184^{a}	0.1219^{b}	2.2405^{abcd}	0.3838^{ab}	0.2442^{abc}	0.6764^{ab}	1.1129^{ab}	0.3062	2.5950^{ab}	1.1823^{abcd}	0.0223^{ab}
Chlamydo	Mixer/Mill	0.038	10.9600^{bc}	6.2355^{bc}	0.0923^{b}	1.2050^{d}	0.1262^{d}	0.1021^{c}	0.1806^{c}	0.2436^{b}	0.3063	0.6847^{d}	1.0194^{bcd}	0.0086^{d}
Chlamydo	Mixer/Mill	6	13.5975^{ab}	8.9830^{abc}	0.1144^{b}	1.6551^{abcd}	0.3100^{abc}	0.1486^{bc}	0.4477^{bc}	0.6724^{ab}	0.3400	1.3701^{bcd}	1.2647^{abcd}	0.0135^{bcd}
Chlamydo	Mixer/Mill	12	13.9875^{ab}	10.5122^{ab}	0.0610^{b}	2.6086^{a}	0.2070^{bcd}	0.3201^{a}	0.3899^{bc}	1.9415^{a}	0.2393	2.3252^{abc}	0.8171^{cd}	0.0189^{abc}
Chlamydo	Soxtec	0.038	8.5700^{c}	5.7432^{c}	0.0532^{b}	1.3424^{cd}	0.1402^{cd}	0.1482^{bc}	0.2470^{c}	0.5943^{ab}	0.2493	0.9909^{cd}	0.6367^{d}	0.0110^{cd}
Chlamydo	Soxtec	6	13.5200^{ab}	10.1397^{abc}	0.0522^{b}	2.4732^{ab}	0.2069^{bcd}	0.3099^{a}	0.4056^{bc}	2.0375^{a}	0.0000	2.2671^{abc}	0.8688^{cd}	0.0193^{abc}
Chlamydo	Soxtec	12	14.8125^{ab}	10.4056^{ab}	0.0817^{b}	2.4037^{abc}	0.2503^{abcd}	0.2888^{ab}	0.4433^{bc}	1.5667^{abc}	0.2114	2.0269^{abcd}	1.0479^{bcd}	0.0186^{abc}

Fig.21. Major fatty acid composition of *C. vulgaris* grown using different CO_2 concentrations.



	Myristoleic (14:1)	Palmitic (16:0)	Palmitoleic (16:1)	Stearic (18:0)	Elaidic (18:1t9)	Oleic (18:1n9)	Vaccenic (18:1n7)	Linoleic (18:2)	Linolenic (ω18:3)	DHA (22:6 ω3)
0.038% CO2	0.0888	1.6180	0.3568	0.1395	0.8976	0.7059	0.3032	1.5577	1.7263	0.0142
6% CO2	0.0926	2.0048	0.3709	0.1834	0.7793	0.8862	0.3413	2.0127	1.3210	0.0151
12% CO2	0.1585	2.2123	0.3929	0.2389	0.7062	1.1594	0.3213	2.6777	1.2479	0.0241

Fig.22. Major fatty acid composition of *C. reinhardtii* grown using different CO_2 concentrations.



	Myristoleic (14:1)	Palmitic (16:0)	Palmitoleic (16:1)	Stearic (18:0)	Elaidic (18:1t9)	Oleic (18:1n9)	Vaccenic (18:1n7)	Linoleic (18:2)	Linolenic (ω18:3)	DHA (22:6 ω3)
0.038% CO2	0.0728	1.2737	0.1332	0.1252	0.2138	0.4190	0.2778	0.8378	0.8281	0.0098
6% CO2	0.0833	2.0642	0.2585	0.2293	0.4267	1.3550	0.1700	1.8186	1.0668	0.0164
12% CO2	0.0714	2.5062	0.2287	0.3045	0.4166	1.7541	0.2253	2.1761	0.9325	0.0188

CHAPTER 2

Comparison of CO_2 uptake by *Chlorella vulgaris* and *Chlamydomonas reinhardtii* in liquid culture and immobilized algal beads

2.1 INTRODUCTION

One of the today's biggest environmental challenges is emission of greenhouse gases (GHG) such as CO_2 from combustion of fuels (Melillo *et al.,* 1993). Common remediation approaches to managing the levels of CO_2 released into the atmosphere include 1) increased efficiency of energy conversion, 2) use of lower carbon energy sources, and 3) various forms of carbon sequestration (Keffer and Kleinheinz, 2002).

Microalgae can fix carbon dioxide from the atmosphere using open-culture systems (lakes or ponds) or closed-culture systems called photo-bioreactors. Open-culture systems are less expensive to build and operate, are more durable than bioreactors and have a large production capacity when compared with closed systems. Photo-bioreactors are advantageous because they are flexible systems that can be optimized according to the biological and physiological characteristics of the algal species being cultivated which allows cultivation of algal species that cannot be grown reliably in open ponds. Mitigation techniques using membrane-type photo-bioreactors have high CO_2 removal efficiency (Cheng *et al*., 2006; Fan *et al*., 2008). However, membrane-type photo-bioreactors have operational problems, which include membrane fouling and high design and operational costs (Cheng *et al*., 2006). Therefore, tubular and bubble column-type photo-bioreactors are commonly applied for use in algal photoreactors (Chiu *et al.,* 2009; de Morais and Costa, 2007a; Fan *et al.,* 2008).

Biological CO_2 sequestration (accomplished by plants and photosynthetic microorganisms) is a promising strategy since the biomass produced can be further utilized for value-added products (de Morais and Costa, 2007a). Sequestration strategies include deep ocean injection, below ground storage of CO_2 in geological formations such as in oil and gas fields or saline aquifers (Stevens, 2000; Nordbotten *et al*., 2005), and biological sequestration (de Morais and Costa, 2007a,b,c). The potential of carbon capture by green plants has been estimated to be 3-6% of fossil fuel emissions (Skjanes *et al.,* 2007). Microalgae have the ability to fix CO_2 during photosynthesis with efficiency 10 to 50 times greater than that of terrestrial plants (Li *et al*., 2008; Usui and Ikenouchi, 1997). According to Sahoo (2010), one ton of algae can fix 0.36

tons of carbon, 0.6 tons of nitrogen and 0.008 tons of phosphorus. Microalgae and cyanobacteria species used for CO_2 mitigation include *Botryococcus braunii* (Yoo *et al.,* 2010), *Chlorella vulgaris* (Chen *et al*., 2010; Cheng *et al*., 2006), *Chlorella kessleri* (de Morais and Costa, 2007b), *Chlorocuccum littorale* (Ota *et al.,* 2009), *Scenedesmus sp.* (de Morais and Costa, 2007a; Ho *et al*., 2010), *Chlamydomonas reinhardtii* (Packer, 2009) and *Spirulina* sp. (de Morais and Costa, 2007a,b,c). Microalgal species that grow well under the natural day–night cycle are suitable for large scale outdoor cultivation systems (Stewart and Hessami, 2005), and strains that can directly use the CO_2 in power-plant flue gas are preferred (Benemann, 1993; de Morais and Costa, 2007c; Maeda *et al*., 1995). However, few *Chlorella* sp. and cyanobacteria species could grow well and achieve high CO_2 fixation ability (500–1800 mg L^{-1} d^{-1}) under a relative high tolerance for temperature or CO_2 concentration (Maeda *et al*., 1995; Ono *et al.,* 2007; Yue and Chen, 2005).

There are several advantages of using microalgae for bio-mitigation purposes. Firstly, microalgae have higher growth and CO_2 fixation rates compared with terrestrial plants (Chisti 2007; Li *et al.* 2008). Secondly, CO_2 can be completely recycled because it can be transformed to biofuel and other bioproducts (Li *et al*. 2008; Huntley and Redalje 2000). Thirdly, microalgae CO_2 fixation is more cost-effective and environmental sustainable when it is combined with other processes such as wastewater treatment (Wang *et al*., 2008). However, to reduce CO_2 levels from industrial waste gas, microalgae have to be tolerant not only to high levels of CO_2, but they would also have to be able to grow in the presence of sulfur dioxides, nitrogen oxides, and volatile organic compounds (VOCs) which exist in the waste gases (Brown, 1996). Some researchers propose isolation of microalgae from lakes or ponds in the area of coal or oil fired thermoelectric power plants to obtain microalgae tolerant to the conditions prevalent in that particular area, in this way the adaptation of exotic strains to the new cultivation conditions existing in a power plant can be avoided (de Morais and Costa, 2007b).

There are several factors that play an important role in controlling the efficacy of CO_2 uptake by microalgae. High light intensity greater than saturation and high oxygen concentration can inhibit photosynthesis and CO_2 fixation (Pope, 1975; Jeon *et al*., 2005). Basic growth nutrients are fundamental for maintaining the physiological integrity of the culture (Anderson, 2005). Temperature can be a decisive factor in the selection of the proper type of microalga with the purpose of CO_2 sequestration (Caron *et al*., 1986). Sakai et al. (1995) isolated *Chlorella* sp. from hot springs in Japan able to grow at temperatures up to 42°C and more than 40% CO_2 in

air. Tolerance to both high temperature and high CO_2 content made this alga potentially suitable for bio-CO_2 mitigation from flue gas. Recent reports (Hsueh *et al.,* 2007; Zhang *et al*., 2002) observed that the CO_2 fixation can be influenced by aeration rate, bubble size, and residence time of the bubble. In general, obtaining an extensive air/liquid interface area is the first vital step to improve the CO_2 mass transfer efficiency (Carvalho and Malcata, 2001; Ryu *et al*., 2009).

Immobilized algae have become a new branch of biotechnology of rapidly growing importance (Hameed and Ebrahim, 2007). An immobilized cell is defined as a cell prevented from moving independently of its neighbors to all parts of the aqueous phase of the system, either by natural or artificial methods (Tampion and Tampion, 1987). The most frequent method used for algae immobilization is entrapment using alginate or carrageenan as polymers, where the cells are free within their compartments and the pores in the material allow substrates and products to diffuse to and from the cells (Mallick, 2002).

Studies on immobilized algae report varying results. Some studies found that the growth rates of immobilized cells are generally lower (Bailliez *et al*., 1985; Robinson *et al*., 1985) than those of the free cell cultures (Chevalier and de la Noue, 1985). Other studies suggest that there is no difference in oxygen evolution between free and immobilized *Chlorella* cells observed under a range of light intensities (Robinson *et al*., 1985). Still other studies found that oxygen evolution was greater in the immobilized state, suggesting a fundamental change of metabolism (Bailliez *et al*., 1988). Leon and Galvan (1995) studied the production of glycerol in *Chlamydomonas reinhardtii* cells immobilized in Ca-alginate and found that the immobilized cells showed a higher production rate in comparison to their free-living counterparts. Other studies demonstrated a decrease in productivity with immobilization. For example, keto-acid production by *Anacystis* and *Chlorella* was reduced by 70–90% (Wilkstrom *et al.,* 1982) following immobilization. The CO_2 fixation rates of algae immobilized in alginate beads are still largely unexplored. The present study is the first report of CO_2 uptake rates by *C. vulgaris* and *C. reinhardtii* alginate beads, using influent CO_2 concentration ranging from 3 to 9% CO_2.

The purpose of this study was to compare CO_2 fixation by *C. vulgaris* and *C. reinhardtii* grown in liquid cultures at ambient (0.038%), 3%, 6%, 9%, and 12% CO_2. The rates of CO_2 fixation on immobilized algal beads with a CO_2 influent of 3%, 6%, and 9% CO_2 were also identified.

2.2. MATERIALS AND METHODS

2.2.1. Consumption of CO_2 in liquid culture

2.2.1.1. Microalgal strain and culture conditions

Algae *Chlorella vulgaris* (UTEX 2714) and *Chlamydomonas reinhardtii* (UTEX 90) were grown at ambient (0.038%) CO_2, 3% CO_2, 6% CO_2, 9% CO_2, and 12% CO_2 for seven days using Wright's cryptophytes (WC-Gly) medium in 250 ml flasks, and stirred continuously. Temperature was maintained at 21˚C and light intensity was maintained at 200 µmol m^{-2} s^{-1} with a photoperiod of 12 h light/12 h dark. The light energy for the photosynthetic fixation of CO_2 by the algae was provided by two GE grow lights source (General Electric, Fairfield, CT, USA).

2.2.1.2. Assay system and CO_2 measurements by gas chromatography

Cells were harvested by centrifugation and the final absorbance was adjusted to OD_{750}=0.050 (See Appendix 1). Three ml of the algal suspensions were added to 100 ml of Wright's Cryptophytes medium in five replicate 160 ml serum bottles and the head space was adjusted to 12% CO_2 (Figure 23). Each bottle corresponded to a specific time (0, 9, 21, 26, and 34 hrs. after inoculation). Another five bottles were not inoculated and used as controls. The bottles were kept under light on four stirrer plates (Cimarec, Thermo Scientific, USA). The experiment was run in duplicate. Three milliliters of gas were sampled using a 5 cc syringe (Becton Dickinson, NJ, USA) at each time point from each bottle, and the amount of CO_2 consumption of gas was monitored by gas chromatography with a Carle AGC Series 100 gas chromatograph (Hach Co., Loveland, CO, USA) connected to a BD40 chart recorder (Kipp & Zonen, Inc., Bohemia, NY, USA). From the same samples, the absorbance was measured at 750 nm using Spectronic 20 Genesys spectrophotometer (Spectronic Instruments, NY).

2.2.2. Consumption of CO_2 flowing through immobilized algal beads

2.2.2.1. Microalgal strain and culture conditions

C. vulgaris (UTEX 2714) and *C. reinhardtii* (UTEX 90) were grown in 250 ml flasks at ambient (0.038%) CO_2 using WC-Gly medium, 21˚C temperature, and 200 µmol $m^{-2}s^{-1}$ light intensity and stirred continuously.

Immobilized algal beads were prepared by pouring a concentrated suspension of algal cells (500 mL, OD_{750}=2.200, See Appendix 1) into an equal volume of 2.5% sodium alginate solution (Mallick, 2002) (Figure 24). The algae/alginate suspension was mixed for 2-3 hours using a stirrer until completely homogenized. Next, the suspension was drawn into a syringe and dripped slowly from the syringe tip into 100mM $CaCl_2$ solution to form the spherical beads. The beads were left overnight to cross-link the alginate molecules with calcium ions, trapping the cells in a matrix of calcium alginate. Next day, the beads were separated from the resulting sodium chloride solution and transferred into two 100 cm Tygon tubes (Thermo Fisher Scientific, Pittsburg, PA, USA) (ID=2.5 cm). For the control columns, beads were prepared in the same manner, without algal cells suspension.

2.2.2.2. Culture system and CO_2 measurements by gas chromatography

Gas in the range of 3% – 9% CO_2 was obtained by mixing compressed air with CO_2 from two tanks (Airgas Mid America, West Virginia, USA) in an stainless steel mixing chamber (20 cm length and an 4 cm internal diameter) (Figure 3). Gas flow through the columns was adjusted to 10-13 mL min^{-1} for 3% and 6% CO_2 and 18-22 mL min^{-1} for 9% CO_2. Influent (3, 6, and 9%) and effluent CO_2 concentrations were measured by gas chromatography with a Carle AGC Series 100 gas chromatograph (Hach Co., Loveland, CO, USA) connected to a BD40 chart recorder (Kipp & Zonen, Inc., Bohemia, NY, USA), over a five day period.

2.3. RESULTS

2.3.1. Consumption of CO_2 in liquid culture

ANOVA analysis showed that both *C. vulgaris* and *C. reinhardtii* had the same maximum uptake rate, but the CO_2 concentration used to grow the cells had a significant impact on uptake (Table 25). Overall, cells grown at higher CO_2 concentration exhibited lower CO_2 uptake compared with cells grown at ambient CO_2 concentration. The highest CO_2 fixation rate was 278 mg CO_2 L^{-1} day^{-1} in case of *C. vulgaris* grown at ambient CO_2 and the lowest CO_2 fixation rate was 117 mg CO_2 L^{-1} day^{-1} displayed by *C. reinhardtii* grown at 12% CO_2 (Table 24).

The OD_{750} also was measured (Fig. 27). Overall *C. vulgaris* grew to higher densities compared with *C. reinhardtii,* and cells grown at lower CO_2 concentration grew better than cells grown at higher CO_2 concentration (Table 25). *C. vulgaris* had higher growth than *C. reinhardtii* at all CO_2 concentrations tested.

2.3.2. Consumption of CO_2 flowing through immobilized algal beads

The percentage of CO_2 reduction by *C. vulgaris* and *C. reinhardtii* was measured as the difference of influent CO_2 and effluent CO_2 measured at both sample ports (See Fig. 25, port D and port F), for each treatment (3%, 6% and 9% CO_2 influent). The difference between influent CO_2 and effluent CO_2 measured for control columns also was taken into account. The percentage of CO_2 reduction was examined at day one versus day five. On day one, immobilized *C. vulgaris* exhibited significantly higher capacity for CO_2 reduction, compared with *C. reinhardtii*. *C. vulgaris* sequestered 34%, 13% and 5% of CO_2 from influent atmospherics containing 3%, 6% and 9% CO_2, respectively. In comparison, *C. reinhardtii* sequestrated 26%, 12% and 4%. Both strains showed higher fixation efficiency on day one. Fixation efficiency declined over time (Table 26, Figure 28, 29, and 32).

The interaction between algae type and influent CO_2 concentration was not significant (Table 26 and 27). However, when rates were estimated without considering controls, higher rates of 6% CO_2 consumption were apparent for both *C. vulgaris* and *C. reinhardtii*. On day one,

rates estimates varied from 35 to 53 mg CO_2 L^{-1} day^{-1} (Table 30). These rates are considerably lower than those observed using liquid batch cultures.

2.4. DISCUSSION

2.4.1. Consumption of CO_2 in liquid culture

Algal cells grown at ambient CO_2 levels were able to fix CO_2 more rapidly than cells grown at higher CO_2 concentrations. Similar findings were reported by Chinnasamy *et al.* (2009). They demonstrated that CO_2 uptake by *C. vulgaris* decreased by 29% under elevated CO_2 (6% CO_2). They reported that algal cells grown at the ambient levels of CO_2 and higher temperature (40°C) exhibited a 23% growth rate of CO_2 uptake. Increase in temperature significantly enhances the process of photorespiration thus causing depletion of intracellular CO_2. To the contrary, DeLucia *et al*. (1985) found that the rate of photosynthesis initially significantly increased for a short period at high CO_2 concentrations followed by a gradual decrease.

Table 24 presents a comparison between the carbon fixation rates indicated in the literature and the results of this study. The differences could be due to different conditions of growth (different media, light intensity, CO_2 concentrations, and pH) and type of reactors (tubular, bubble column, membrane, and air-lift) used in the various studies. Badger and Price (1994) explained that the efficiency of dissolved inorganic carbon (DIC) utilization for photosynthesis in low-CO_2 grown cells was higher due to the activity of carbonic anhydrase (an enzyme that catalyzes the interconversion of CO_2 and HCO^{3-}). Carbonic anhydrase activity in low-CO_2 grown cells was higher than that of cells grown in high-CO_2. Also, cells grown in low-CO_2 exhibited lower photorespiration, a lower CO_2 compensation point, and low O_2 inhibition of photosynthesis.

A reason for lower CO_2 consumption rates obtained in the present study might be due to difficulty of mixing all the batch culture on the stirrer plates, and unequal distribution of light. As presented in the previous chapter, light intensity can affect photosynthesis and algal growth. Mixing is important for better diffusion of carbon dioxide in the medium, and preventing sedimentation of algal cells. Algae can fix carbon dioxide from carbon dioxide and

hydrogencarbonate dissolved in their aqueous surroundings, but must overcome the low rate of carbon dioxide diffusion in water. The key enzyme that catalyzes the fixation of carbon dioxide, ribulose-1, 5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco) is localized in the chloroplast pyrenoid (center of carbon dioxide fixation within the chloroplast). CO_2 is the substrate required by Rubisco and not hydrogencarbonate, therefore the accumulated HCO^{3-} must be converted to CO_2 before inorganic carbon fixation takes place. The carbonic anhydrase catalyses the interconversion of hydrogencarbonate into carbon dioxide and results in a high local concentration of carbon dioxide, which Rubisco can use rapidly before the CO_2 has a chance to leak out of the cell (Moroney and Somachi, 1999).

Another method to improve the CO_2 fixation rate could be changing the light cycle from 12 h light/12 h dark to 24 h light/0 h dark. Jacob-Lopes *et al.* (2009) reported an increase rate of CO_2 fixation from 562 mg CO_2 L^{-1} day^{-1} to 1,440 mg CO_2 L^{-1} day^{-1} when such a change was implemented.

2.4.2. Consumption of CO_2 flowing through immobilized algal beads

The CO_2 reduction efficiency of alginate-immobilized *C. vulgaris* and *C. reinhardtii* decreased as the CO_2 influent was increased. One factor that confounds this result is the different flow rate at the different CO_2 concentrations tested. The columns flows were 10-13 mL min^{-1} for 3% and 6% CO_2 and 18-22 mL min^{-1} for 9% CO_2. Unfortunately, this was unavoidable due to technical difficulties in achieving a stable 9% CO_2. Similarly, Doucha *et al.* (2005) reported a 10-50% decrease in CO_2 mitigation by *Chlorella* sp. in an outdoor open thin-layer photobioreactor due to increasing flue gas rate. Also, often it was not possible to maintain a constant CO_2 concentration for 24 hrs, therefore it was necessary to recalibrate the gas composition each morning. For future experiments a more stable flow rate and CO_2 concentrations will give a better idea of the CO_2 fixation efficiency of immobilized algal beads.

On day one *C. vulgaris* had a significant higher uptake compared with *C. reinhardtii,* but on day five there was not a significant difference.

Only one study has evaluated CO_2 capture by immobilized algae. Rooke *et al.* (2011) studied immobilized *C. vulgaris* and *Botryococcus braunii* cells in low sodium silica gel, and demonstrated growth inhibition due to competition for space. They also reported that the mesoporosity of the gels enabled diffusion of nutrients and gases, and promoted the light and

dark photosynthetic reactions. Furthermore, the activity and the viability of encapsulated cells were for at least 80 days. During this time, the efficiency of the photosynthetic bioreactor in terms of CO_2 remediation was monitored and suggested the capability for long term productivity of living gels. However, they noticed a decrease in photosynthetic yield. They assumed that this was an indicator of nutrient stress and suggested the need for improvement of gas diffusion, replenishment of nutrients, or cell acclimatization prior immobilization. Interestingly, also they noticed a differentiation in the durability of living gels in term of algal species. *C. vulgaris* cells were more viable and consistent to the matrix than *Botryococcus braunii.*

When was observed the experimental columns on day five, a general decrease in bead diameter. This might happen because air flowing through the columns dried out the beads, shrinking the beads and decreasing the space available to living algal cells. In future experiments it would be beneficial to flow the influent gas through sterile liquid media to maintain the alginate beads in a high humidity atmosphere.

Future studies could include the efficient removal of wastewater nitrogen and phosphorus by alginate-immobilized *C. vulgaris* and *C. reinhardtii.* Chevalier and de la Noue (1985) reported that immobilized *Scenedesmus* was capable of removing 90% of the ammonium (within four hours) and 100% of phosphate (within two hours) from a typical effluent, suggesting possible uses in the tertiary treatment of wastewaters. Similar findings were reported by Tam *et al*. (2000) who used *Chlorella vulgaris* cells immobilized in alginate beads for removing of ammonia and phosphate from wastewater. They also suggested that immobilized *Chlorella vulgaris* can be used as a secondary treatment process for domestic wastewater. Wang and Huang (2003) co-immobilized *Chlorella pyrenoidosa* and activated sludge for nitrate and phosphate removal. They reported 80% nitrate removal and 88% phosphate removal.

2.5. CONCLUSION

CO_2 fixation was explored using two approaches: 1) consumption of known quantities of CO_2 in sealed serum bottles, 2) consumption of CO_2 flowing through immobilized algal beads. In liquid culture, the cells grown at higher CO_2 concentration exhibited lower CO_2 uptake compared with algae grown at ambient CO_2 concentration. In the immobilized algae study, a significant decrease in CO_2 fixation from day one to day five and a decrease in CO_2 uptake with increasing the influent CO_2 concentration were evident. Both cases showed a similar trend in CO_2 fixation rate based on increasing CO_2 concentration level. The fixation rate decreased with increasing CO_2 concentration.

CO_2 consumption generally decreased over the five day experiment. The maximum CO_2 consumption rate observed using immobilized algae was only 20% of the maximum obtained in liquid culture. Clearly, additional work is needed to optimize and sustain CO_2 uptake in alginate beads.

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Figure 23. Sealed serum bottles used for CO_2 consumption and growth measurements inoculated with *Chlorella vulgaris.*

Figure 24. Production of immobilized algal beads.

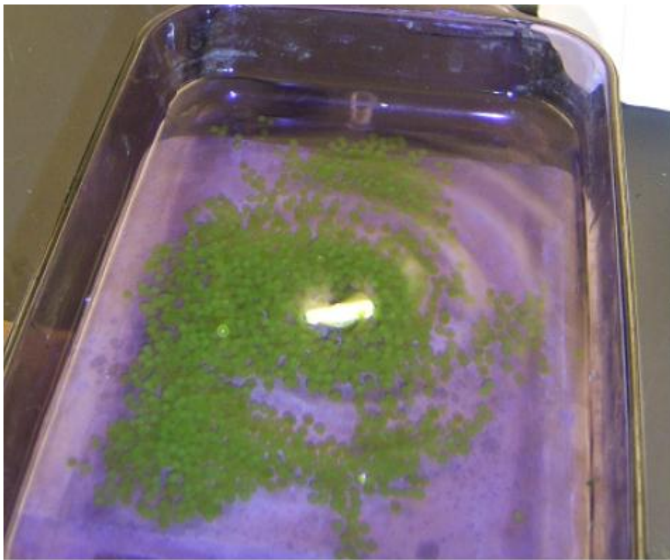


Figure 25. The experimental system designed for CO_2 consumption measurements of immobilized algae beads.

A- CO_2 tank

B- air tank

C- gas mixing chamber

D- CO_2 influent gas sample port*

E- Tygon tubes containing immobilized algae

F- CO_2 effluent gas sample port*

* - CO_2 measured by gas chromatography

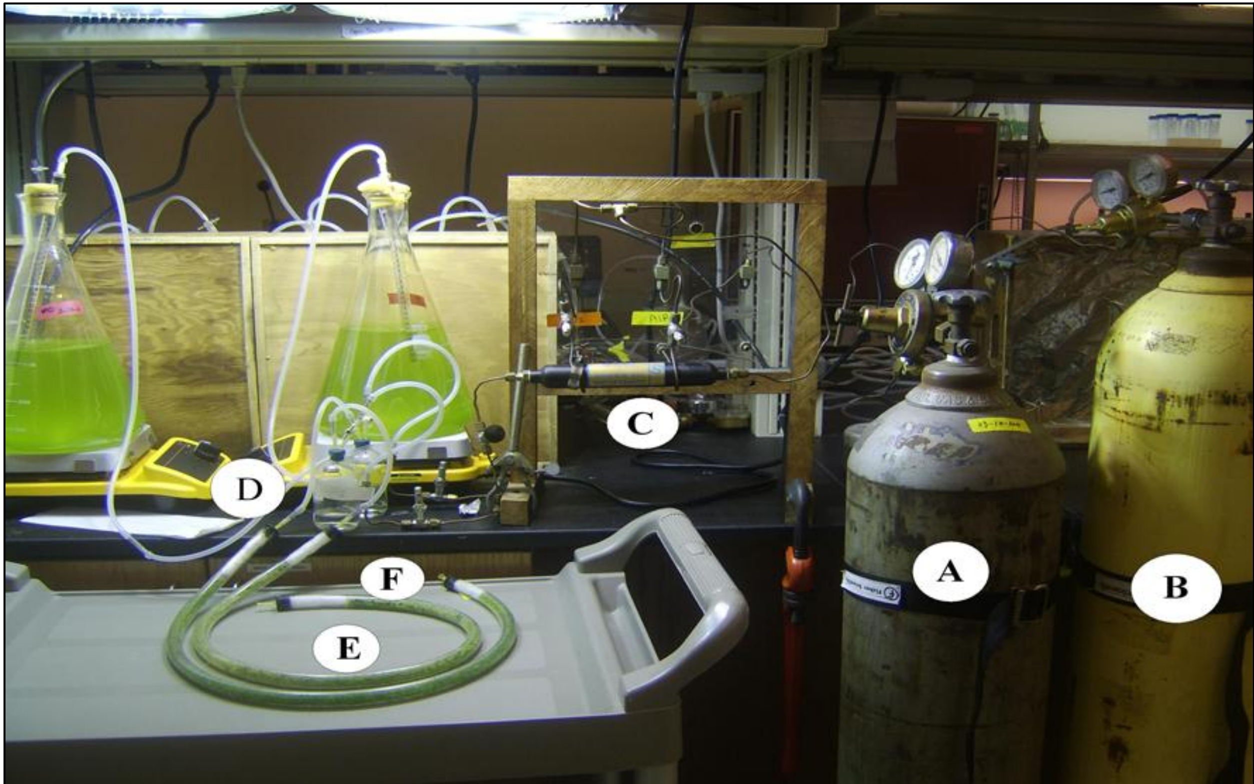
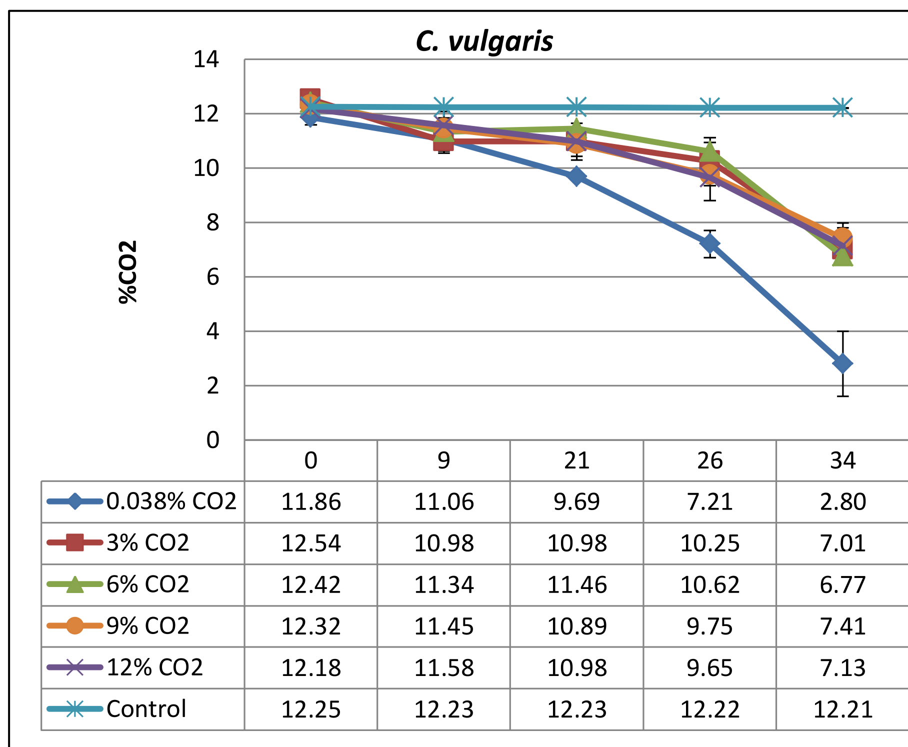
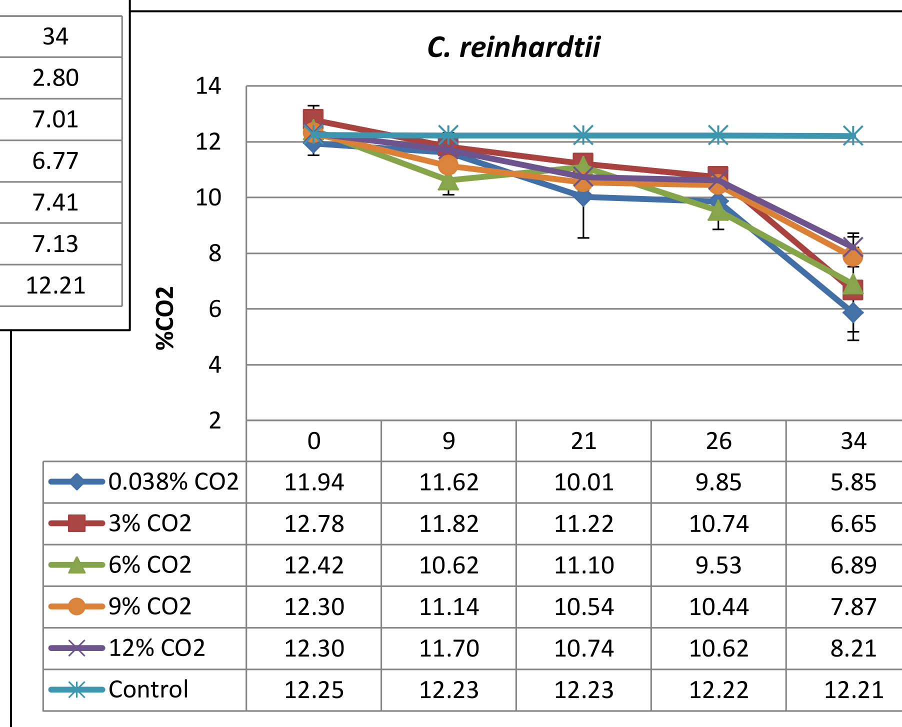


Figure 26. Consumption of 12% atmospheric CO_2 and growth over a 34-hrs period by *C. vulgaris* and *C. reinhardtii* using algal inoculum grown initially at ambient (0.038%) CO_2, 3% CO_2, 6% CO_2, 9% CO_2, and 12% CO_2.

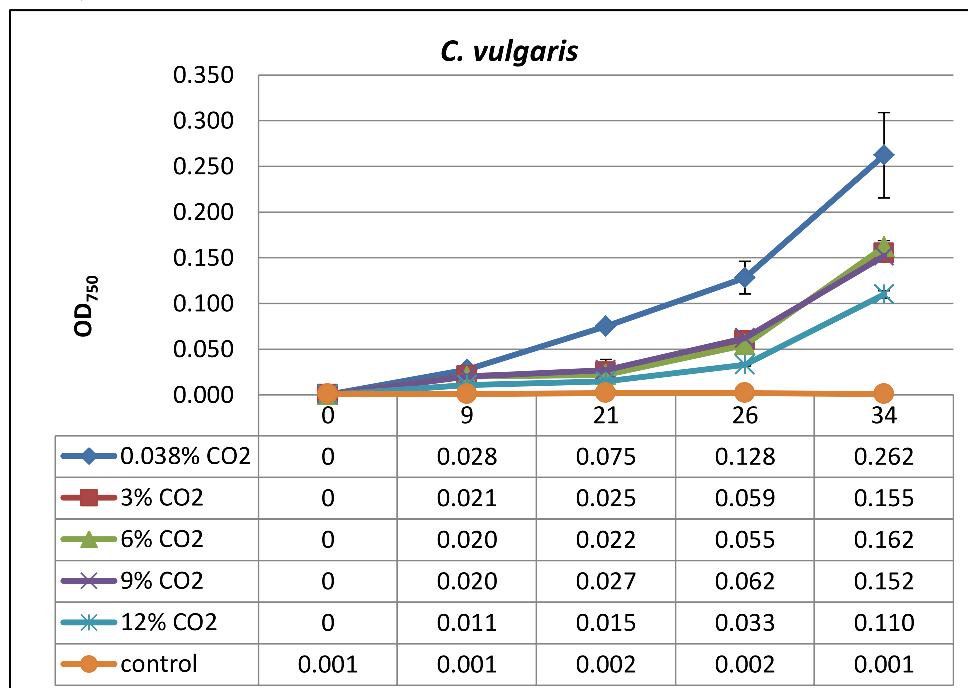


	0	9	21	26	34
0.038% CO2	11.86	11.06	9.69	7.21	2.80
3% CO2	12.54	10.98	10.98	10.25	7.01
6% CO2	12.42	11.34	11.46	10.62	6.77
9% CO2	12.32	11.45	10.89	9.75	7.41
12% CO2	12.18	11.58	10.98	9.65	7.13
Control	12.25	12.23	12.23	12.22	12.21

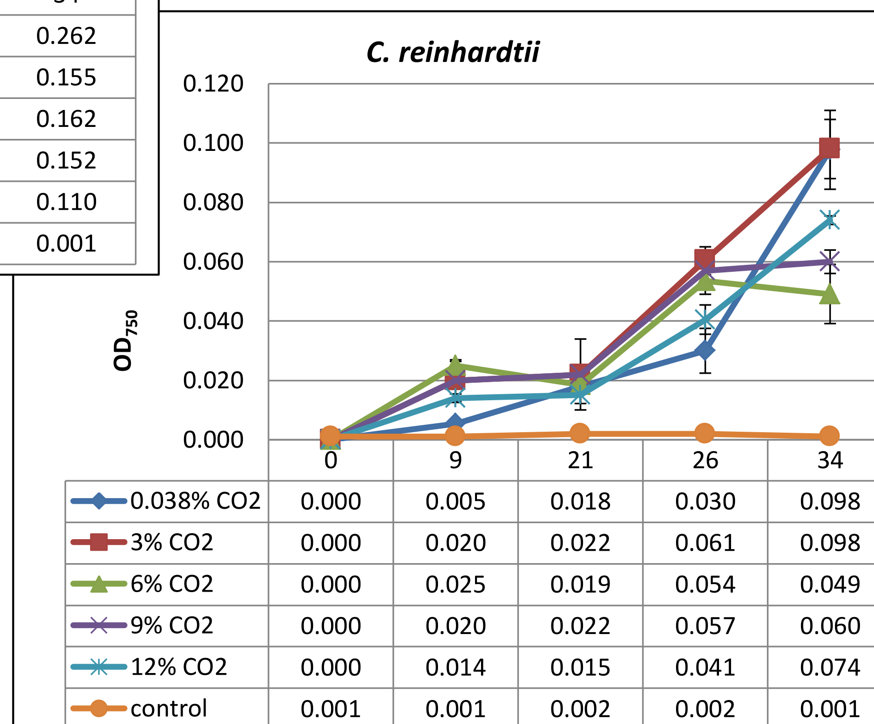


	0	9	21	26	34
0.038% CO2	11.94	11.62	10.01	9.85	5.85
3% CO2	12.78	11.82	11.22	10.74	6.65
6% CO2	12.42	10.62	11.10	9.53	6.89
9% CO2	12.30	11.14	10.54	10.44	7.87
12% CO2	12.30	11.70	10.74	10.62	8.21
Control	12.25	12.23	12.23	12.22	12.21

Figure 27. Growth of *C. vulgaris* and *C. reinhardtii* in serum bottle filled with 12% atmospheric CO_2 using algal inoculum grown initially at ambient (0.038%) CO_2, 3% CO_2, 6% CO_2, 9% CO_2, and 12% CO_2



	0	9	21	26	34
0.038% CO2	0	0.028	0.075	0.128	0.262
3% CO2	0	0.021	0.025	0.059	0.155
6% CO2	0	0.020	0.022	0.055	0.162
9% CO2	0	0.020	0.027	0.062	0.152
12% CO2	0	0.011	0.015	0.033	0.110
control	0.001	0.001	0.002	0.002	0.001



	0	9	21	26	34
0.038% CO2	0.000	0.005	0.018	0.030	0.098
3% CO2	0.000	0.020	0.022	0.061	0.098
6% CO2	0.000	0.025	0.019	0.054	0.049
9% CO2	0.000	0.020	0.022	0.057	0.060
12% CO2	0.000	0.014	0.015	0.041	0.074
control	0.001	0.001	0.002	0.002	0.001

Table 24. CO_2 fixation rates (mg_{CO2} L^{-1} day^{-1}) of 12%CO_2 atmospheric *C. vulgaris* and *C. reinhardtii* cells grown at ambient, 3%, 6%, 9%, and 12% CO_2.

Microalgal species	CO_2(%)	CO_2 consumption rate (mg L^{-1} d^{-1})	Reference
C. vulgaris	0.038	278	This work
C. vulgaris	3	149	This work
C. vulgaris	6	152	This work
C. vulgaris	9	146	This work
C. vulgaris	12	152	This work
C. reinhardtii	0.038	176	This work
C. reinhardtii	3	169	This work
C. reinhardtii	6	154	This work
C. reinhardtii	9	121	This work
C. reinhardtii	12	117	This work
Chlorella sp.	10	1767	Sung *et al.* (1999)
Chlorella sp.	20	1316	Sakai *et al.* (1995)
C. vulgaris	0.038	75	Scragg *et al*. (2002)
C. vulgaris	10	612	Jin *et al.* (2006)
C. vulgaris	0.8–1	6240	Cheng *et al.* (2006)
C. vulgaris	0.09	3450	Fan *et al.* (2008)
Chlorella sp.	2	857	Chiu *et al.* (2008)
Chlorella sp.	10	717	Chiu *et al.* (2009)
Chlorella sp.	5	700	Ryu *et al*. (2009)
C. vulgaris	5	251	Sydney *et al.* (2010)

Table 25. Analysis of variance (ANOVA) table of CO_2 uptake in liquid culture after 34 hrs.

Source of Variation	dF	Sum of Squares	F Ratio	Prob > F
Model	9	31.1224	5.2915	0.0078
Algae type	1	2.5590	3.9020	0.0765
CO_2 growth	4	23.4958	8.9567	0.0024
Algae type* CO_2 growth	4	5.1775	1.9737	0.1747
Error	10	6.5582		
Total	19	37.7906		

Table 26. Analysis of variance (ANOVA) table of OD_{750} in liquid culture after 34 hrs.

Source of Variation	dF	Sum of Squares	F Ratio	Prob > F
Model	9	0.0607	14.8536	0.0001
Algae type	1	0.0400	88.1924	<.0001
CO_2 growth	4	0.0132	7.3160	0.0051
Algae type* CO_2 growth	4	0.0074	4.0564	0.0330
Error	10	0.0045		
Total	19	0.0652		

Figure 28. Consumption of 3%, 6%, and 9% influent CO_2 flowing through immobilized *C. vulgaris* algal beads.

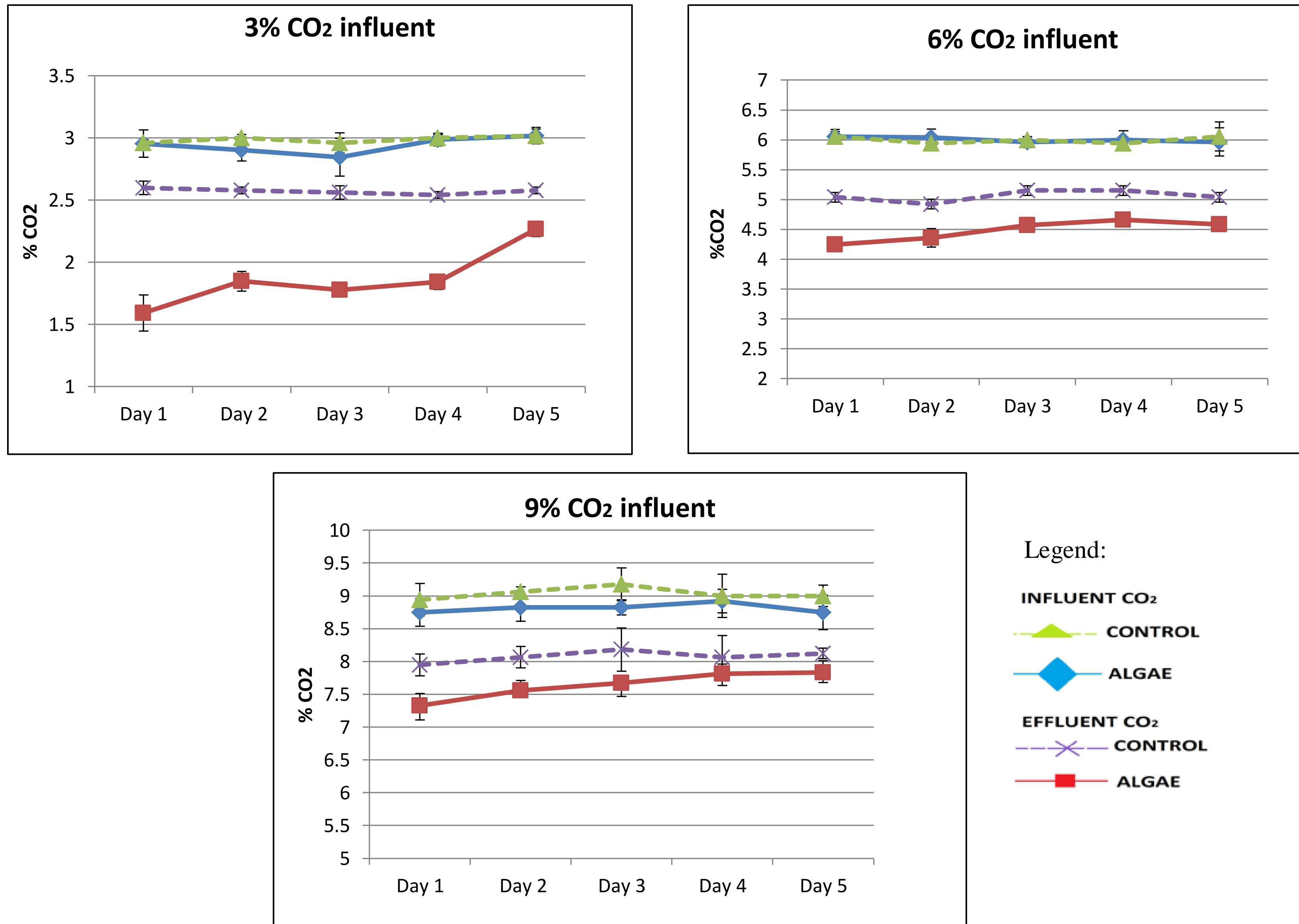


Figure 29. Consumption of 3%, 6%, and 9% influent CO_2 flowing through immobilized *C. reinhardtii* algal beads.

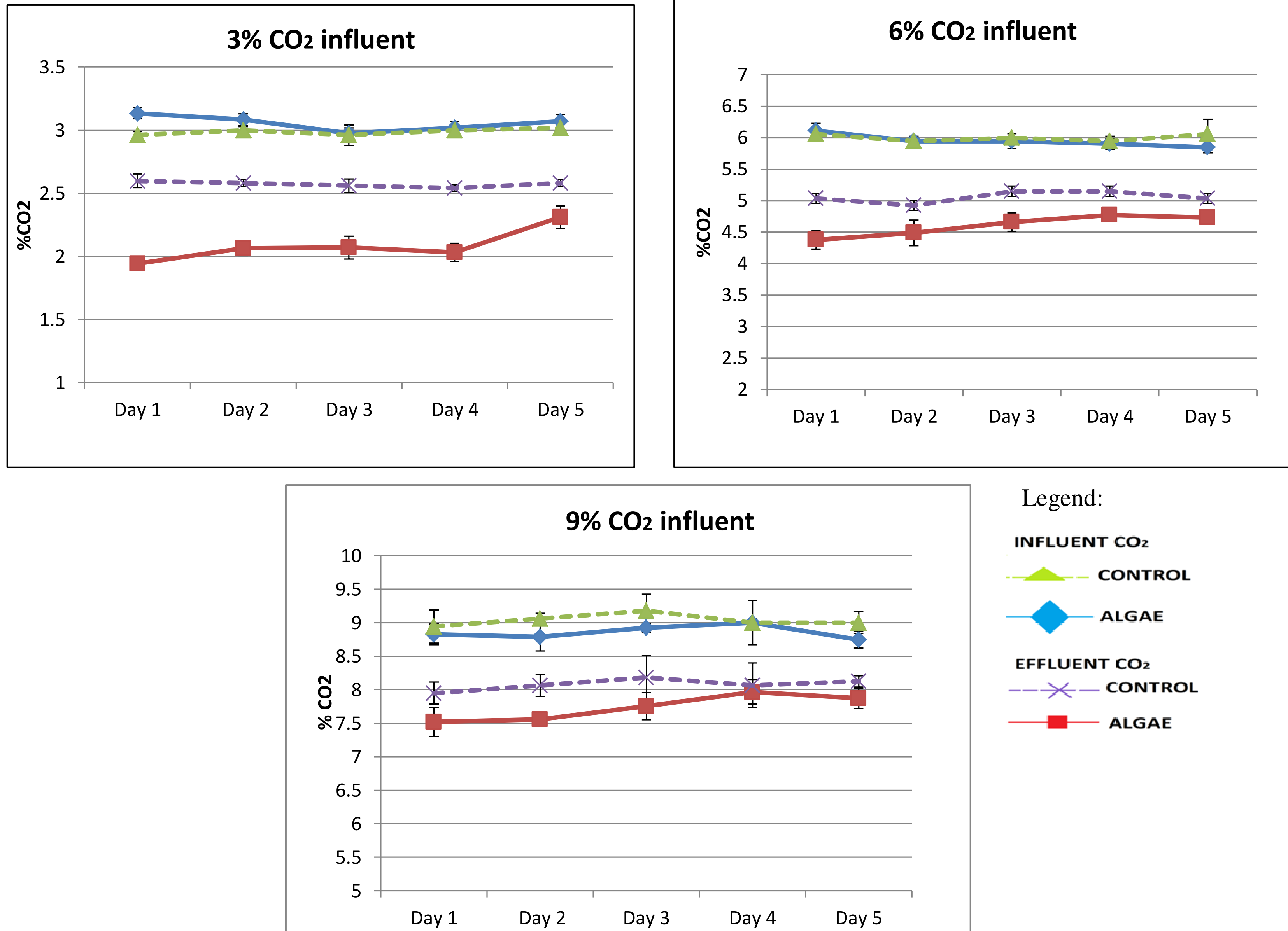
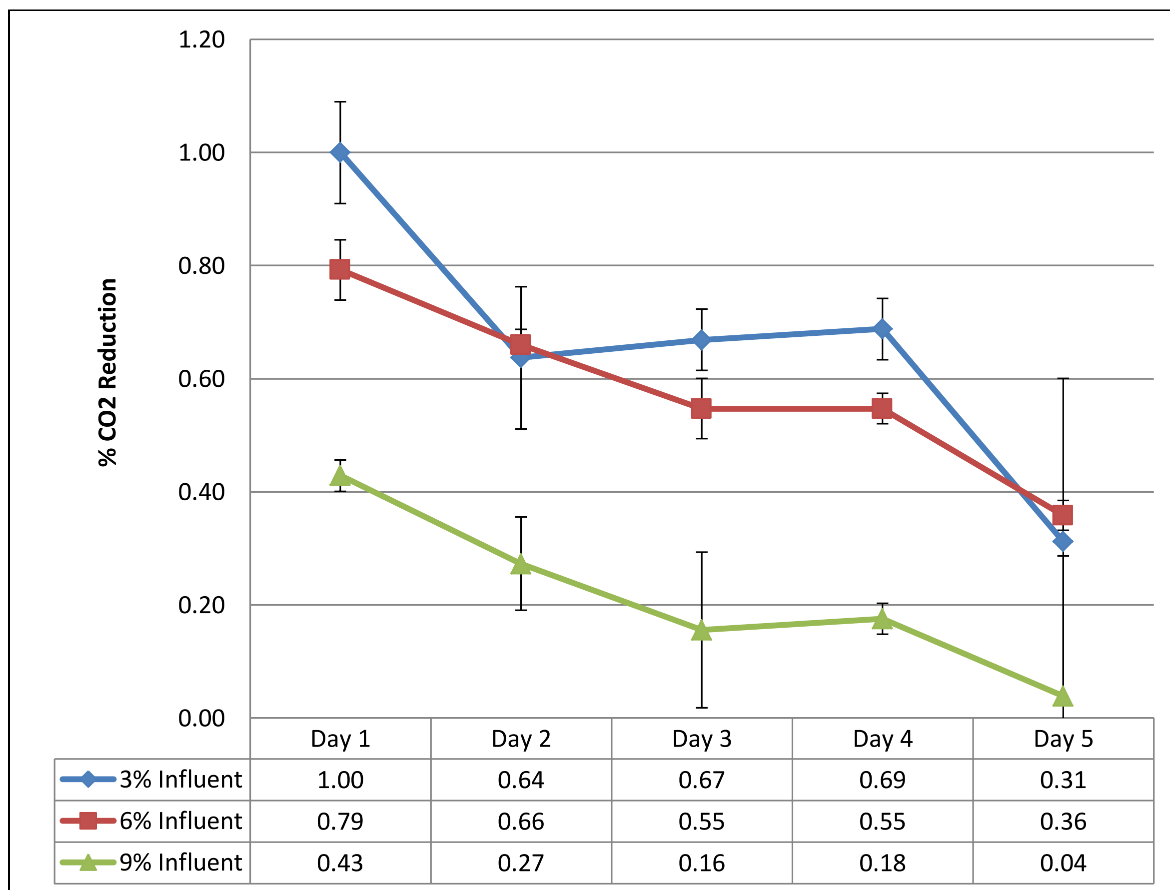
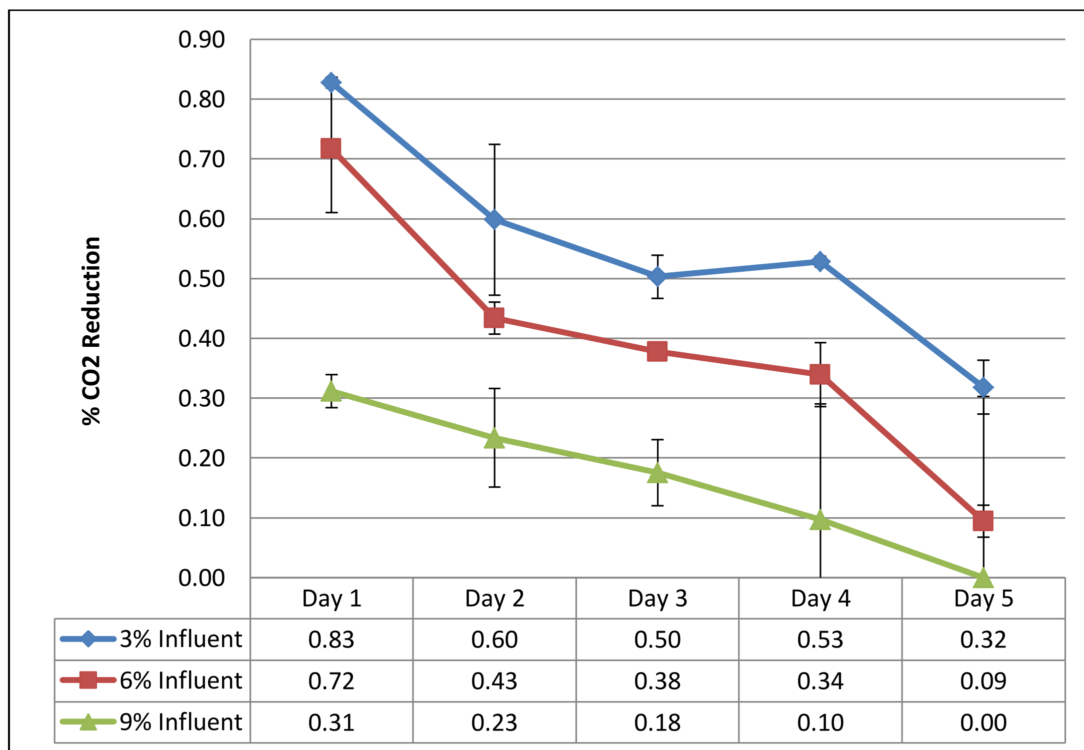


Figure 30. *C. vulgaris* % CO_2 reduction over a 5-day period at different %CO_2 influent.



	Day 1	Day 2	Day 3	Day 4	Day 5
3% Influent	1.00	0.64	0.67	0.69	0.31
6% Influent	0.79	0.66	0.55	0.55	0.36
9% Influent	0.43	0.27	0.16	0.18	0.04

Figure 31. *C. reinhardtii* % CO_2 reduction over a 5-day period at different %CO_2 Influent.



	Day 1	Day 2	Day 3	Day 4	Day 5
3% Influent	0.83	0.60	0.50	0.53	0.32
6% Influent	0.72	0.43	0.38	0.34	0.09
9% Influent	0.31	0.23	0.18	0.10	0.00

Table 27. Analysis of variance (ANOVA) table of %CO_2 reduction by algal beads on day one.

Source of Variation	dF	Sum of Squares	F Ratio	Prob > F
Model	5	0.6722	34.6232	0.0002
Algae type	1	0.0456	11.7511	0.0140
% CO_2 Influent	2	0.6216	80.0365	<.0001
Algae type* % CO_2 Influent	2	0.0050	0.6459	0.5571
Error	6	0.0233		
Total	11	0.6956		

Table 28. Analysis of variance (ANOVA) table of %CO_2 reduction by algal beads on day five.

Source of Variation	dF	Sum of Squares	F Ratio	Prob > F
Model	5	0.1613	1.4750	0.3222
Algae type	1	0.0225	1.0305	0.3492
% CO_2 Influent	2	0.0910	2.0812	0.2058
Algae type* % CO_2 Influent	2	0.04771	1.0911	0.3943
Error	6	0.1312		
Total	11	0.2925		

Table 29. Influent and effluent CO_2 concentration of control column and column containing algal beads.

A) Control column

%CO2	3% CO_2		6% CO_2		9% CO_2	
Day	Influent	Effluent	Influent	Effluent	Influent	Effluent
Day 1	2.9618 ± 0.03	2.5987 ± 0.05	6.0566 ± 0.08	5.0377 ± 0.08	8.9416 ± 0.25	7.9481 ± 0.17
Day 2	3.0000 ± 0.03	2.5796 ± 0.08	5.9434 ± 0.08	4.9245 ± 0.08	9.0584 ± 0.08	8.0649 ± 0.17
Day 3	2.9618 ± 0.08	2.5605 ± 0.05	6.0000 ± 0.01	5.1509 ± 0.08	9.1753 ± 0.25	8.1818 ± 0.33
Day 4	3.0000 ± 0.03	2.5414 ± 0.08	5.9434 ± 0.08	5.1509 ± 0.08	9.0000 ± 0.33	8.0649 ± 0.33
Day 5	3.0191 ± 0.05	2.5796 ± 0.08	6.0566 ± 0.24	5.0377 ± 0.08	9.0000 ± 0.17	8.1234 ± 0.08

B) *C. vulgaris* column

%CO2	3% CO_2		6% CO_2		9% CO_2	
Day	Influent	Effluent	Influent	Effluent	Influent	Effluent
Day 1	2.9554 ± 0.11	1.5923 ± 0.15	6.0566 ± 0.11	4.2453 ± 0.07	8.7468 ± 0.21	7.3247 ± 0.19
Day 2	2.9045 ± 0.09	1.8471 ± 0.08	6.0377 ± 0.14	4.3585 ± 0.15	8.8247 ± 0.21	7.5584 ± 0.16
Day 3	2.8471 ± 0.15	1.7771 ± 0.04	5.9622 ± 0.09	4.5660 ± 0.06	8.8247 ± 0.11	7.6753 ± 0.09
Day 4	2.9873 ± 0.05	1.8408 ± 0.06	6.0000 ± 0.15	4.6604 ± 0.09	8.9221 ± 0.18	7.8117 ± 0.15
Day 5	3.0191 ± 0.07	2.2675 ± 0.06	5.9623 ± 0.24	4.5849 ± 0.10	8.7468 ± 0.26	7.8312 ± 0.18

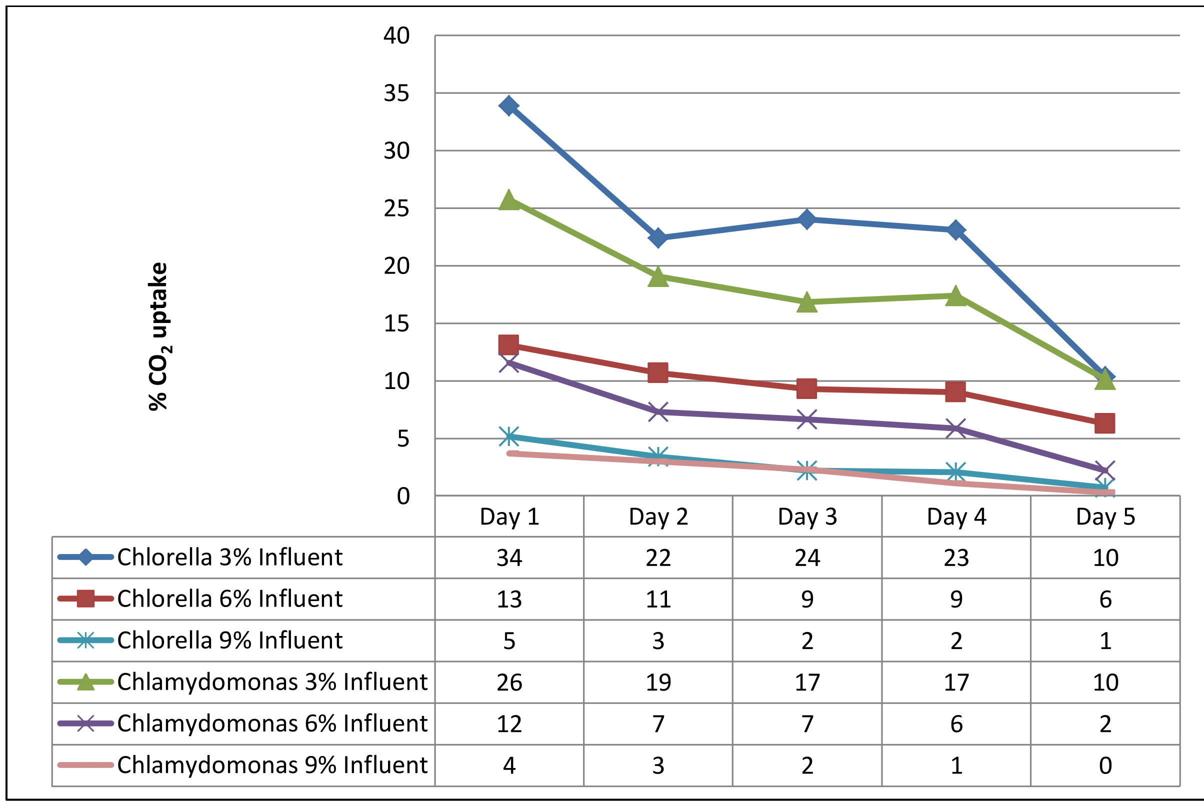
C) *C. reinhardtii* column

%CO2	3% CO_2		6% CO_2		9% CO_2	
Day	Influent	Effluent	Influent	Effluent	Influent	Effluent
Day 1	3.1338 ± 0.04	1.9427 ± 0.04	6.0566 ± 0.11	4.2453 ± 0.07	8.8247 ± 0.16	7.5195 ± 0.21
Day 2	3.0828 ± 0.05	2.0637 ± 0.06	6.0377 ± 0.14	4.3585 ± 0.15	8.7857 ± 0.21	7.5584 ± 0.09
Day 3	2.9745 ± 0.04	2.0701 ± 0.09	5.9623 ± 0.09	4.5660 ± 0.07	8.9221 ± 0.07	7.7532 ± 0.21
Day 4	3.0191 ± 0.05	2.0318 ± 0.07	6.0000 ± 0.15	4.6604 ± 0.07	9.0000 ± 0.07	7.9675 ± 0.18
Day 5	3.0701 ± 0.06	2.3121 ± 0.09	5.9623 ± 0.24	4.5849 ± 0.10	8.7468 ± 0.12	7.8701 ± 0.15

Table 30. Rates of CO_2 uptake (mg L^{-1} day^{-1}) by alginate-immobilized *C. vulgaris* and *C. reinhardtii* at 3%, 6% and 9% CO_2 influent.

% CO_2 Influent	**3% CO_2**		**6% CO_2**		**9% CO_2**	
Time	*C. vulgaris*	*C. reinhardtii*	*C. vulgaris*	*C. reinhardtii*	*C. vulgaris*	*C. reinhardtii*
Day 1	40.28	35.28	53.65	51.41	42.12	38.66
Day 2	31.32	30.18	49.74	43.03	37.50	36.35
Day 3	31.69	26.79	41.35	38.00	34.04	34.62
Day 4	33.96	29.24	39.68	33.53	32.89	30.578
Day 5	22.26	22.45	40.79	32.97	27.12	25.96

Figure 32. *C. vulgaris* and *C. reinhardtii* % CO_2 uptake at different influent CO_2%.



	Day 1	Day 2	Day 3	Day 4	Day 5
Chlorella 3% Influent	34	22	24	23	10
Chlorella 6% Influent	13	11	9	9	6
Chlorella 9% Influent	5	3	2	2	1
Chlamydomonas 3% Influent	26	19	17	17	10
Chlamydomonas 6% Influent	12	7	7	6	2
Chlamydomonas 9% Influent	4	3	2	1	0

Appendix 1.

Fig. 1. *C. vulgaris* standard curve for conversion of OD_{750} values to cell numers.

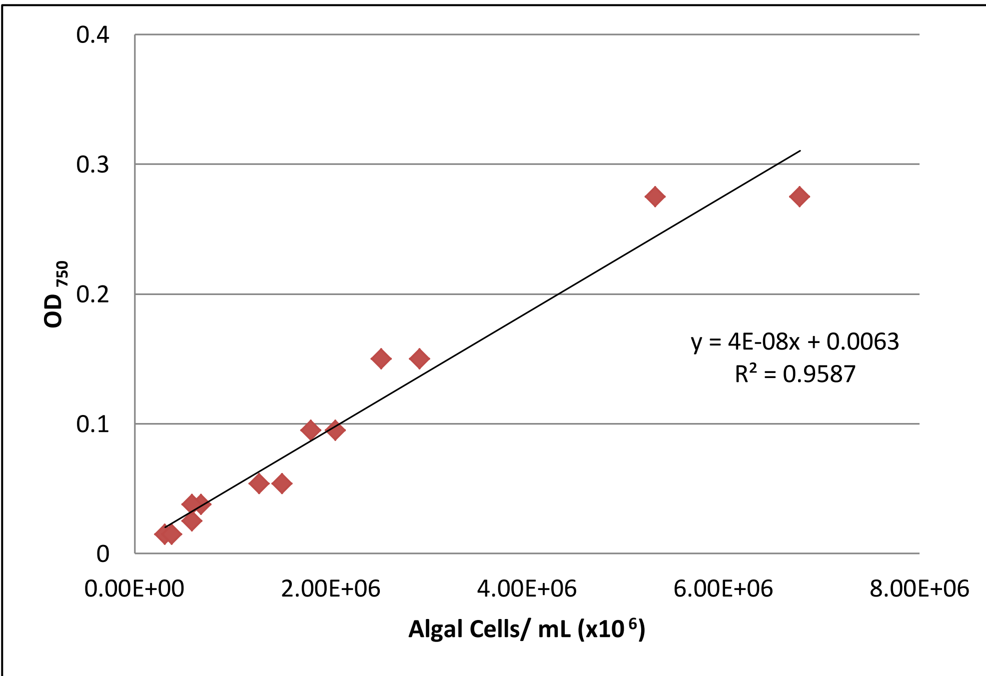


Fig. 2. *C. reinhardtii* standard curve for conversion of OD_{750} values to cell numers.

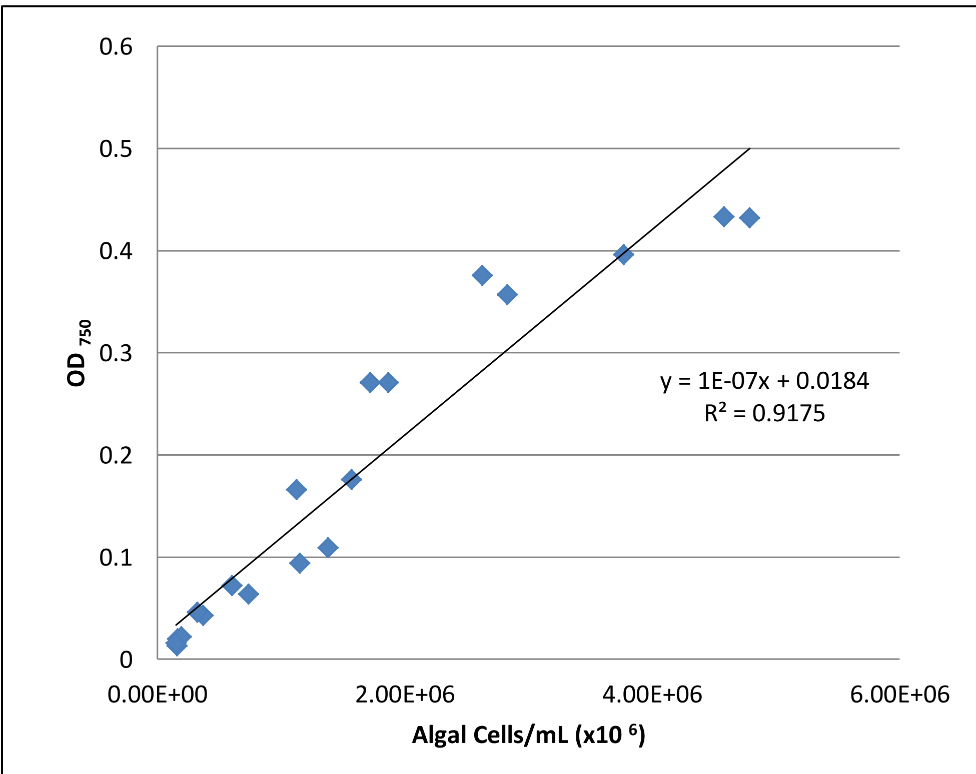


Table 1. Conversion of OD_{750} values to cell numers.

Experiments	Inoculum OD_{750}	# Cell mL^{-1}	
		C. vulgaris	*C. reinhardtii*
Chapter 1 (varying growth medium, pH, light, and CO_2 concentration)	0.250	6.09E+06	2.32E+06
Chapter 2 Consumption of CO_2 in liquid culture	0.050	1.09E+06	0.32E+06
Chapter 2 Consumption of CO_2 flowing through immobilized algal beads	2.200	5.48E+07*	2.18E+07*

* # cells cm^{-3}