EVALUATION OF NITORGEN AND CARBON SUPPLEMENTATION STRATEGIES FOR OPTIMIZING BIOMASS GENERATION DURING CULTIVATION OF *CHLORELLA SOROKINIANA*, STRAIN SLA-04

by

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DEDICATION

In memoriam of my Grandmother, Pamma (Betty Schardien).

Also, to my Mom (Dr. Bette Jackson) and Dad (Dr. Jerome Jackson); my friends, Marie Abraham Ralston and Cole Conners; and my dogs, Mr. Bear, Kona, and Copper.

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ABSTRACT

Algal cultivation requires significant nitrogen and carbon inputs, which are expensive and can offset benefits associated with biofuel production. This research investigates growth of an alkali-tolerant *Chlorella sorokiniana*, strain SLA-04, using different nitrogen and carbon regimes to improve physiological knowledge of this novel organism, and improve biomass production and resource demand. Nitrate, ammonium, and urea were used efficiently by SLA-04, however pH changes during utilization of nitrate and ammonium impacted inorganic carbon availability (species and concentration). Generation of OH^- during use of nitrate increased pH, increasing mass transfer of CO_2 into solution and increasing the ratio of HCO_3^-/CO_2. Ammonium utilization resulted in proton generation, lowering pH and inhibiting growth. When bicarbonate, rather than CO_2, was provided, productivity improved for the urea and mixed nitrogen conditions. This likely resulted from upregulation of genes related to nitrogen and carbon assimilation in the presence of bicarbonate, however Na^+ cotransport with urea and nitrate is required in some organisms. It is possible that Na^+ was insufficient when CO_2 was provided, but not in conditions with bicarbonate since it was added as $NaHCO_3^-$. The impact of Na^+, as well as other ions, on nitrogen and carbon utilization is not well understood, but it may alter gene regulation. Bicarbonate and CO_2 both promoted increased growth relative to cultures without inorganic carbon supplementation. The highest productivities were observed when carbon supplementation, either as continued CO_2 augmentation to the air sparge or as a 50mM bicarbonate amendment, was provided during nitrogen deplete growth. Glucose availability improved productivity for conditions without CO_2 supplementation. The use of urea or a combination of nitrogen sources with bicarbonate, instead of CO_2, was promising due to (a) the low cost of urea, relative to the other nitrogen sources; (b) the potential for using wastewater containing a mix of nitrogen sources; and (c) the low cost and easy transport of bicarbonate. Future research should evaluate changes in SLA-04 gene expression resulting from the supply of different nutrients, including nitrogen and carbon sources, as well as other ions essential for growth.

CHAPTER ONE

INTRODUCTION

Fossil fuel dependence has created issues related to environmental degradation due to acquisition, transportation, refining, and end use of ancient subsurface hydrocarbons. As a result, there has been a significant increase in interest in the development of alternative energy technologies including biofuels (Chisti, 2007; Gavrilescu & Chisti, 2005). Algal biofuels are of particular interest due to the general algal characteristics of increased productivity and reduced land and water requirements relative to plant-based biofuels (Chisti, 2007). In addition, algae can be cultivated using low quality waste streams on otherwise arid land (Abdel-Raouf et al., 2012; Cuellar-Bermudez et al., 2017). When compared to biofuels produced from food crops, algal biofuel production has the added benefit of avoiding competition with food production (Chisti, 2007). Beyond biofuels, generation of a number of other high-value products from algal biomass or its derivatives is possible, including pigments, animal feed, fertilizers, bioplastics, and nutraceuticals (Gavrilescu & Chisti, 2005; Griffiths et al., 2016; Sahoo & Seckbach, 2015; Torzillo et al., 2003). In addition, the use of microalgae cultivation as a bioremediation process for air and water, with the added benefit of bioproduct generation, has gained increased attention. The high carbon fixation rates achievable by many algae strains has been investigated for carbon capture and sequestration (CCS) to address the rising concentration of CO_2 in the atmosphere (Chi et al., 2013; Kadam, 2002; Richard, 2010). Similarly, the high rate of nitrogen and phosphorus (as well as other contaminant) assimilation observed for many microorganisms has been exploited as a method for treating various industrial, agricultural, and domestic waste streams (Bohutskyi et al., 2015). For most applications, optimization of growth, biomass

composition, and nutrient uptake is dependent on algal physiology, growth, biomass composition, and resource demand and costs (yield from resources).

Currently, numerous constraints exist for expanded commercialization of algal biotechnology related to (1) the identification and isolation of organisms suitable for rapid production of desired products (Bohutskyi et al., 2015; Mutanda et al., 2011; Sheehan & National Renewable Energy, 1998), (2) environmental constrains of cultivation using large, open-air culturing systems (Torzillo et al., 2003; Vonshak & Richmond, 1988), and (3) limited understanding of the physiology of diverse algae (Borowitzka et al., 2016). Further, optimization of product generation or process performance hinges on understanding resource utilization by organisms or consortia cultivated. To address these constraints, significant work has been done. Bioprospecting for organisms that are able to achieve high productivity of biomass containing desired products is an ongoing effort, with significant interest being directed towards extreme environments, where physiological adaptations by organisms might be solicited to address current limitations related to nutrient demand and contamination during open air cultivation, and to otherwise expand the range of potential operating conditions for production (Jorquera et al., 2019; Rai et al., 2001; Rathinam & Sani, 2018; Varshney et al., 2015). Large-scale outdoor cultivation is essential for expansion of algal based biotechnology applications but is currently constrained due to a still developing understanding of how to mitigate for environmental factors impacting open culturing systems, such as climate, homogenous light and nutrient distribution in large culture volumes, and culture contamination (Torzillo et al., 2003; Vonshak & Richmond, 1988). In addition, the current understanding of algal physiology is limited for many organisms

and as a result, optimization of culturing conditions for enhancement of production and composition of algal biomass can be strain dependent (Borowitzka et al., 2016; Rai et al., 2001).

Nitrogen and carbon are essential nutrients for cultivation of algae, and macro-nutrient availability (concentration and source) can have profound impacts on algal growth and biomass composition (Canon-Rubio et al., 2016; Eustance et al., 2013; Guihéneuf et al., 2008; Li et al., 2008; Mokashi et al., 2016; Pal et al., 2011; Tu et al., 2018), as well as solution chemistry (Eustance et al., 2013; Lachmann et al., 2019). Numerous strategies attempting to optimize algal biomass productivity through selective nutrient (N and C) supplementation have been investigated with varying levels of success (Canon-Rubio et al., 2016; Mokashi et al., 2016; Nayak et al., 2018; Ramanna et al., 2014; Ribeiro et al., 2020). As a result, optimization of growth should be approached holistically, considering not only the demand of individual resources, but also how their presence and utilization by algae impacts the system as a whole.

Bioavailability of Nitrogen and Carbon Sources

In general, algae exhibit significant flexibility in their ability to utilize different sources of nitrogen and carbon for growth, with many organisms being able to use a multitude of these nutrient resources interchangeably (Eustance et al., 2013; Gardner, Lohman, et al., 2012). A large number of nitrogen sources are biologically available to most algal species, including inorganic nitrogen sources such as nitrate and ammonium, as well as organic nitrogen forms such as urea (Borowitzka et al., 2016; Eustance et al., 2013). Ammonium is the source of exogenous nitrogen utilized by algae and is integrated into biomass as amino acids via condensation with glutamate to form glutamine in a reaction catalyzed by glutamine synthetase (Lachmann et al.,

2019; Miflin & Lea, 1980). It is noteworthy that ammonium (NH_4^+) and ammonia (NH_3) both exist in solution, with the relative abundance of NH_3 increasing with pH (Collos & Harrison, 2014). NH_3 is volatile and can escape solution, is able to passively diffuse across cell membranes, and is toxic to cells. Therefore, the use of NH_3 during cultivation at high-pH is inherently problematic. In contrast, NH_4^+ is unable to passively diffuse across cell membranes due to its charge and must be actively transported (Collos & Harrison, 2014). Nitrate (NO_3^-) and nitrite (NO_2^-) are also commonly available to many algal strains, with NO_3^- going through successive reduction steps, first to NO_2^- and then NH_4^+ once intracellular (Eustance et al., 2013; Hellebust & Ahmad, 1989; Lachmann et al., 2019; Li et al., 2008; Podevin et al., 2015; Sanz-Luque et al., 2015). Utilization of NO_3^- is therefore more energy intensive and requires activity of nitrate and nitrite reductase (Lachmann et al., 2019). Urea has also been proposed by a number of researchers as a promising nitrogen source for algal cultivation due to its relatively high availability and low cost (Berman & Chava, 1999; Eustance et al., 2013; Kumbhar et al., 2020; Podevin et al., 2015; Ramanna et al., 2014; Ribeiro et al., 2020). Urea metabolism in algae occurs via one of two pathways, either via urease or via urea amidolyase. The urea amidolyase system utilizes two enzymes for urea metabolism, urea carboxylase, which converts urea to allophanate by carboxylation using HCO_3^-, and allophanate hydrolase, which hydrolyzes allophanate to form HCO_3^- and NH_4^+ (Strope et al., 2011; Tu et al., 2018). Each urea molecule generates 2 NH_4^+ and 1 HCO_3^-.

Both organic carbon and inorganic carbon can be used by algae, with different organisms displaying variable affinity for different inorganic and organic carbon sources. Inorganic carbon as CO_2 is the functional carbon source used by RuBisCO during photosynthesis (Moroney &

Somanchi, 1999). Due to the low affinity of RuBisCO for CO_2, as well as the relatively low concentration of CO_2 in the atmosphere, RuBisCO is generally less than half saturated for most algal organisms (Giordano et al., 2005; Moroney & Somanchi, 1999; Singh, 2014). The problem with low CO_2 saturation is exacerbated by the high affinity of RuBisCO to bind with oxygen (Moroney & Somanchi, 1999; Singh, 2014). To address this issue, many algal strains have developed carbon concentrating mechanisms (CCMs) to increase the concentration of inorganic carbon, as HCO_3^-, in close proximity to the photocenters of the cell (Giordano et al., 2005; Singh, 2014). Near the active sites of RuBisCO, HCO_3^- is converted to CO_2 by carbonic anhydrases prior to use by the cell (Moroney & Somanchi, 1999). The presence of CO_2 significantly represses the activity of CCMs in eukaryotic algae (Giordano et al., 2005). Inorganic carbon as CO_2/H_2CO_3 and HCO_3^- are the primary sources of inorganic carbon taken up by algae for photosynthesis (John et al., 2008; Mokashi et al., 2016), however many species/strains are capable of using exogenous and endogenous organic carbon via respiration to generate biomass (Perez-Garcia et al., 2011; Pipes & Gotaas, 1960). Interestingly, no literature was found where CO_3^-, the dominant form of inorganic carbon at pH>10.3, was investigated as an inorganic carbon source for algae cultivation. This is likely because pathways for CO_3^{2-} metabolism are not well understood. Diffusive transport of CO_3^{2-} is not possible at pH values where carbonate speciation is relevant, and no active transport mechanisms are known (Li et al., 2018). The affinity to utilize organic carbon varies between organisms and strains, with some organisms being capable of growth under fully heterotrophic conditions (Lee, 2001; Lowrey et al., 2015; Perez-Garcia et al., 2011). Growth using both inorganic and organic carbon (mixotrophic cultivation) is common during commercial cultivation for many applications (Lee,

2001; Zhang et al., 2017). Some algal strains have displayed synergistic growth under mixotrophic conditions, where productivity is enhanced beyond what is achievable during phototrophic (inorganic carbon only) and heterotrophic (organic carbon only) cultivation combined (Lee, 2001; Lowrey et al., 2015; Zhang et al., 2017). In contrast, there is also evidence of a negative interaction between organic carbon and CO_2/H_2CO_3 metabolism for some algal strains with respect to lipid generation (lower lipid productivities have been observed during mixotrophic cultivation for some algal strains; Cheirsilp and Torpee, 2012), however to the best of the author's knowledge, this has not been observed for cultures where HCO_3^- is the dominant form of inorganic carbon.

Nutrient Interactions and Solution Chemistry

Assimilation of nitrogen from NH_4^+ and NO_3^- both impact growth medium pH, albeit in opposing ways. The utilization of nitrogen from NH_4^+ results in the release of a single proton for each nitrogen. This results in a reduction in culture pH and alkalinity, which can negatively impact growth and ultimately inhibit photosynthesis and other cellular processes (Collos & Harrison, 2014; Eustance et al., 2013; Lachmann et al., 2019; Miflin & Lea, 1980; Ribeiro et al., 2020; Wolf-Gladrow et al., 2007). NH_4^+ availability is subject to solution pH and is converted to NH_3 as pH is increased(Collos & Harrison, 2014). The pK_A of NH_4^+/NH_3 under standard conditions is 9.3 (Sutton, 2009). NH_3 is problematic for algal cultivation due to its toxicity and ability to volatilize out of solution (Collos & Harrison, 2014). In contrast to NH_4^+, assimilation of nitrogen from nitrate results in the generation of a single OH^- ion for each nitrogen, resulting in an increase in system pH (Eustance et al., 2013; Lachmann et al., 2019; Wolf-Gladrow et al.,

2007). The subsequent impacts on pH from nitrate or ammonium can in turn impact carbon speciation.

Dissolved inorganic carbon (DIC) is significantly impacted by pH, alkalinity, and the partial pressure of CO_2 gas at its interface with the growth solution (Michałowski & Asuero, 2012). At high pH, OH^- in solution can react directly with CO_2 gas to form HCO_3^-, which can be concentrated within algal cells via CCMs (Giordano et al., 2005). During conversion of HCO_3^- to CO_2 by carbonic anhydrases OH^- is released, becoming available to bind with a new CO_2 molecule (Giordano et al., 2005; Mokashi et al., 2016; Tu et al., 2018).

High-Alkalinity Algae Cultivation

Alkaliphilic environments are generally inhospitable to most organisms due to oxidative, ion, osmotic, and pH stresses (Mir et al., 2018), however numerous microorganisms have adaptations that not only allow them to survive under these harsh conditions, but to thrive. Significant research has been conducted on alkaliphilic bacteria, and to a lesser degree on cyanobacteria, but studies investigating alkaliphilic eukaryotic algae are far more limited (Gardner et al., 2011; Moll et al., 2014; Rai et al., 2001). In recent years however, increased interest has been shown in understanding the potential exploitation of alkaliphilic algae for the generation of high-value products due to the high productivity observed for these organisms and their ability to grow at a broader range of operating conditions (Jorquera et al., 2019; Rai et al., 2001; Rathinam & Sani, 2018). In addition, the use of HCO_3^- for cultivation of microalgae has received increased attention due to (1) increased mass transfer of inorganic carbon under high pH conditions, where OH^- is able to react directly with CO_2 gas to generate HCO_3^- in solution

(Canon-Rubio et al., 2016); (2) reduced competition and predation by organisms that are intolerant to high-pH and/or high-alkalinity conditions (Peng et al., 2016; Peng et al., 2015; Varshney et al., 2015); and (3) improved lipid synthesis caused by enhanced photosynthesis, resulting in increased carbon fixation, as well as differential gene regulation promoting lipid accumulation from starch and protein degradation (Blaskovich, 2014; Mokashi et al., 2016; Peng et al., 2015; Qu & Miao, 2021; Vadlamani, 2016). Variability exists in the potential for different algal strains to grow in high-pH and/or high-alkalinity conditions, with organisms that are able to grow at pH values >9, but that grow optimally at circumneutral pH or below, described as alkali-tolerant, and organisms that grow optimally at pH values between 10 and 11 described as alkaliphilic (Rai et al., 2001) .

Cultivation of *Chlorella* sp., strain LPF, using bicarbonate amended media with different nitrogen sources was performed by Tu et al. (2018) to evaluate the impact of bicarbonate supplementation on gene expression. During that study genes associated with carbon fixation (RBsC) and HCO_3^- transport (SLC4) were upregulated (3-4 fold and 1-5 fold upregulation for RBsC and SLC4, respectively) when HCO_3^- was added to the growth medium. Genes related to NO_3^- conversion to NH_4^+ (nitrate and nitrite reductase) and urea conversion to NH_4^+ (urea carboxylase, UC) were upregulated (1.8-5 fold and 25-34 fold upregulation for NR and UC, respectively) when HCO_3^- was added to cultures supplemented with nitrate and urea as the sole nitrogen source, respectively. In addition, expression of genes related to assimilation of NH_4^+ into amino acids (glutamine synthetase, GS) was upregulated (4-47 fold) when HCO_3^- was added to medium containing either NO_3^-, NH_4^+, or urea (Tu et al., 2018). Collectively these changes in gene expression suggest that HCO_3^- can significantly enhance both carbon fixation and nitrogen

assimilation. During a separate study, transcriptomic analysis of *Chlorella sp*, strain BLD, identified a number of differences in the regulation of genes related to carbon flow during cultivation at pH 7.5 and pH 10 (Qu & Miao, 2021). From their investigation, Qu and Miao (2021) concluded that changes in gene regulation redirected carbon flow away from protein and starch synthesis, and towards cell wall metabolism, organic acid synthesis, and lipid accumulation.

Rationale for Current Study

The current study investigates the use of various nitrogen and carbon supplementation strategies to improve biomass production and resource requirements for production during cultivation of *Chlorella sorokiniana*, strain SLA-04. Strain SLA-04, is an isolate from Soap Lake in Washington state, that has been investigated for biofuel production due to its high productivity using slightly saline, high alkalinity, and high pH conditions (Halverson, 2014). In addition, it is able to grow well in low alkalinity, circumneutral pH, and low salt media; as well as in media supplemented with organic carbon (Vadlamani et al., 2017). Its ability to grow under a wide range of conditions makes it a promising organism for elucidating physiological responses during cultivation using high- and low-alkalinity media, and high- and circumneutral-pH media. As noted previously, extensive research has been conducted to evaluate the impact of nitrogen and carbon availability on growth, biomass (and biomass component) productivity, and nitrogen removal by eukaryotic algae, however research focusing on nitrogen and carbon utilization by alkaliphilic or alkali-tolerant algal strains is more limited. To this end, the research presented here investigates the impact of nitrogen (nitrate, ammonium, and urea) and carbon (CO_2, HCO_3^-,

and glucose) sources on cell growth, biomass production, and growth media chemistry, to identify culturing parameters that optimize biomass production and resource demand.

CHAPTER TWO

METHODS

Strain Maintenance and Experimental Conditions

Chlorella sorokiniana strain SLA-04 was isolated from Soap Lake in the state of Washington and identified as described by (Vadlamani et al., 2017). Cellular growth was evaluated here using four nitrogen and five inorganic carbon supplementation strategies. Three nitrogen sources (nitrate, ammonium, and urea) were evaluated independently and in combination at equal nitrogen ratios to comprise the four nitrogen supplementation strategies. Each of the four nitrogen strategies were evaluated using Bold's Basal Medium (BBM) titrated to pH 8.7 with 1N KOH and modified to contain 3.5 mM of nitrogen using the different nitrogen sources analyzed. In addition, the nitrogen supplementation strategies were evaluated in BBM modified with 50 mM sodium bicarbonate ($NaHCO_3$) to understand how high-alkalinity conditions impact nitrogen utilization. Inorganic carbon supplementation strategies included supplementation with (1) air at 0.4 $L*min^{-1}$ (Air), (2) 5% CO_2 to 0.4 $L*min^{-1}$ air supply continuously during the photoperiod for nitrogen replete and deplete growth stages (5% CO_2 2S), (3) 5% CO_2 to 0.4 $L*min^{-1}$ air supply continuously during the photoperiod during nitrogen replete growth (5% CO_2 NR), (4) 5% CO_2 to 0.4 $L*min^{-1}$ air supply intermittently, 5 $min*hour^{-1}$, during the photoperiod for nitrogen replete and deplete growth stages (5% CO_2 INT), and (5) 50 mM HCO_3^- added to the growth medium prior to inoculation (50 mM HCO_3^-). The inorganic carbon conditions were cultivated using BBM titrated to pH 8.7 with 1N KOH. Nitrate was used as the sole nitrogen source provided for the inorganic carbon conditions. The Air, 5% CO_2 2S,

5% CO_2 INT, and 50 mM HCO_3^- conditions were also evaluated in BBM supplemented with 725 mg*L^{-1} glucose prior to inoculation for characterization of mixotrophic growth. The addition of a 50 mM HCO_3^- amendment at nitrogen limitation was also evaluated for all nitrogen and high-alkalinity nitrogen conditions, the 5% CO_2 2S, 5% CO_2 NR, 5% CO_2 INT, and 50 mM HCO_3^- conditions, and the glucose supplemented 5% CO_2 2S, 5% CO_2 INT, and 50 mM HCO_3^- conditions. Independent inoculum cultures for each condition were prepared using BBM titrated to pH 8.7 using KOH. For the nitrogen and high alkalinity nitrogen conditions, inoculum medium was prepared with the respective nitrogen source(s) that would be available in the experimental reactors. For conditions containing 50 mM HCO_3^- at inoculation, inoculum cultures were prepared with 50 mM $NaHCO_3$ present in the medium and were not titrated using KOH. Inoculation of the experimental system was conducted while inoculum cultures were in late exponential growth phase and nitrogen concentrations in the inoculum were 0.5-1.0mM.

Each condition was evaluated using a minimum of three experimental replicates in batch cultures using 70 × 500 mm glass tubes containing 1.25 L medium submersed in a water bath to control temperature at 24 °C ± 1 °C. Filtered air (0.5 μm pore size filter) was supplied at 0.4 L*min^{-1} via a glass tube extending to the bottom of the culture vessel and escaped through a filtered exhaust (1.0 μm filter) at the top. Fluorescent light banks (T5) were positioned vertically behind the aquarium system and provided 400 $\mu mol_{Photons}*m^{-2}*s^{-1}$ light on a 14:10 light–dark cycle for the duration of the study.

Growth media was prepared for all conditions without the addition of nitrogen. Reactors were filled with 1.15 and 1.00 L of media for conditions without and with 50 mM HCO_3^-, respectively. Reactors were then sealed, and autoclaved for 60min at 120°C and 1 atm. The

reactors were allowed to cool and subsequently placed in the aquarium water-baths maintained at 24 °C ± 1 °C. Gas lines were connected and gas sparge was initiated approximately four hours prior to inoculation in order to allow for CO_2 saturation and stabilization of medium pH. Filter sterilized (0.5 μm filter) BBM (100 mL) containing the nitrogen required for a 3.5mM final nitrogen concentration in each reactor was added after the gas sparge was initiated. For conditions requiring 50 mM HCO_3^-, 150 mL of BBM containing 1.25mM of HCO_3^- was also filter sterilized into the autoclaved reactor systems. To facilitate dissolution of $NaHCO_3$ at 1.25 mM (slightly above saturation), media was heated to 30 °C on a stir plate as it was added. This potentially facilitated off-gassing of CO_2, but the solution was added to the reactors 4 hours prior to inoculation to allow for equilibration.

Culture Monitoring

Cell Concentration

Cell concentration was quantified using an optical hemocytometer with a minimum of 400 cells counted per sample for statistical reliability. Specific growth rates, μ, were determined using Equation 1, where $[X]_A$ and $[X]_B$ represent the cell concentration at times t_A and t_B, respectively.

$$\mu = \frac{(ln[X]_B - ln[X]_{A.})}{t_B - t_A} \qquad \text{Equation 1}$$

Maximum specific growth rate, μ_{max}, was determined as the maximum μ observed between two sequential time points.

Nitrogen Removal

Nitrogen concentration was monitored daily using colorimetric assays; NAS Szecherome for nitrate (Polysciences, Inc.), 2-Phenylphenol Method for ammonium (Rhine et al., 1998), and a modified Jung's Assay for urea (Zawada et al., 2009). Nitrogen concentrations and the absence of other nitrogen compounds were verified by HACH Total Nitrogen Test'N'Tube kits (Hach).

Nitrogen removal rates, r_N, were calculated using Equation 2, where $[N]_{Inoc}$ and $[N]_{NL}$ are the concentration of nitrogen at inoculation and nitrogen limitation, respectively, and t_{NL} and t_{Inoc} are the time of nitrogen limitation and inoculation, respectively.

$$r_N = \frac{([N]_{Inoc} - [N]_{NL.})}{t_{NL} - t_{Inoc}} \qquad \text{Equation 2}$$

Dissolved Inorganic Carbon (DIC) and pH

DIC and pH were measured daily; pH was measured immediately after sample collection using a pH meter. Samples for DIC analysis were filtered after collection using a 0.2 μm syringe filter and diluted within the linear standard range of 0.100 to 100.0 mM carbon using deionized water prior to analysis. DIC analysis was performed as soon as possible on a Skalar Formacs series TOC/TON analyzer.

Biomass Analysis

Biomass samples were collected at nitrogen limitation ([N]<1.0mM) and 72 hours following nitrogen limitation. Biomass samples were prepared for dry weight and compositional analysis by centrifuging three 50mL centrifuge tubes containing 50mL of culture from each reactor at 4939xg for 20 minutes. Following centrifugation, the supernatant was removed and the

pellet was stored at -80 for a minimum of 24 hours to ensure complete freezing. After 24 hours moisture was removed via lyophilization for 48 hours.

Biomass Yield from Nitrogen, Y_N

Yield from nitrogen was calculated for biomass during nitrogen replete and two-stage growth using Equation 3. Where $Y_{biomass\text{-}N}$ is the yield of biomass from nitrogen; $[X]_A$ and $[X]_B$ are the concentrations of biomass at the beginning and end of the growth phase of interest, respectively; and $[N]_A$ and $[N]_B$ are the concentrations of nitrogen remaining at the beginning and end of the growth phase of interest, respectively.

$$Y_{X-N} = \frac{[X]_B - [X]_A}{([N]_A - [N]_B)} \quad \text{Equation 3}$$

Yield from nitrogen was not calculated for nitrogen deplete growth alone because nitrogen availability was negligible during this growth phase.

Biomass Productivity

Productivity of biomass was calculated for nitrogen replete, nitrogen deplete, and two-stage growth using Equation 4, where $[X]_A$ and $[X]_B$ are the concentrations of biomass, at time t_A and t_B. Time points t_A and t_B are the beginning and end of the growth phase of interest (nitrogen replete, nitrogen deplete, or two-stage growth), respectively. Biomass concentrations were assumed to be negligible at inoculation.

$$P = \frac{[X]_B - [X]_A}{t_B - t_A} \quad \text{Equation 4}$$

Statistical Analysis

A combination of ANOVA and two-tailed t-testing (paired and unpaired) was performed on data sets to determine statistical significance with a null hypothesis that all conditions were similar at 95% confidence. ANOVA testing was performed using a general linear model for comparison of data sets from the four nitrogen conditions when equal variances were observed between data sets. Individual comparisons of conditions were subsequently performed using Tukey's test to determine significance. When unequal variances were observed, a one-way ANOVA was performed using Welch's test, followed by Games-Howell Pairwise comparisons. For direct comparison of two conditions t-tests were utilized. Unpaired two-tailed tests were performed for comparisons of data with replicates that do not match. Paired two-tailed t-testing was performed when corresponding replicates were compared from the same condition for two time points.

CHAPTER THREE

RESULTS AND DISCUSSION

Impact of Inorganic Nitrogen Sources on Cultivation of SLA-04

Cell Growth and Nitrogen Utilization

Cell growth was similar for all nitrogen conditions (Figure 1 A). A lag period of one day was observed before an increase in cell concentration occurred. This was followed by exponential growth. The maximum specific cell growth rate, μ_{max}, was observed between 48 and 72 hours after inoculation for all conditions. For the nitrate, ammonium, urea, and mixed nitrogen conditions μ_{max} was 1.68±0.55, 2.08±0.34, 1.81±0.54, and 2.13±0.29 day^{-1}, respectively, with no significant difference between treatments ($p=0.292$ based on general linear ANOVA model). During previous research, Podevin et al. (2015) evaluated the impact of nitrogen sources on specific growth rates for *C. sorokiniana*, strain SAG 1.80, a freshwater isolate, during three successive cultivations to understand the impact of acclimatization to available nitrogen sources by the algae. They observed similar specific growth rates during cultivation using sodium nitrate ($NaNO_3$), ammonium chloride (NH_4Cl), and urea for all nitrogen source conditions during the second and third cultivations except for the second cultivation using urea, where a significantly higher growth rate was observed in the presence of urea than was observed for cultures provided with sodium nitrate (Podevin et al., 2015).

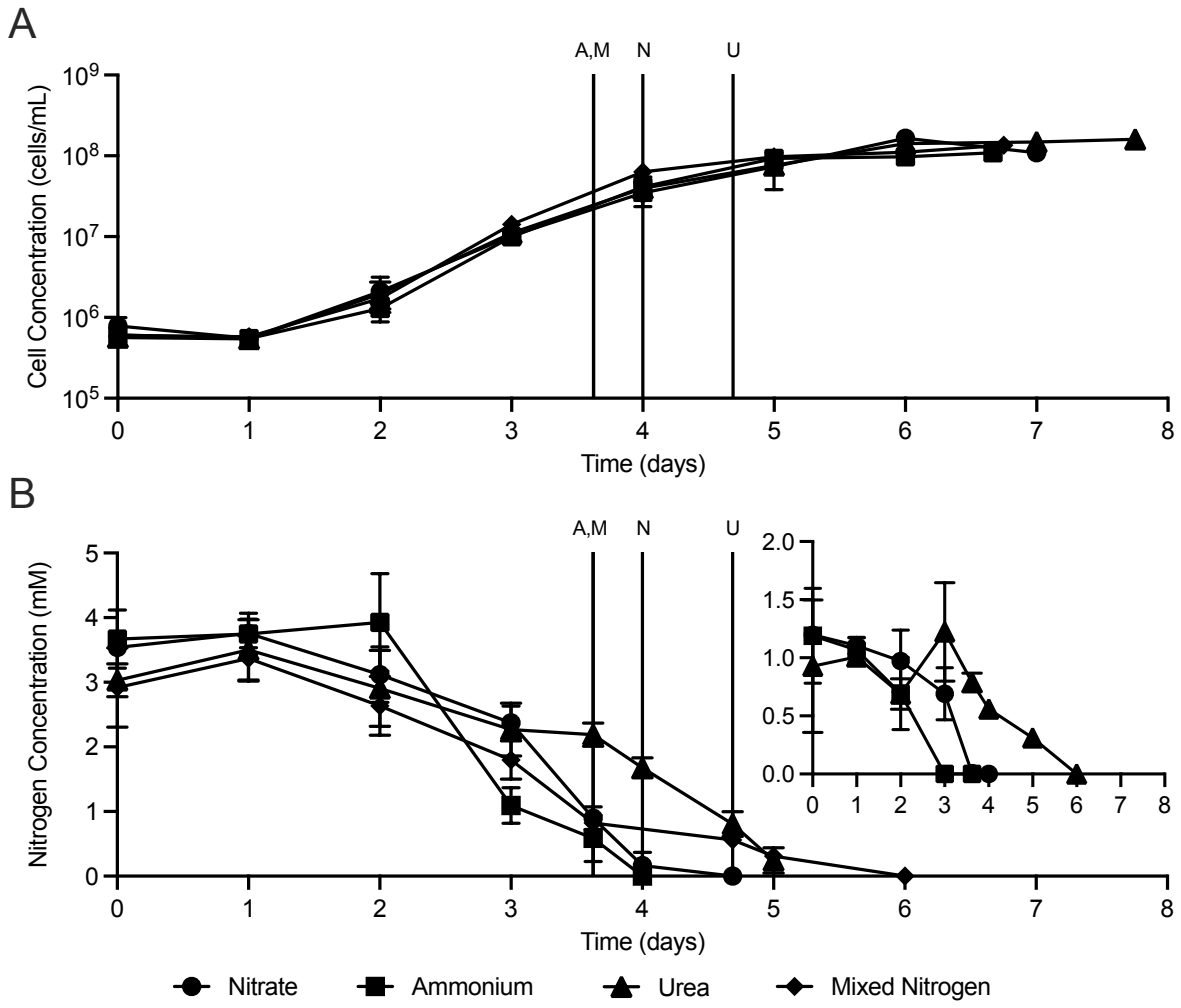

Figure 1: (A) Cell concentration (cells*mL^{-1}) and (B) nitrogen concentration (mM) as a function of time during growth using different nitrogen sources. Vertical bars represent time of nitrogen limitation for the different conditions (N=nitrate, A= ammonium, U=urea, M=mixed nitrogen). Inset graph in B is nitrogen concentration for the individual nitrogen species in the mixed nitrogen condition. Error bars are one standard deviation of three experimental replicates. Changes in cell concentration as a function of time were similar for all conditions. Changes in nitrogen concentration varied between the different nitrogen conditions.

During the current study, a gradual decrease in cell growth rate was observed 72 hours after inoculation for all conditions, but cell concentration continued to increase until the end of the 2-stage growth process for all conditions except the nitrate condition. The cell growth rate for the nitrate condition also began to gradually decrease after 72 hours but by the final time point of

the experiment the cell concentration had stopped increasing. Continued cell replication during nitrogen deplete growth has been observed previously for *C. sorokiniana*, strain KAN 1228, a freshwater isolate, during cultivation using NH_4Cl (Negi et al., 2016). During that study, rapidly growing cultures were transferred to either nitrogen replete or nitrogen deplete growth media to evaluate the impact of nitrogen limitation on growth. They found that cell growth rates remained similar for cultures regardless of nitrogen availability. During the current study, 3.32±0.11, 3.29±0.09, 2.05±0.24, and 3.10±0.44 cell doublings were observed between nitrogen limitation and the end of the nitrogen deplete growth phase for the nitrate, ammonium, urea, and mixed nitrogen conditions, respectively.

Cell concentration at nitrogen limitation was significantly higher for the urea condition than the nitrate and ammonium conditions, but not significantly different than the mixed nitrogen condition ($3.18*10^7±7.75*10^6$, $4.13*10^7±1.33*10^7$, $7.63*10^7±1.00*10^7$, and $6.33*107±1.20*10^7$ cells*mL^{-1} for the nitrate, ammonium, urea, and mixed nitrogen conditions, respectively; p=0.002, 0.013, and 0.479 for comparison of urea to the nitrate, ammonium, and mixed nitrogen conditions, respectively). The cell concentration for the mixed nitrogen condition was significantly greater than the concentration for the nitrate condition, but not the ammonium condition (p=0.016 and 0.120 for comparison of mixed nitrogen to the nitrate and ammonium conditions, respectively). Cell concentrations were lowest for the nitrate condition, significant to all conditions except the ammonium condition (p=0.665 for comparison of the nitrate and ammonium condition). The higher cell concentration observed for the urea condition is a result of the similar growth curves for the different conditions and the slower nitrogen removal rate observed for the urea condition. The slower nitrogen removal rate increased the duration of the

nitrogen replete growth phase, allowing more time for cell replication. The lower cell concentrations for the nitrate and ammonium conditions at nitrogen limitation are suspected to be caused by the high DIC concentration and pH observed, relative to the urea and mixed nitrogen conditions, during nitrogen replete growth (Figure 2). The high pH results in HCO_3^- being the dominant form of DIC present in solution. In previous work, HCO_3^- has been shown to reduce cell replication and increase lipid generation of different algal strains (Gardner, Cooksey, et al., 2012; Gardner, Lohman, et al., 2012; Li et al., 2018) The final cell concentration of the urea condition was also significantly greater than the concentrations in the nitrate and ammonium conditions ($1.10*10^8 \pm 6.36*10^6$, $1.09*10^8 \pm 1.11*10^7$, and $1.61*10^8 \pm 8.50*10^6$ cells*mL^{-1} for the nitrate, ammonium, and urea conditions, respectively; p=0.007 and 0.004 for comparison of the urea condition with the nitrate and ammonium conditions, respectively), however the final cell concentration of the mixed nitrogen condition was not significantly different than any of the other conditions ($1.35*10^8 \pm 1.52*10^7$ cells*mL^{-1}, p=0.145, 0.092, and 0.106 for comparison of mixed nitrogen with the nitrate, ammonium, and urea conditions, respectively). The relatively low cell concentrations of the nitrate and ammonium conditions are again suspected to be caused by the higher pH and DIC of these conditions.

Nitrogen concentrations and cultivation times at nitrogen limitation were 0.16±0.20 mM at 4 days, 0.59±0.36 mM at 3.67 days, 0.81±0.19 mM at 4.75 days, and 0.82±0.13 mM at 3.75 days for the nitrate, ammonium, urea, and mixed nitrogen conditions, respectively (Figure 1 B). The highest nitrogen removal rate was observed in the ammonium condition, followed by the nitrate, mixed nitrogen, and urea conditions (0.85±0.17, 0.84±0.08, 0.57±0.17, and 0.47±0.08 mM*day^{-1} for the ammonium, nitrate, mixed nitrogen, and urea condition, respectively). The

nitrogen removal rates for the urea and mixed nitrogen conditions were significantly slower than for the nitrate ($p=0.004$ and 0.040 when compared to urea and mixed nitrogen, respectively) and ammonium conditions ($p=0.001$ and 0.017 when compared to urea and mixed nitrogen, respectively). A previous evaluation of nitrogen removal rates for a different strain of *C. sorokiniana*, strain SAG 1.80, found no significant preference between nitrogen from $NaNO_3$, NH_4Cl, $(NH_4)_2CO_3$, and urea; however the authors noted a significant variability in cell growth and nitrogen removal rates between the replicates for the urea condition (Podevin et al., 2015). During the present study, the highest variability in nitrogen removal rates was also observed for the urea condition, however similar variability was observed for the nitrate and urea conditions with respect cell growth rate. Nitrogen sources in the mixed nitrogen condition were sequentially removed with the seemingly same respective rates as in the single nitrogen source conditions. In the mixed nitrogen medium removal of nitrogen sources happened sequentially, with availability of each nitrogen source being removed to low concentrations before significant removal of the next nitrogen source was observed. Thus, NH_4^+ was nearly deplete before significant NO_3^- removal was observed, and urea removal was not observed until NO_3^- approached limitation (Figure 1B). It is well established that NH_4^+ negatively impacts NO_3^- assimilation at the transcriptional and post transcriptional levels, especially in the presence of elevated CO_2 (Fernandez et al., 1989; Hellebust & Ahmad, 1989; Sanz-Luque et al., 2015). Less established, but still identified is inhibition of NO_3^- uptake by urea, which was described for *Chlorella fusca* (Molloy & Syrett, 1988).

There was no apparent impact of the varying nitrogen removal rates on cell growth, with all conditions exhibiting similar growth curves. The higher final cell concentration observed in the urea conditions is suspected to be a result of its longer experimental run time relative to the other conditions, caused by the slower nitrogen utilization rate. If cell concentrations five days after inoculation (prior to the end of nitrogen deplete growth for all conditions) are compared, rather than cell concentrations at the end of nitrogen deplete growth, which temporally varies for the different conditions, cell concentrations are similar for all of the conditions ($p=0.352$ based on ANOVA).

Growth Media Chemistry

During nitrogen replete growth, changes in DIC concentration were similar for the urea and mixed nitrogen condition, where DIC concentration remained fairly stable initially, but then began to decrease as nitrogen limitation was approached (Figure 2A). The decline in DIC concentration observed as nitrogen limitation was approached resulted from the increased carbon demand of the growing biomass surpassing the mass transfer of inorganic carbon in the air sparge into the growth medium, even with 5% CO_2 supplementation. In addition, the pH of the urea and mixed nitrogen conditions was gradually reduced during nitrogen replete growth, with both conditions being below pH 6.3, the lower pK_A for H_2CO_2/HCO_3^- under standard conditions, at nitrogen limitation (pH 6.27±0.09 and 6.25±0.11 for the urea and mixed nitrogen conditions, respectively, at nitrogen limitation). In the nitrate condition a gradual increase in DIC concentration was observed throughout nitrogen replete growth, however the pH remained stable. The assimilation of nitrate into biomass results in the generation of a single hydroxyl ion for each nitrogen assimilated (Eustance et al., 2013; Wolf-Gladrow et al., 2007). An increase in

hydroxyl ions increases solution pH, which increases the rate of CO_2 mass transfer into the growth medium and shifts inorganic carbon speciation toward HCO_3^- and CO_3^{2-} (Wolf-Gladrow et al., 2007). The lack of change in pH is caused by sufficient CO_2 mass transfer to compensate for hydroxyl ion generation during nitrogen assimilation from nitrate. As a result, during nitrogen replete growth a 3.77±0.35mM increase in DIC was observed for the nitrate condition (Figure 2A).

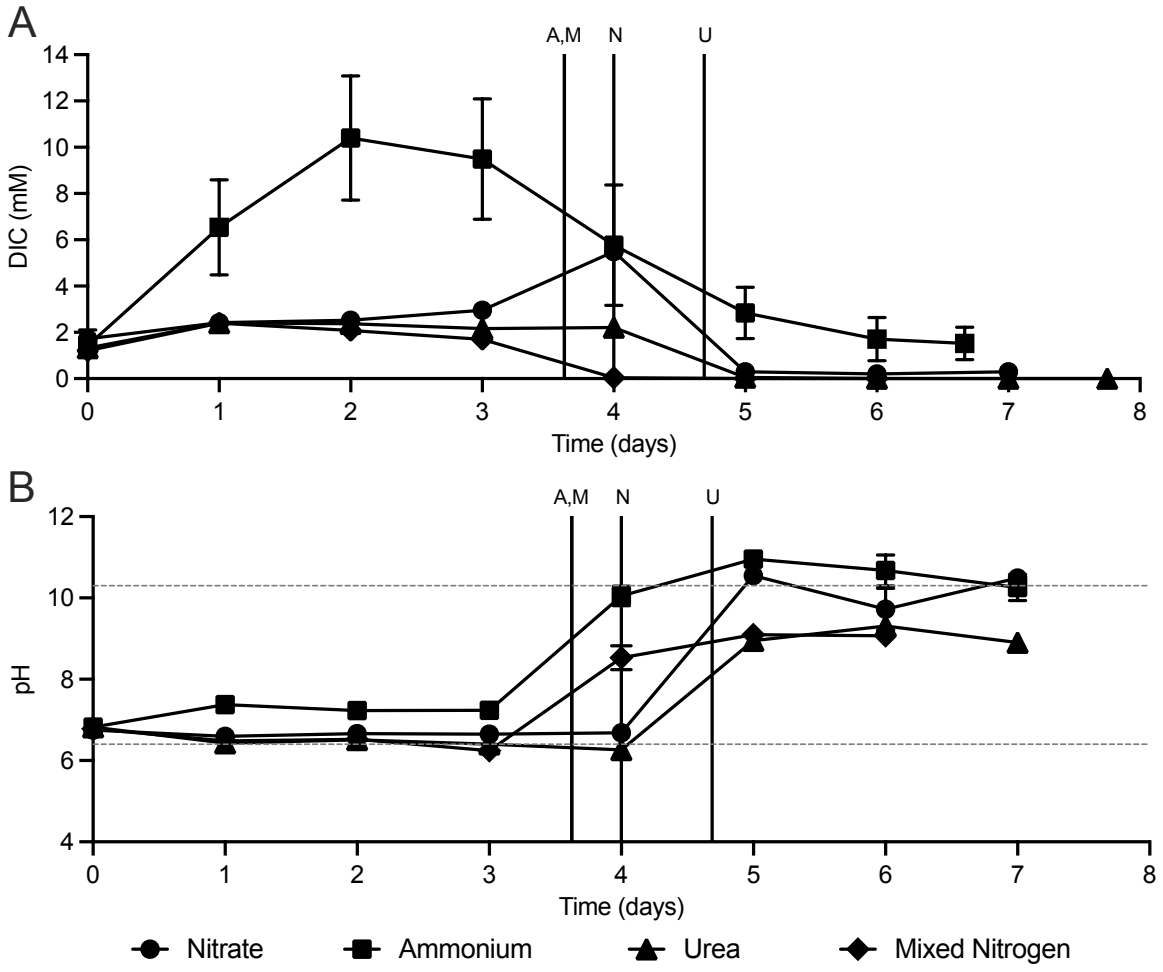

Figure 2: (A) DIC (mM) and (B) pH as a function of time during growth of *C. sorokiniana* SLA-04 in the presence of different nitrogen sources. Vertical bars represent time of nitrogen limitation for the different conditions (N=nitrate, A= ammonium, U=urea, M=mixed nitrogen). Horizontal dotted lines designate the two pK_A of H_2CO_3/HCO_3^- and HCO_3^-/CO_3^{2-} under standard temperature and pressure, 6.4 and 10.3, respectively. The pK_A values for this system are a function of other experimental parameters including temperature, salinity, other media components that contribute to alkalinity, and bioproducts that contribute to alkalinity. Error bars are one standard deviation of the data. Increased DIC concentrations observed for the ammonium condition during nitrogen replete growth are caused by overcompensation by the pH control system used to counter proton generation during nitrogen assimilation from NH_4^+. This highlights the difficulties associated with pH control of actively growing cultures. Increased DIC concentrations were also observed for the nitrate condition, resulting from the generation of hydroxyl ions during nitrogen assimilation from nitrate. Following nitrogen depletion and cessation of CO_2 supplementation, a reduction in DIC and increase in pH was observed for all conditions.

An increase in DIC also occurred in the ammonium condition, however not due to hydroxyl ion generation, but rather through the addition of excess KOH by the pH control system necessary to compensate for the generation of protons during the assimilation of NH_4^+ by the algae. In addition to the increased DIC concentration, in the ammonium condition an increase in pH was observed. Without the pH control system, a rapid drop in pH occurred when NH_4^+ was supplied as the sole nitrogen source, resulting in cessation of cell growth and ultimately, loss of pigmentation of cells. The negative impact of reduced pH resulting from the assimilation of nitrogen from NH_4^+ has been described previously (Eustance et al., 2013; Podevin et al., 2015; Ribeiro et al., 2020). As described by Eustance et al. (2013), a completely balanced pH control system is practically impossible in actively growing algal cultures since pH measurements are slow to stabilize and result in over-titration with base. During previous work investigating cultivation of freshwater *Chlorella sorokiniana*, strain Embrapa_LBA#39, using a mix of NO_3^-, NH_4^+, and urea, a reduction of pH from 6.8 to 2.8 was observed during the first 75 hours of cultivation, presumably due to proton generation during assimilation of nitrogen from NH_4^+. There was no impact on growth observed as a result of the pH reduction which was attributed to a potential evolutionary adaptation to low pH conditions (Ribeiro et al., 2020). During the current study, there was no significant pH reduction observed for the mixed nitrogen condition during the period where NH_4^+ was being removed from the system, however pH values were measured daily, as opposed to at 5 minute intervals as was done in the study by Ribeiro et al. (2020). As a result, it is possible that a reduction in pH occurred during assimilation of nitrogen from NH_4^+ in the current study that was not apparent at the time pH measurements were conducted, however this seems unlikely.

During nitrogen replete growth, the difference in DIC concentrations between the conditions, with higher DIC concentrations measured in the nitrate and ammonium conditions (Figure 2A), as well as the small reduction in pH observed for the urea and mixed nitrogen conditions (Figure 2B), has significant implications for the speciation of inorganic carbon available to the cultures. In the urea and mixed nitrogen conditions the coinciding reduction in DIC concentration and pH suggests that there is low alkalinity in these conditions and as a result, inorganic carbon availability is likely dominated by CO_2/H_2CO_3. The slight increase in DIC concentration and stable pH (average pH 6.67±0.05 during nitrogen replete growth) observed for the nitrate condition during nitrogen replete growth results in a higher ratio of HCO_3^-/H_2CO_3 present in solution, relative to the urea and mixed nitrogen conditions. The relative availability of HCO_3^- and CO_2/H_2CO_3 to algal cells cannot easily be determined since both can be assimilated into biomass, and CO_2 available in the air sparge can easily replenish HCO_3^- as it is consumed. In the ammonium condition the increase in DIC concentration during nitrogen replete growth was more significant than what was observed in the nitrate condition, and a significant increase in pH was also observed. This resulted in a further increase in the relative availability of HCO_3^- for this condition. The differences in inorganic carbon speciation are suspected to be the primary cause for the differences in cell concentrations observed at nitrogen limitation, with either increased availability of HCO_3^- suppressing cell replication in the nitrate and ammonium conditions, or metabolic dependence on CO_2(aq) promoting cell replication for the urea and mixed nitrogen condition. Li et al. (2018) investigated the impacts of $NaHCO_3$ supplementation at different concentrations on cell growth by *Chlorella vulgaris*, strain UTEX 2714, and

concluded that increased HCO_3^- concentrations led to suppression of cell growth during their investigation.

During nitrogen deplete growth DIC concentration decreased for all conditions relative to nitrogen replete growth, with the nitrate, urea, and mixed nitrogen conditions decreasing to below quantifiable limits (0.100 mM; Figure 2A). Despite being below quantifiable limits, analysis of DIC for the nitrate and urea conditions still produced a signal suggesting the presence of inorganic carbon at a concentration below 0.100 mM. Measurements of DIC concentration do not fully capture DIC availability. The relatively low, but detectable presence of DIC suggests that algal consumption of inorganic carbon is balanced with mass transfer of CO_2 into solution. It is not clear whether carbon availability is sufficient for optimal growth or if the small amount of DIC detected in the system is at scarce enough concentrations that it cannot be more rapidly assimilated (scavenging limitations). In Figure 2A approximation of DIC concentrations that were below the instruments quantifiable range are included for these conditions. The sudden decrease in DIC availability for these conditions resulted from cessation of 5% CO_2 supplementation to the air sparge at nitrogen limitation. In the ammonium condition a decrease in DIC was observed during nitrogen deplete growth, stabilizing at a final DIC concentration of 1.52±0.70 mM.

Due to mass transfer of CO_2 into or out of sample solution during filtration and the delay between sample collection and analysis, it is likely that DIC values measured represent the DIC values at the sample's equilibrium point for alkalinity rather than the instantaneous DIC concentration at the time of sample collection. For nitrate and ammonium condition samples collected during this study, DIC would more likely enter solution following collection due to the

high pH, rather than off gas, which suggests that measured DIC concentrations are greater than the actual DIC concentrations. For the urea and mixed nitrogen conditions, a loss of inorganic carbon during sample processing is more likely due to the low pH, and relatively high concentrations of inorganic carbon as CO_2. It is important to note that DIC concentrations do not fully represent the available inorganic carbon pool for the different conditions, as the increased alkalinity in the nitrate and ammonium conditions promotes more rapid replenishment of inorganic carbon in solution, in addition to improving the size of the overall carbon pool. This highlights the complex nature of carbon availability as a function of system alkalinity described previously (Vadlamani, 2016; Wolf-Gladrow et al., 2007).

An increase in pH was observed for all conditions following nitrogen limitation as a result of cessation of 5% CO_2 supplementation, algal consumption of inorganic carbon from HCO_3^- in solution, and the resulting release of hydroxyl ions (Figure 2B). The pH of the urea and mixed nitrogen conditions increased to 8.90±0.23 and 9.07±0.0.07, respectively, at the time the DIC decreased below quantifiable levels (<0.100 mM). The pH for the nitrate and ammonium conditions increased to 10.50±0.08 and 10.68±0.38, respectively, during nitrogen deplete cultivation. The higher pH reached by the ammonium and nitrate conditions reflects the increased alkalinity for these conditions. Since DIC concentrations were near the lower quantifiable limit of the methods used (0.100 mM) for the nitrate, urea, and mixed nitrogen conditions, hydroxyl alkalinity was dominant, as opposed to carbonate alkalinity. Carbon that is brought into the system is likely assimilated into algal biomass rapidly, resulting in replenishment of hydroxyl ions. Ultimately, the elevated pH in the nitrate and ammonium conditions is associated with a higher rate of mass transfer of inorganic carbon present in the gas

sparge into solution, however speciation of inorganic carbon may be negatively impacted by the higher pH, as an increase in the ratio CO_3^-/HCO_3^- is increased. Pathways for CO_3^{2-} metabolism are not well understood for algae, however diffusive transport is not possible at pH values where carbonate speciation is relevant, and no active transport mechanisms are known (Li et al., 2018).

Biomass Generation

Figure 3A presents biomass concentration generated during the nitrogen replete growth phase (day 0 to day 4, 3.67, 4.75, and 3.75 for the nitrate, ammonium, urea, and mixed nitrogen conditions, respectively) and the nitrogen deplete growth phase (the 72 hours following nitrogen limitation). The stacked bars present the biomass concentration generated during the 2-stage cultivation process. At nitrogen limitation the biomass concentration in the nitrate condition (0.47 ± 0.02 g*L^{-1}) was greater than in the ammonium (0.34 ± 0.03 g*L^{-1}; $p=0.006$) and urea (0.36 ± 0.08 g*L^{-1}; $p=0.032$) conditions. Biomass generated in the mixed nitrogen condition (0.43 ± 0.04 g*L^{-1}) was not significantly different than the other conditions when a 95% confidence interval is used ($p= 0.530$, 0.061, and 0.274 when compared to the nitrate, ammonium, and urea conditions, respectively), however the difference between the nitrate and mixed nitrogen conditions is significant when a 90% confidence interval is used. Due to the lower cell concentration and similar or higher biomass concentration observed for the nitrate condition at nitrogen limitation, relative to the other nitrogen conditions, the average cell mass for the nitrate condition was significantly larger than the cell mass observed in the other conditions (15.4 ± 3.10, 8.77 ± 2.66, 4.43 ± 1.47, and 6.69 ± 1.06 fg*$cell^{-1}$ for the nitrate, ammonium, urea, and mixed nitrogen conditions respectively; $p=0.021$, 0.001, and 0.004 for comparison of the nitrate condition with the ammonium, urea, and mixed nitrogen conditions, respectively). The

difference in cell mass of the nitrate condition is suspected to be a result of the increased presence of HCO_3^- in solution causing reduced cell replication and increased lipid production (Li et al., 2018). The lower cell mass of the ammonium condition, relative to the nitrate condition, is a result of lower biomass generation, not increased cell concentration, as cell concentrations for the two conditions were similar. There were no significant differences observed in cell mass for the other nitrogen conditions. It is unclear why the ammonium condition failed to generate as much biomass as the nitrate condition, but it is likely a result of the higher pH of the ammonium condition, as well as the resulting speciation of inorganic carbon in solution. In addition, it is possible that the generation of protons inside the cell during NH_4^+ assimilation impacts cell physiology with respect to maintenance of cell neutrality. Even during cultivation at high pH, algal cells generally maintain a circumneutral internal pH (Rai et al., 2001). Since biomass concentration alone does not account for cultivation time or nutrient (nitrogen and carbon) availability, comparisons with biomass concentrations observed during other studies is problematic. In addition, optimization of biomass concentration is unlikely to coincide with optimization of nutrient or time requirements.

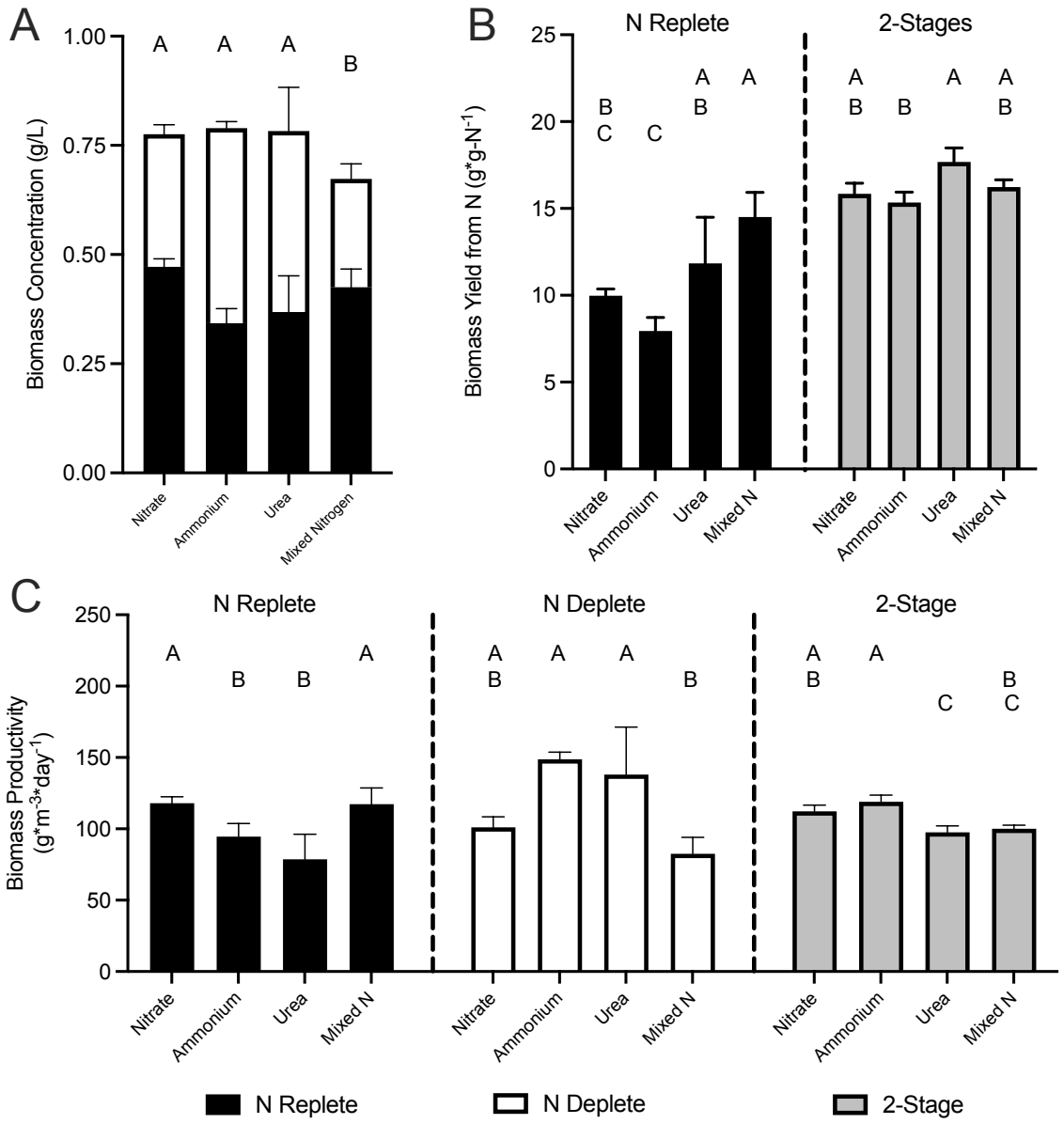

Figure 3: Biomass (A) concentration (mg*L^{-1}), (B) yield from nitrogen ($g_{biomass}$*$g_{nitrogen}^{-1}$), and (C) productivity (mg*L^{-1}*day^{-1}). Biomass concentration is separated as the biomass generated during the nitrogen replete (N Replete) and deplete (N Deplete) growth phases. Biomass productivity is presented separately for both growth phases, and as a weighted average for the duration of the study. Biomass yield from nitrogen is presented for biomass collected at nitrogen limitation and at the end of the study. Error bars are one standard deviation of the data. The error bars for biomass concentration are for the two independent growth phases (error bars for the combined 2-stage cultivation process are not presented).

Biomass yield from nitrogen, which standardizes the amount of biomass generated by the amount of nitrogen required for growth, is a better parameter to evaluate for optimizing nitrogen requirements for growth. During the current study, biomass yield from nitrogen during nitrogen replete growth was greater for the urea and mixed nitrogen conditions than the ammonium condition (11.8±2.66, 14.5±1.41, and 7.95±0.77 $g_{biomass}*g_{nitrogen}^{-1}$ for the urea, mixed nitrogen, and ammonium conditions, respectively; p=0.003 and <0.001 when the ammonium condition is compared to the urea and mixed nitrogen conditions, respectively). Biomass yield from nitrogen for the nitrate condition (9.98±0.39 $g_{biomass}*g_{nitrogen}^{-1}$) was less than what was observed for the mixed nitrogen condition (p=0.002) but was similar to the ammonium and urea conditions (p=0.256 and 0.330 for comparison with the ammonium and urea conditions, respectively). The lower biomass yield from nitrogen observed for the ammonium condition is related to loss of nitrogen as NH_3 to the environment and the use of nitrogen removed from growth medium instead of nitrogen assimilated into biomass for the calculation for yield. The average measured pH for the ammonium condition during nitrogen replete growth was 7.17±0.25, with a maximum average pH measurement at a single time point for all replicates of 7.38±0.12 two days after inoculation. Thus, the measured pH for the ammonium condition was always significantly lower than the pK_A for NH_4^+/NH_3, pH 9.3. This would suggest that nitrogen losses as NH_3 were limited, however the addition of KOH for pH control was not instantly homogenous throughout the reactor systems, and the actual pH of growth medium at times when the pH control system was active varied as a function of reactor mixing, time since addition, and distance from the point where the base was added to the reactor, with media at or near the location of base addition likely experiencing higher pH values than what were measured. In addition, pH measurements

were taken daily at the end of the 14 hour period when the reactors were illuminated and there was continuous 5% CO_2 supplementation to the 4 $L*min^{-1}$ air sparge. During the dark phase cessation of the CO_2 supplementation likely resulted in an increase in pH, potentially above pH 9.3. This highlights the value of continuous pH monitoring during cultivation, which was not conducted during the current study.

For the ammonium condition a comparison of nitrogen removed from growth medium (3.82±0.36 mM) and nitrogen present in product biomass (3.29±0.19 mM based on elemental analysis) confirms a significant loss of nitrogen to the environment (p=0.016). If biomass yield from nitrogen for the ammonium condition is calculated using nitrogen assimilated into biomass instead of nitrogen removed from the growth medium, there are no significant differences observed between biomass yield from nitrogen for the different nitrogen conditions (p=0.102 based on ANOVA). The use of nitrogen assimilated into biomass for calculation of biomass yield from nitrogen is problematic as it does not fully capture the nitrogen demand for the system, since loss of NH_3 is an inherent property of the carbon supply regime and buffering capacity of the medium used. There were no significant differences between nitrogen removed and nitrogen assimilated for the other nitrogen conditions (3.67±0.12 mM, 3.28±0.32 mM, and 3.22±0.38 mM nitrogen removed from the nitrate, urea, and mixed nitrogen conditions, respectively; 3.47±0.27 mM, 3.41±0.17 mM, and 3.51±0.21 mM nitrogen assimilated into biomass for the nitrate, urea, and mixed nitrogen conditions, respectively; p=0.264, 0.414, and 0.143 comparing nitrogen removed and assimilated for the nitrate, urea, and mixed nitrogen conditions, respectively). This suggests that nitrogen loss as NH_3 was not significant for the mixed nitrogen.

Biomass productivity during nitrogen replete growth was greater for the nitrate and mixed nitrogen conditions (118±4.61 and 117±11.4 $g*m^{-3}*day^{-1}$ for nitrate and mixed nitrogen, respectively) than the ammonium (94.6±9.21 $g*m^{-2}*day^{-1}$; p=0.039 and 0.023 when compared to the nitrate and mixed nitrogen conditions, respectively) and urea (78.6±17.7 $g*m^{-3}*day^{-1}$; p=0.001 and p<0.001 when compared to the nitrate and mixed nitrogen conditions, respectively; Figure 3C) conditions, respectively. The lower productivity for the urea condition is a result of the slower nitrogen removal rate observed, which prolonged the nitrogen replete growth stage. For the ammonium condition the cause of the reduced productivity during nitrogen replete growth is likely reduced nitrogen availability due to volatilization of NH_3 from the system as discussed previously.

During nitrogen deplete growth an additional 0.30±0.02 $g*L^{-1}$, 0.44±0.02 $g*L^{-1}$, 0.41±0.10 $g*L^{-1}$, and 0.25±0.03 $g*L^{-1}$ of biomass were produced for the nitrate, ammonium, urea, and mixed nitrogen conditions, respectively. The biomass generated in the ammonium and urea conditions during this growth phase was significantly greater than what was produced in the mixed nitrogen condition (p=0.016 and 0.037 for ammonium and urea conditions, respectively). The nitrate and mixed nitrogen conditions both generated less biomass during nitrogen deplete growth than during nitrogen replete growth (p=0.010 and 0.002, respectively), while the ammonium condition generated significantly more biomass following nitrogen limitation (p=0.004). No significant difference was observed between the biomass generated in the urea condition during the two growth stages (p=0.870). The increased biomass generation observed for the ammonium condition during nitrogen deplete growth, with respect to nitrogen replete growth, but not observed for the other nitrogen conditions, is suspected to be caused by the

higher DIC concentrations observed in this condition during nitrogen deplete growth, specifically the increased availability of HCO_3^- . Since the higher DIC concentration is related to poor precision of the pH control system, rather than growth or metabolism of the organism, the increase in biomass generation observed should not be attributed to the use of NH_4^+ for cultivation.

The higher biomass concentration generated during nitrogen deplete growth for the urea condition is largely a result of its higher cell concentration, rather than an increase in cell mass. Interestingly, cell masses at the end of 2-stage cultivation were similar for the urea and mixed nitrogen conditions (4.67±0.13 and 4.93±0.42 fg*cell^{-1}, respectively; p=0.802), as well as for the nitrate and ammonium conditions (7.18±0.14 and 7.26±0.47 fg*cell^{-1}, respectively; p=0.993), but cell masses for the nitrate and ammonium conditions were significantly larger than cell masses in the urea and mixed nitrogen conditions ($p<0.001$ and $p=0.001$ for comparison of the nitrate conditions with the urea and mixed nitrogen conditions, respectively; $p<0.001$ for comparison of the ammonium condition with both the urea and mixed nitrogen conditions). During nitrogen deplete growth a reduction in cell mass was observed for the nitrate condition, resulting in smaller cells at the end of the 2-stage process than at the end of nitrogen replete growth (15.4±3.10 and 7.18±0.14 fg*cell^{-1} for nitrogen replete growth and 2-stage cultivation, respectively; p=0.013). Cell masses were not significantly different at the end of nitrogen replete and 2-stage growth for the other nitrogen conditions (p=0.434, 0.801, and 0.116 comparing nitrogen replete and 2-stage growth for the ammonium, urea, and mixed nitrogen conditions respectively). The reduction in cell mass observed for the nitrate condition during nitrogen deplete growth was likely a result of reduced inorganic carbon as HCO_3^- relative to nitrogen

replete growth but may also be a result of the high pH observed. As noted, DIC concentrations in the nitrate condition drop rapidly following nitrogen limitation to below quantifiable levels (<0.100 mM). Simultaneously an increase in pH to 10.3±0.13, the pK_A of HCO_3^-/CO_3^{2-} at standard conditions, was observed, reducing the ratio of HCO_3^- present in the smaller inorganic carbon pool present.

During nitrogen deplete growth biomass productivity increased to 149±5.00 $g*m^{-3}*day^{-1}$ for the ammonium condition ($p<0.001$ when compared to nitrogen replete growth) but was similar during both growth stages for the nitrate, urea, and mixed nitrogen conditions ($p=0.363$, 0.175, and 0.106 when comparing biomass productivity during nitrogen replete and deplete growth for the nitrate, urea, and mixed nitrogen conditions, respectively). Higher productivity during nitrogen deplete growth for the ammonium condition is likely the result of the higher DIC availability during the nitrogen deplete growth phase (average of 2.88±1.27 mM DIC during nitrogen deplete growth) relative to the other conditions (average DIC concentration of 0.27±0.05, 0.02±0.01, and 0.01±0.02 mM for the nitrate, urea, and mixed nitrogen conditions, respectively; $p=0.016$, 0.006, and 0.006 when comparing biomass productivity during nitrogen deplete growth for the ammonium condition to the nitrate, urea, and mixed nitrogen conditions, respectively). In addition, the relatively low biomass productivity observed during nitrogen replete growth for the ammonium condition impacted this comparison. When biomass productivity during nitrogen deplete growth is compared for the different nitrogen conditions, a significantly lower biomass productivity was observed in the mixed nitrogen condition relative to the ammonium and urea conditions ($p=0.016$ and 0.037 for comparison with the urea and mixed nitrogen conditions, respectively). The reduced biomass productivity for the mixed nitrogen

condition is not well understood. It is hypothesized that the sequential elimination of nitrogen species may result in a reduced impact of nitrogen limitation for this condition. This assumes that cells sense nitrogen availability as a function of source rather than as total nitrogen present. To the best knowledge of the author, this has not been investigated previously.

Final biomass concentrations for the single source nitrogen conditions were similar (0.79±0.03, 0.79±0.03, and 0.75±0.03 $g*L^{-1}$ for the nitrate, ammonium, and urea conditions, respectively; p=0.369 from ANOVA), but significantly less for the mixed nitrogen condition relative to the other conditions (0.66±0.02 $g*L^{-1}$, p=0.009, 0.005, 0.029 for comparison with the nitrate, ammonium, and urea conditions, respectively). The generation of additional biomass during nitrogen deplete growth in all conditions increased biomass yield from nitrogen since no additional nitrogen was added to the conditions during this growth phase. At the end of both growth stages yield from nitrogen was similar for all conditions (15.4±0.80, 16.5±1.02, 17.4±1.08, and 14.5±2.05 g-biomass*g-$nitrogen^{-1}$ for nitrate, ammonium, urea, and mixed nitrogen, respectively; p=0.155 based on ANOVA), despite the loss of nitrogen as NH_3 from the ammonium condition during nitrogen replete growth. Biomass productivity for the two-stage growth system was greater for the nitrate (112±4.34 $g*m^{-3}*day^{-1}$) and ammonium (119±4.65 $g*m^{-3}*day^{-1}$) conditions than for the urea condition (97.6±4.45 $g*m^{-3}*day^{-1}$; p=0.022 and 0.002 compared to the nitrate and ammonium conditions, respectively). Biomass productivity in the ammonium condition during 2-stage cultivation was also significantly greater than productivity in the mixed nitrogen condition (100±2.58 $g*m^{-3}*day^{-1}$; p=0.003). Greater DIC availability during both growth stages is suspected to have contributed to the increased total productivity for the nitrate and ammonium conditions. During nitrogen deplete growth the urea and mixed

nitrogen conditions were carbon limited. In addition, the slower nitrogen utilization in the urea condition, which increased the cultivation time, and the generation of less biomass in the mixed nitrogen condition are believed to have contributed to the lower productivities observed for these conditions.

High-Alkalinity Culturing Using Different Nitrogen Sources

High-alkalinity and/or high-pH algae cultivation has been posited to reduce the costs of biomass production by increasing mass transfer of inorganic carbon from the atmosphere into growth media (Canon-Rubio et al., 2016; Chi et al., 2011; Chi et al., 2013; Gardner, Lohman, et al., 2012; Mokashi et al., 2016; Nayak et al., 2018). In addition, cultivation using HCO_3^- has been identified as a promising strategy to mitigate predation and competition (Bartley et al., 2014; Peng et al., 2016; Peng et al., 2015). To understand the impact of high-alkalinity cultivation on nitrogen availability to, and metabolism by, *C. sorokiniana,* strain SLA-04, changes in cell growth, media chemistry, and biomass production were evaluated for the four nitrogen conditions assessed previously (nitrate, ammonium, urea, and mixed N) under high-alkalinity conditions (50mM HCO_3^- supplementation to the growth medium prior to inoculation). In addition, the impact of a 50mM bicarbonate amendment at nitrogen limitation on cell growth, media chemistry, and biomass generation was evaluated for the nitrogen and high-alkalinity nitrogen conditions. Lipid synthesis has been shown to be stimulated by a timely addition of bicarbonate prior to nitrogen depletion for some algal strains (Gardner, Cooksey, et al., 2012). For clarity, experimental conditions that received supplementation with $NaHCO_3$ at inoculation

will be described as “high-alkalinity,” and conditions where $NaHCO_3$ was supplemented at nitrogen limitation will be described as “bicarbonate amended.”

Cell Growth and Media Chemistry

A comparison of cell growth and nitrogen removal are presented for the nitrogen and high-alkalinity nitrogen conditions in Figure 4. Maximum specific cell growth rates for the high-alkalinity nitrate and mixed nitrogen conditions ($2.18\pm0.15 day^{-1}$ and $2.65\pm0.08 day^{-1}$, respectively) were greater than observed in their respective standard nitrogen conditions ($p=0.050$ and 0.002 for the nitrate and mixed nitrogen conditions, respectively), however maximum growth rates for the high-alkalinity ammonium and urea conditions ($1.84\pm0.14 day^{-1}$ and $2.07\pm0.18 day^{-1}$, respectively) were not significantly different than the standard nitrogen conditions with the same nitrogen sources ($p=0.132$ and 0.270 for the ammonium and urea conditions, respectively; Figure 4A). The increased cell growth rate observed during high-alkalinity cultivation for the nitrate and mixed nitrogen conditions is contrary to what is expected, since the presence of HCO_3^- has been found to reduce cell replication (Li et al., 2018). It may be that the higher availability of inorganic carbon or higher pH promotes cell replication for SLA-04 since it was isolated from a high-alkalinity environment, however this is contrary to what was observed for the nitrate and ammonium conditions during cultivation in standard BBM.

An alternative hypothesis for the reduced cell replication observed for the standard nitrate and ammonium conditions, relative to the standard urea and mixed nitrogen conditions, is that transport of HCO_3^- into cells was limited due to insufficient availability of cations for cotransport. The maintenance of intracellular pH neutrality at high pH requires the active maintenance of high proton concentrations within the cell, relative to the growth medium. HCO_3^-

is transported into algal cells via cotransport or counter transport systems, where either an anion is displaced from the cell or cation moved into the cell. Extrusion of hydroxyl ions generated from carbonic anhydrase conversion of HCO_3^- to CO_2 and OH^- may contribute to counter transport, however this leaves a limited capacity for concentrating inorganic carbon within the cell (one in, one out). The use of cotransport is limited in low salinity high pH media due to the limited availability of protons or cations. When alkalinity is added to the systems as $NaHCO_3$, equimolar concentrations of Na^+ and HCO_3^- are present. As a result, Na^+ for cotransport is abundant. The increased pH and DIC observed for the standard nitrate and ammonium conditions was not coupled with increased availability of cations, and therefore it stands to reason that HCO_3^- present in the media for these conditions was not as easily accessible to the algal cells as it was when $NaHCO_3^-$ was added to the growth medium. This hypothesis, if confirmed, may explain the higher cell masses observed for the standard nitrate condition, relative to the standard ammonium condition. Previous research where cell replication was reduced in the presence of HCO_3^- was not conducted using alkaliphilic organisms, and it is likely that the adaptations to a high-alkalinity environment have resulted in significant differences in the physiology of SLA-04 relative to other organisms. If confirmed, this alternative hypothesis would suggest that anion availability is vital for optimal growth of SLA-04 during cultivation at high-pH. It is also possible that the continuous supplementation of 5% CO_2 results in repression of CCM activity, despite low concentrations of dissolved CO_2 in solution. Repression of CCM activity by CO_2 in solution is well established (Giordano et al., 2005; John et al., 2008; Moroney & Somanchi, 1999; Spalding, 2008), however the impact of undissolved CO_2 on CCM is not well understood, particularly in alkaliphilic green algae.

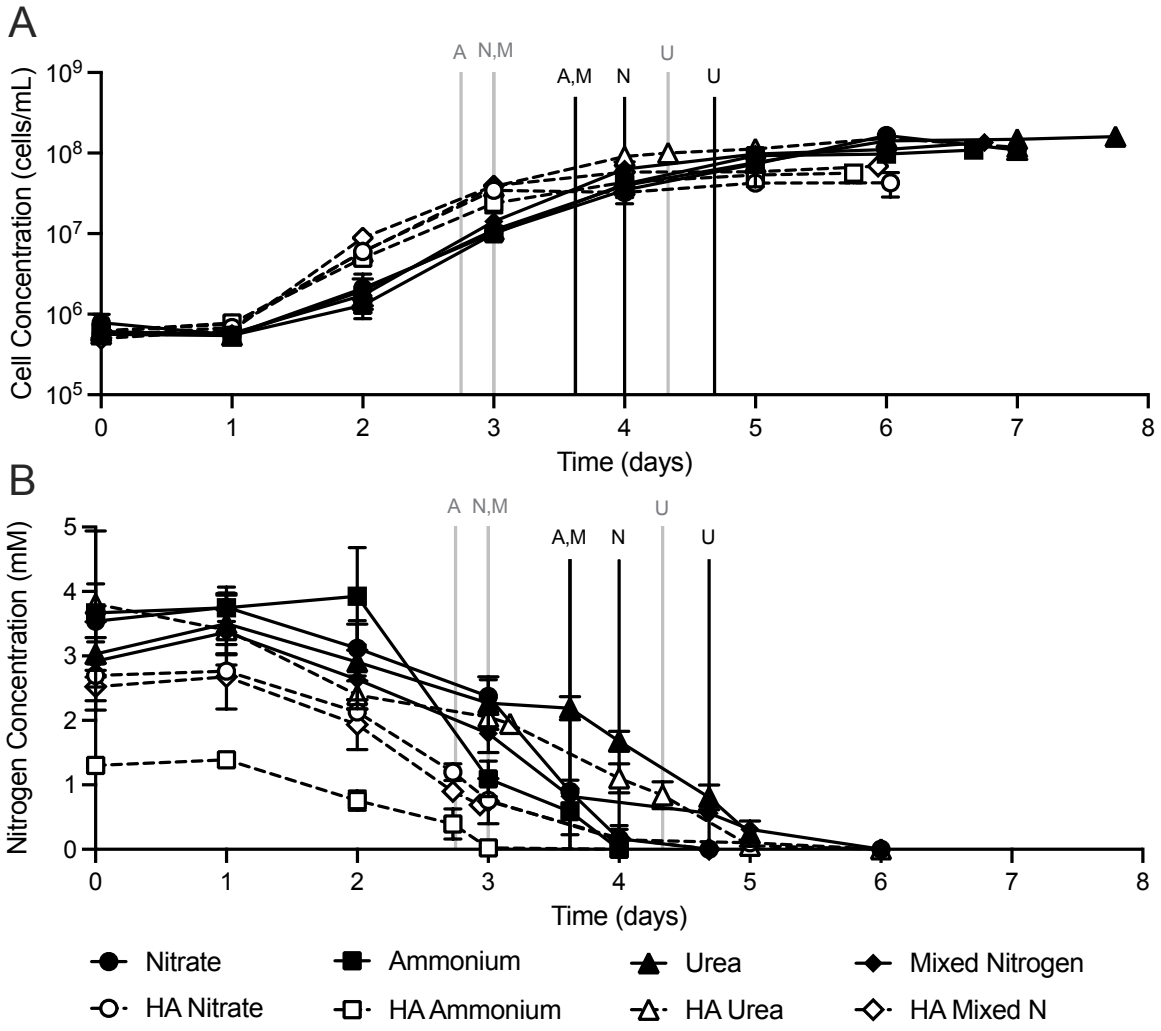

Figure 4: Cell (A) and nitrogen (B) concentrations (mM) as a function of time for the standard (solid lines, solid symbols) and high-alkalinity (dashed lines, open symbols) nitrogen conditions. Vertical black and grey lines represent the approximate time of nitrogen limitation for the nitrogen and high-alkalinity nitrogen conditions, respectively. Error bars are one standard deviation of data. Increased alkalinity resulted in a reduction in the lag period following inoculation. In addition, a reduction in the time between inoculation and nitrogen limitation was observed, however nitrogen removal rates were reduced relative to the standard nitrogen conditions. Thus, the reduced time prior to nitrogen limitation is caused by the shorter lag period and potentially, loss of nitrogen as NH_3 to the atmosphere for the ammonium and mixed nitrogen conditions where the measured pH was greater than the pK_A of NH_4^+/NH_3 (pH 9.3) for all sampling points (Figure 5B).

When the high-alkalinity nitrogen conditions are compared to each other, the high-alkalinity mixed nitrogen condition had a significantly faster maximum specific cell growth rate than the other conditions ($p<0.001$ for comparisons of high alkalinity mixed nitrogen with all other high-alkalinity nitrogen conditions), and the high-alkalinity ammonium condition had a significantly slower maximum specific cell growth rate ($p=0.002$, 0.041, and <0.001 relative to the high-alkalinity nitrate, urea, and mixed nitrogen conditions, respectively). For all high-alkalinity nitrogen conditions the maximum specific cell growth rate was observed 24-48 hours after inoculation, 24 hours sooner than for the standard nitrogen conditions. This suggests that SLA-04 grew more rapidly due to the greater availability of inorganic carbon in the high-alkalinity conditions, resulting in a shorter lag period before exponential cell growth. During nitrogen deplete growth, cell replication continued for the high-alkalinity conditions, however there were fewer cell doublings relative to the standard nitrogen conditions (1.06±0.20, 3.31±0.40, 1.11±0.19, and 2.91±0.39 cell doublings for the high-alkalinity nitrate, ammonium, urea, and mixed nitrogen conditions). As a result, cell concentrations at the end of the 2-stage growth process were significantly lower for all high-alkalinity nitrogen conditions except for urea ($4.28*10^7±1.43*10^7$, $5.63*10^7±1.36*10^7$, $1.18*10^8±3.45*10^7$, and $6.91*10^7±1.60*10^7$ cells*mL^{-1} for the high-alkalinity nitrate, ammonium, urea, and mixed nitrogen conditions, respectively; $p=0.009$, 0.007, 0.108, and 0.007 for comparison of the standard nitrogen and high-alkalinity nitrogen nitrate, ammonium, urea, and mixed nitrogen conditions, respectively).

Nitrogen removal time for the high-alkalinity nitrogen conditions occurred in the same order as was observed during cultivation under standard conditions, however it was reached sooner for all nitrogen regimes during high-alkalinity cultivation (3.00, 2.73, 4.33 and 3.00 days

after inoculation for the high-alkalinity nitrate, ammonium, urea, and mixed nitrogen conditions, respectively). The reduced lag period before nitrogen removal may be related to increased Despite the shorter length of the nitrogen replete growth phase, high-alkalinity cultivation caused a reduction in the nitrogen removal rates for the nitrate and ammonium conditions (0.65±0.08 $mM*day^{-1}$ and 0.33±0.06 $mM*day^{-1}$; $p=0.005$ and $p<0.001$ for the high-alkalinity nitrate and ammonium conditions, respectively). No significant difference was observed as a result of high-alkalinity cultivation in nitrogen removal rates for the urea and mixed nitrogen conditions (0.68±0.29 $mM*day^{-1}$ and 0.62±0.10 $mM*day^{-1}$; $p=0.117$ and $p=0.585$ for the high-alkalinity urea and mixed nitrogen conditions, respectively). The earlier time of nitrogen limitation observed in the high-alkalinity nitrogen conditions is suspected to be at least partially driven by the shorter lag period prior to exponential cell growth that was observed. In addition, the initial nitrogen concentrations measured for the high-alkalinity nitrate, ammonium, and urea conditions were less than was measured in the standard nitrogen conditions. For the ammonium and mixed nitrogen conditions, nitrogen from NH_4^+ is believed to have escaped the system as NH_3 gas due to the higher pH observed in the high-alkalinity conditions (Figure 5B), which causes a shift towards NH_3 in the NH_4^+/NH_3 equilibrium and increases the potential for NH_3 volatilization (Collos & Harrison, 2014).

Initial DIC concentrations (Figure 5A) and pH (Figure 5B) were greater for the high-alkalinity nitrogen conditions ([DIC]=43.8±1.34, 43.3±1.69, 77.8±2.91, and 44.2±1.40 mM; pH=9.67±0.04, 9.66±0.04, 8.84±0.02, and 9.69±0.02 for the high-alkalinity nitrate, ammonium, urea, and mixed nitrogen conditions, respectively) than the standard nitrogen conditions ([DIC]=1.72±0.39, 1.43±0.30, 1.33±0.24, and 1.20±0.19 mM; pH=6.75±0.10, 6.82±0.10,

6.82±0.10, and 6.79±0.12 for the nitrate, ammonium, urea, and mixed nitrogen conditions, respectively). In the high-alkalinity conditions DIC concentrations gradually decreased following inoculation, throughout the nitrogen replete growth stage, and then essentially stabilized (Figure 5A). A small increase in DIC concentration was observed during the nitrogen deplete growth stage for all high-alkalinity conditions. Final DIC concentrations were 29.7±0.52, 27.7±2.86, 37.8±1.41, and 29.2±0.61 mM for the high-alkalinity nitrate, ammonium, urea, and mixed nitrogen conditions, respectively. Reduction in DIC during nitrogen replete growth is due to the shifting balance between influx of CO_2 into the medium from the air sparge and carbon consumption by algae, caused by increasing demand of the growing biomass. The reduction of DIC concentration coincides with a gradual increase in system pH during the nitrogen replete growth stage, with pH for all conditions surpassing pH 10.3, the upper pK_A for HCO_3^-. Culture pH peaked within 48 hours following nitrogen limitation (max pH 12.2±0.08 , 11.6±0.36, 11.5±0.17, and 12.2±0.11 for the nitrate, ammonium, urea, and mixed nitrogen conditions, respectively) followed by a decline in pH until the end of the nitrogen deplete growth phase (final pH=11.0±0.13, 10.0±0.04, 10.8±0.24, and 10.7±0.64 for the nitrate, ammonium, urea, and mixed nitrogen conditions, respectively). With pH>10.3, the dominant form of DIC for these conditions during the nitrogen deplete growth phase was CO_3^{2-}, however HCO_3^- and dissolved CO_2 are still available due to equilibrium considerations and redissolution of CO_2 from the air. The general stabilization of DIC availability and pH following nitrogen limitation indicates that the net influx of CO_2 from the air sparge balances the DIC consumption by algal biomass. Further, if inorganic carbon consumption and net mass transfer with the air sparge are balanced

at a stable pH and DIC concentration, the rate of carbon assimilation into biomass must also be generally stable.

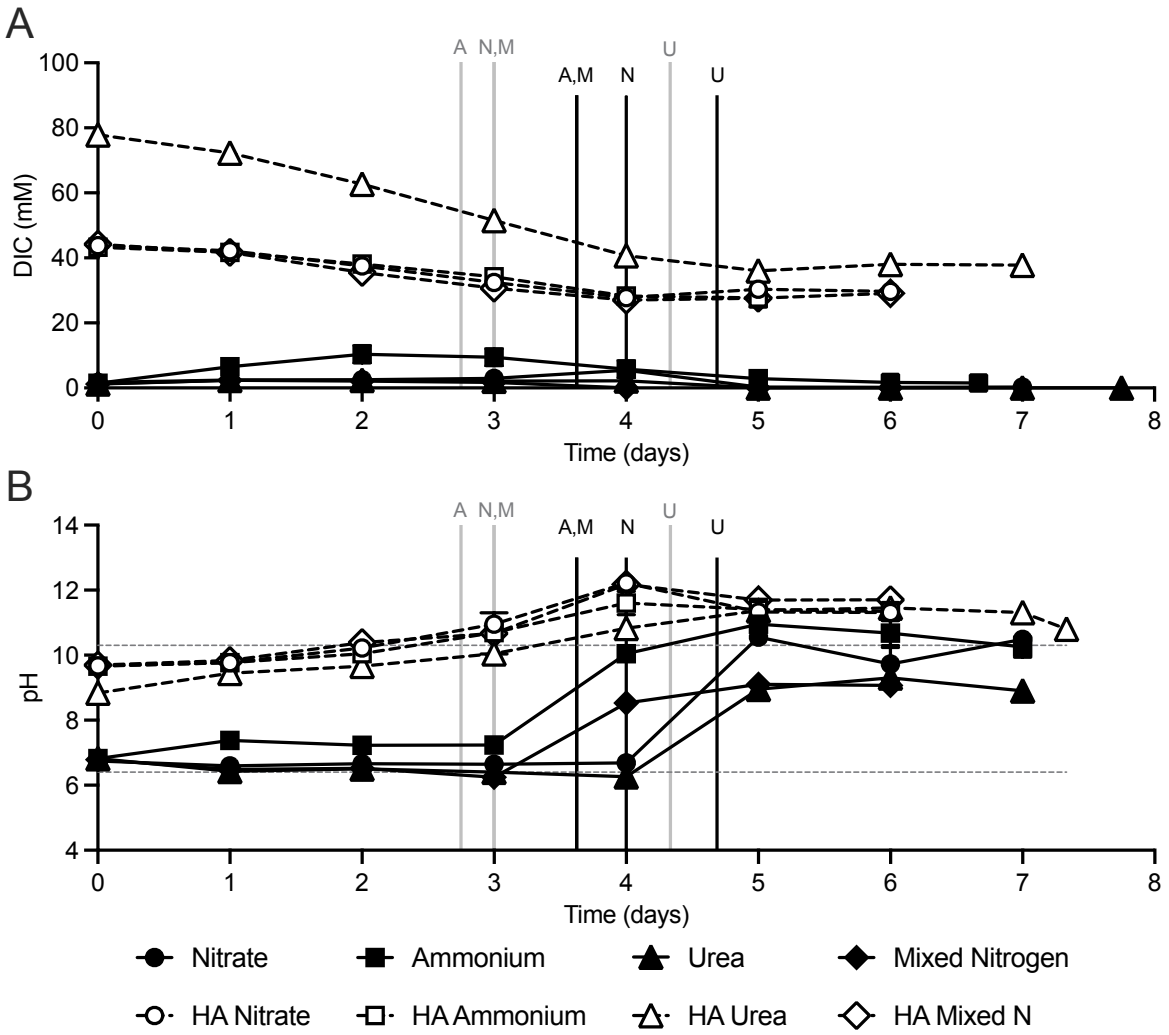

Figure 5: Media (A) DIC concentration (mM) and (B) pH for the standard (solid lines, solid symbols) and high-alkalinity (dashed lines, open symbols) nitrogen conditions, as a function of time. Vertical black and grey lines represent the time of nitrogen limitation for the standard and high-alkalinity nitrogen conditions, respectively. Horizontal dotted lines indicate the two pK_A values for the $H_2CO_3/HCO_3^-/CO_3^{2-}$ system under standard conditions (low salinity, ~20°C), 6.4 and 10.3. Error bars are one standard deviation of data. Throughout the duration of the study, the high-alkalinity nitrogen conditions maintained higher DIC concentrations than the standard nitrogen conditions. A higher pH was also observed for the high-alkalinity conditions, and the pH gradually increased for these conditions throughout the nitrogen replete growth phase. The pH for the high-alkalinity conditions surpassed pH 10.3, the pK_A of HCO_3^-/CO_2 equilibrium, prior to nitrogen limitation. In contrast, a sharp increase in pH was observed following nitrogen

limitation for the standard nitrogen conditions, resulting from cessation of the 5% CO_2 supplementation to the air supply during the nitrogen replete growth stage.

Biomass Generation

Biomass concentrations for the nitrogen and high alkalinity nitrogen conditions are compared in Figure 6, where the total biomass generated during 2-stage cultivation (full bars) is separated into biomass generated during the two growth phases (black and grey bars representing nitrogen replete and nitrogen deplete growth, respectively). Statistically significant differences in biomass concentration for the 2-stage growth process are indicated by letters, with conditions that do not share a common letter being significantly different. Differences in biomass concentrations generated during the two growth stages (nitrogen replete and deplete growth) are not indicated in Figure 6.

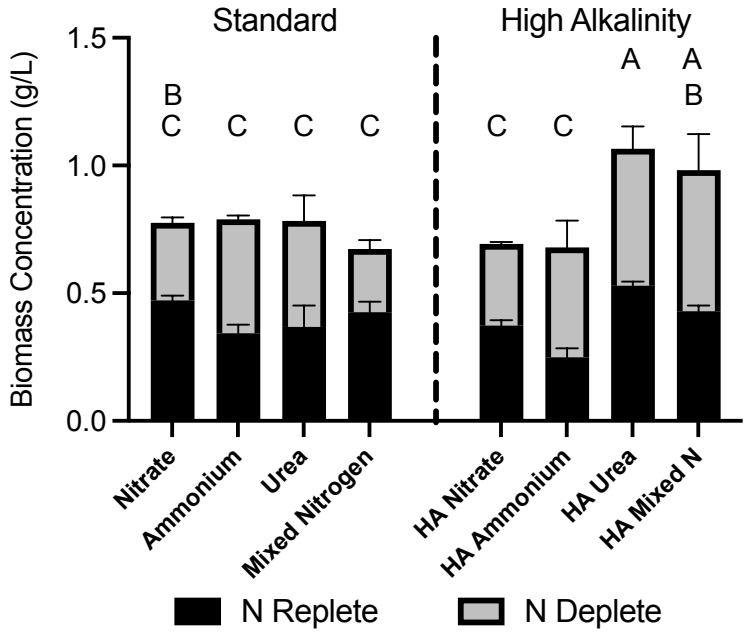

Figure 6: Biomass generated ($g*L^{-1}$) during nitrogen replete (black bars) and nitrogen deplete (grey bars) growth for the nitrogen (left of dashed line) and high alkalinity nitrogen (right of dashed line) conditions. Total biomass generated during the 2-stage growth process is equal to the combination of biomass generated independently in the two growth stages. Letters above

individual bars indicated significance of differences in biomass concentration generated during the 2-stage growth process, with conditions that are not significantly different sharing a common letter. Significance comparisons are for all eight conditions (standard and high-alkalinity nitrogen conditions). Error bars are one standard deviation of biomass concentration for the nitrogen replete growth stages. An increase in the amount of biomass generated as a result of high alkalinity cultivation was observed for the urea and mixed nitrogen conditions, but not for NO_3^- and NH_4^+ amended conditions.

During nitrogen replete growth high-alkalinity cultivation resulted in less biomass being generated when NO_3^- (0.37±0.02 $g*L^{-1}$; $p<0.001$ compared with the standard nitrate condition) and NH_4^+ (0.25±0.03 $g*L^{-1}$; $p=0.047$ compared with the standard ammonium condition) were provided as the only nitrogen sources. For the urea condition, high-alkalinity cultivation resulted in more biomass being generated prior to nitrogen limitation (0.53±0.02 $g*L^{-1}$; $p=0.006$ compared with standard urea condition). High-alkalinity cultivation did not impact the amount of biomass generated during nitrogen replete growth using mixed nitrogen (0.53±0.02 $g*L^{-1}$; $p=0.828$ compared with standard mixed nitrogen condition). Loss of nitrogen, as NH_3, to the environment due to high pH for the high-alkalinity ammonium condition is suspected to have contributed to the reduction in biomass generated for this condition, however the loss of nitrogen as NH_3 from the high-alkalinity mixed nitrogen condition did not have a significant impact on the amount of biomass generated. A comparison of nitrogen removed and nitrogen assimilated confirms the loss of nitrogen as NH_3 for the ammonium condition (1.27±0.18 and 0.90±0.17 mM removed and assimilated nitrogen, respectively, $p=0.005$). There were no differences observed between nitrogen removed and nitrogen assimilated for the other nitrogen conditions ($p=0.663$, 0.177, and 0.827 mM for the nitrate, urea, and mixed nitrogen conditions, respectively), however the total amount of nitrogen added to the growth medium was lower for all high-alkalinity conditions when compared to the standard nitrogen conditions. This likely contributed to the

lower biomass concentration observed at the end of the nitrogen replete growth stage for the high-alkalinity nitrate condition when compared to the standard nitrate condition. Biomass concentration was significantly different between the high-alkalinity nitrogen conditions at the end of nitrogen replete growth ($p<0.001$ for all conditions based on ANOVA).

During nitrogen deplete growth there was no significant difference in the change in biomass concentration between the four high-alkalinity nitrogen conditions (0.32±0.01, 0.43±0.11, 0.54±0.09, and 0.55±0.14 $g*L^{-1}$ for the high-alkalinity nitrate, ammonium, urea, and mixed nitrogen conditions, respectively; $p=0.065$ based on ANOVA), or between high-alkalinity and standard conditions for the individual nitrogen regimes ($p=0.500$, 0.815, 0.211, 0.068 for nitrate, ammonium, urea, and mixed nitrogen comparing standard and high-alkalinity conditions). For the 2-stage process high-alkalinity cultivation caused an increase in the final biomass concentrations observed for the urea and mixed nitrogen conditions (1.07±0.07 and 0.97±0.12 $g*L^{-1}$, respectively; $p=0.018$ and 0.049 when comparing standard and high-alkalinity cultivation for the urea and mixed nitrogen conditions, respectively). When the eight standard and high-alkalinity nitrogen conditions are compared for the 2-stage growth process, the amount of biomass generated was significantly greater for the high-alkalinity urea condition than for all other conditions except for the high-alkalinity mixed nitrogen condition ($p=0.409$). The second highest amount of biomass generated was observed for the high-alkalinity mixed nitrogen condition, which had a significantly greater biomass concentration than all conditions except for the nitrate (0.785±0.03 $g*L^{-1}$; $p=0.066$) and high-alkalinity urea conditions. Due to differences in culturing conditions, and the lack of previous research investigating utilization of different nitrogen sources under high-alkalinity conditions, it is not possible to compare the findings from

the high-alkalinity nitrogen experiments with existing literature. In addition, differences in cultivation systems and media constituents used between studies make comparisons of biomass concentrations difficult. However, the significantly increased biomass under the urea and mixed N conditions with high-alkalinity warrant future investigation.

Biomass productivity and biomass yield from nitrogen are presented in Figure 7A and B, respectively, for the nitrogen and high-alkalinity nitrogen conditions. For the nitrate conditions, high-alkalinity culturing did not have a significant impact on biomass productivity during either growth stage or for the combined 2-stage process (118±4.61 and 124±7.17 $g*m^{-3}*day^{-1}$ for nitrogen replete growth, p=0.123; 101±7.31 and 107±2.45 $g*m^{-3}*day^{-1}$ for nitrogen deplete growth, p=0.925 based on Games-Howell pairwise comparison due to unequal variances; 111±4.34 and 116±3.66 $g*m^{-3}*day^{-1}$ for 2-stage growth, p=0.468). During standard cultivation, the use of NO_3^- as the sole nitrogen source resulted in a 3.77±0.35 mM increase in alkalinity as a result of the net release of a single hydroxyl ion during conversion of NO_3^- to NH_4^+ and its subsequent integration into algal biomass. It is suspected that this small increase in alkalinity was sufficient to improve mass transfer of inorganic carbon into the system to meet consumption demands of the algal culture. This is supported by maintenance of a detectable DIC presence in the standard nitrate condition during the nitrogen deplete growth stage. It is likely that the relatively similar biomass production for the nitrate condition during standard and high alkalinity cultivation is a result of sufficient inorganic carbon already being available in the system. Biomass yield from nitrogen was improved during growth using NO_3^- by high-alkalinity cultivation during the nitrogen replete growth stage (9.98±0.39 and 14.3±0.82 $g_{biomass}*g_N^{-1}$ for standard and high-alkalinity cultivation, respectively; p<0.001 based on Games-Howell pairwise

comparison due to unequal variances), but there was no impact for the 2-Stage process (15.4±0.80 and 19.0±0.60 $g_{biomass}*g_N^{-1}$ for standard and high-alkalinity cultivation, p=0.152). The increased availability of cations, namely Na^+ from $NaHCO_3$, may facilitate improved HCO_3^- transport into the cell, as discussed previously, and as a result, increase the rate of carbon fixation during nitrogen replete growth.

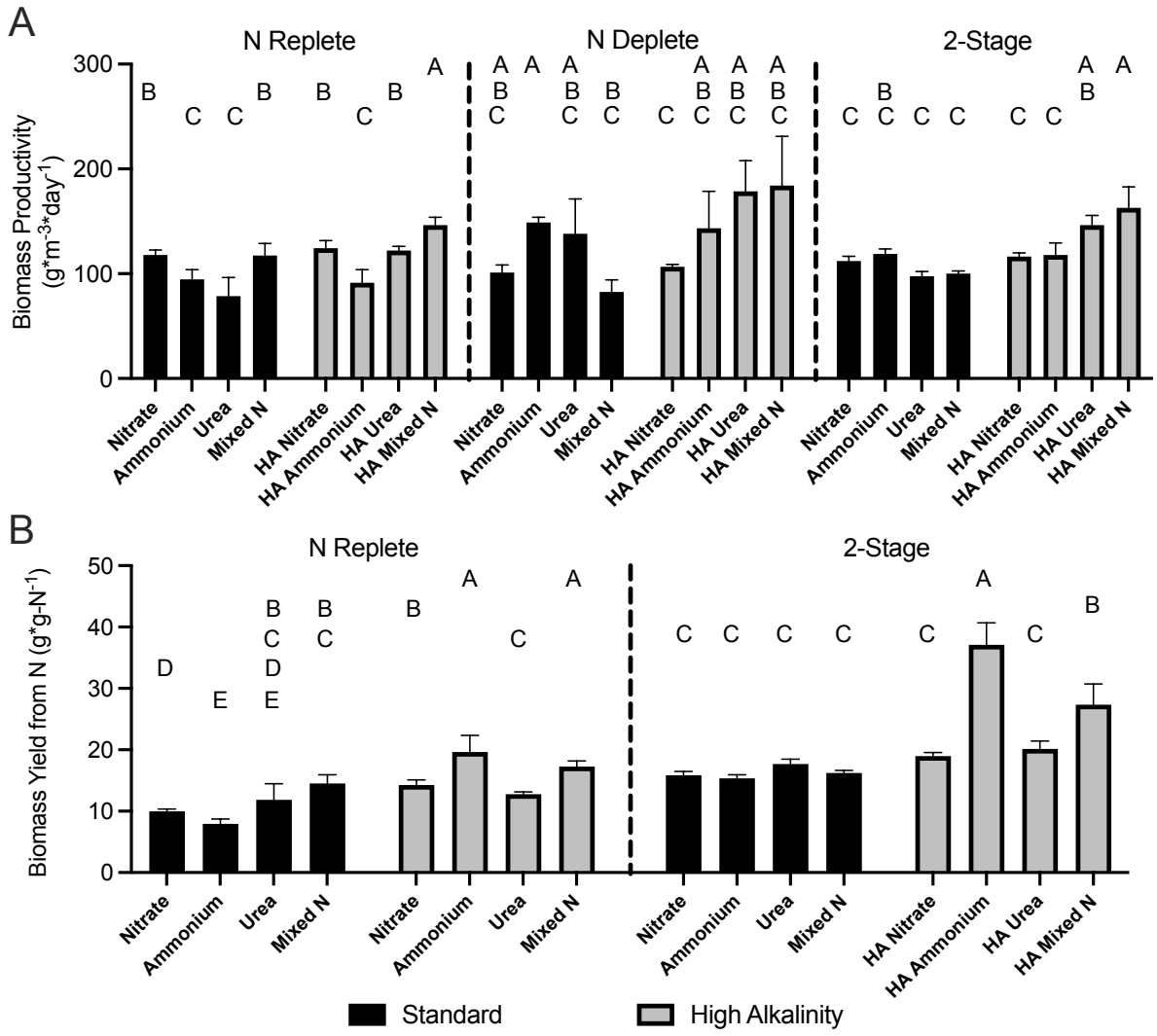

Figure 7: (A) Biomass productivity ($g*m^{-3}*day^{-1}$) and (B) yield from nitrogen (g-biomass*g-nitrogen^{-1}) for the nitrogen and high-alkalinity nitrogen conditions. Productivity is presented for the nitrogen replete and deplete growth phases, as well as a weighted average for the 2-stage cultivation process. Yield from nitrogen is presented for nitrogen replete growth and the

combined 2-stage growth system. Error bars are one standard deviation of data. Statistical significance for comparisons for all standard and high-alkalinity nitrogen conditions for each growth phase independently are presented using letters above each column. Conditions that share a common letter are not significantly different. High-alkalinity cultivation improved biomass productivity for the urea and mixed nitrogen conditions, and improved biomass yield from nitrogen for the ammonium and mixed nitrogen conditions.

Despite significant nitrogen losses resulting from conversion of NH_4^+ to NH_3 in the high-alkalinity ammonium condition, biomass productivity during high-alkalinity cultivation was not significantly different than in the standard nitrogen condition (94.6±9.21 and 91.3±12.66 $g*m^{-3}*day^{-1}$ for standard and high-alkalinity nitrogen replete growth, respectively, p=0.999; 149±5.00 and 143±35.2 $g*m^{-3}*day^{-1}$ for standard and high-alkalinity nitrogen deplete growth, respectively, p=1.000 based on Games-Howell pairwise comparison due to unequal variances; 119±4.65 and 118±11.4 $g*m^{-3}*day^{-1}$ for standard and high-alkalinity 2-stage growth, respectively, p=1.000). Biomass yield from nitrogen was greater during high alkalinity cultivation for this condition during nitrogen replete growth and for the 2-stage growth system (8.09±0.93 and 20.0±3.53$g_{biomass}*g_N^{-1}$ for standard and high-alkalinity nitrogen replete growth, respectively, p=0.001 based on Games-Howell pairwise comparison due to unequal variances; 16.45±1.02 and 36.0±3.20$g_{biomass}*g_N^{-1}$ for standard and high-alkalinity 2-stage growth, p<0.001). The biomass yield from nitrogen calculation for this condition may be inflated as the initial (at time of inoculation) concentration of nitrogen measured was less than half of the expected nitrogen concentration based on what was added to the growth medium. This lower nitrogen concentration is suspected to be the result of off gassing of NH_3 from the high pH medium.

High-alkalinity cultivation caused an increase in biomass productivity for the urea and mixed nitrogen conditions, relative to their standard nitrogen counterparts, during nitrogen

replete growth (78.6±17.7 and 122±3.82 $g*m^{-3}*day^{-1}$ for urea and high-alkalinity urea, respectively, $p<0.001$; 117±11.4 and 146±7.65 $g*m^{-3}*day^{-1}$ for mixed nitrogen and high-alkalinity mixed nitrogen, respectively; $p=0.001$), but not during nitrogen deplete growth (138±33.2 and 179±29.3 $g*m^{-3}*day^{-1}$ for urea and high-alkalinity urea, respectively, $p=0.746$ based on Games-Howell pairwise comparison due to unequal variances; 82.6±11.5 and 184±47.0 $g*m^{-3}*day^{-1}$ for mixed nitrogen and high-alkalinity mixed nitrogen, respectively; $p=0.249$ based on Games-Howell pairwise comparison due to unequal variances). Biomass yield from nitrogen was not impacted by high-alkalinity cultivation for the urea condition during nitrogen replete growth or the 2-stage process (12.2±3.58 and 15.0±6.41 $g_{biomass}*g_N^{-1}$ for standard and high-alkalinity nitrogen replete growth, $p=0.373$ based on Games-Howell pairwise comparison due to unequal variances; 17.4±1.08 and 24.9±9.46 $g_{biomass}*g_N^{-1}$ for standard and high-alkalinity 2-stage growth, $p=0.762$). There was also no difference observed in biomass yield from nitrogen for the mixed nitrogen condition during nitrogen replete growth (15.7±4.94 and 17.2±3.84 $g_{biomass}*g_N^{-1}$ for standard and high-alkalinity nitrogen replete growth, $p=0.249$ based on Games-Howell pairwise comparison due to unequal variances), however high-alkalinity cultivation improved biomass yield from nitrogen for the 2-stage process (14.5±2.05 and 24.9±2.94 $g_{biomass}*g_N^{-1}$ for standard and high-alkalinity cultivation; $p<0.001$).

Of all standard and high-alkalinity nitrogen conditions, high-alkalinity urea and high-alkalinity mixed nitrogen exhibited the highest biomass productivity for the 2-stage process. Productivity in the standard ammonium condition was not significantly different than the high-alkalinity urea condition, but all other nitrogen conditions had significantly lower biomass productivities. Increased biomass productivity for the urea condition could be associated with the

potential upregulation of genes associated with urea carboxylase due to the high concentration of HCO_3^- (Tu et al., 2018). The highest biomass yield from nitrogen for the 2-stage process was observed in the high-alkalinity ammonium and high-alkalinity mixed nitrogen conditions, with yield for these conditions being significantly greater than all other conditions. Due to nitrogen losses as NH_3 from the ammonium condition during cultivation, as discussed previously, it is likely that the biomass yield from nitrogen for this condition is inaccurate.

Media Chemistry – Bicarbonate Amendment at Nitrogen Limitation

For the standard nitrogen conditions the bicarbonate amendment at nitrogen limitation resulted in an immediate increase in DIC followed by a gradual decline during the nitrogen deplete growth stage (Figure 8A). Due to cessation of the 5% CO_2 supplementation to the air sparge at nitrogen limitation, as well as DIC consumption by algae and CO_2 off-gassing from the system, the pH for the bicarbonate amended conditions increased sharply in the 24 hours following nitrogen limitation towards pH 10.3, the HCO_3^-/CO_3^{2-} pKa. Without the bicarbonate amendment the pH also increased, however DIC concentration rapidly decreased. The unamended urea and mixed nitrogen conditions stabilized at a lower pH around 8.3 due to the lower alkalinity of these conditions relative to the unamended nitrate and ammonium conditions.

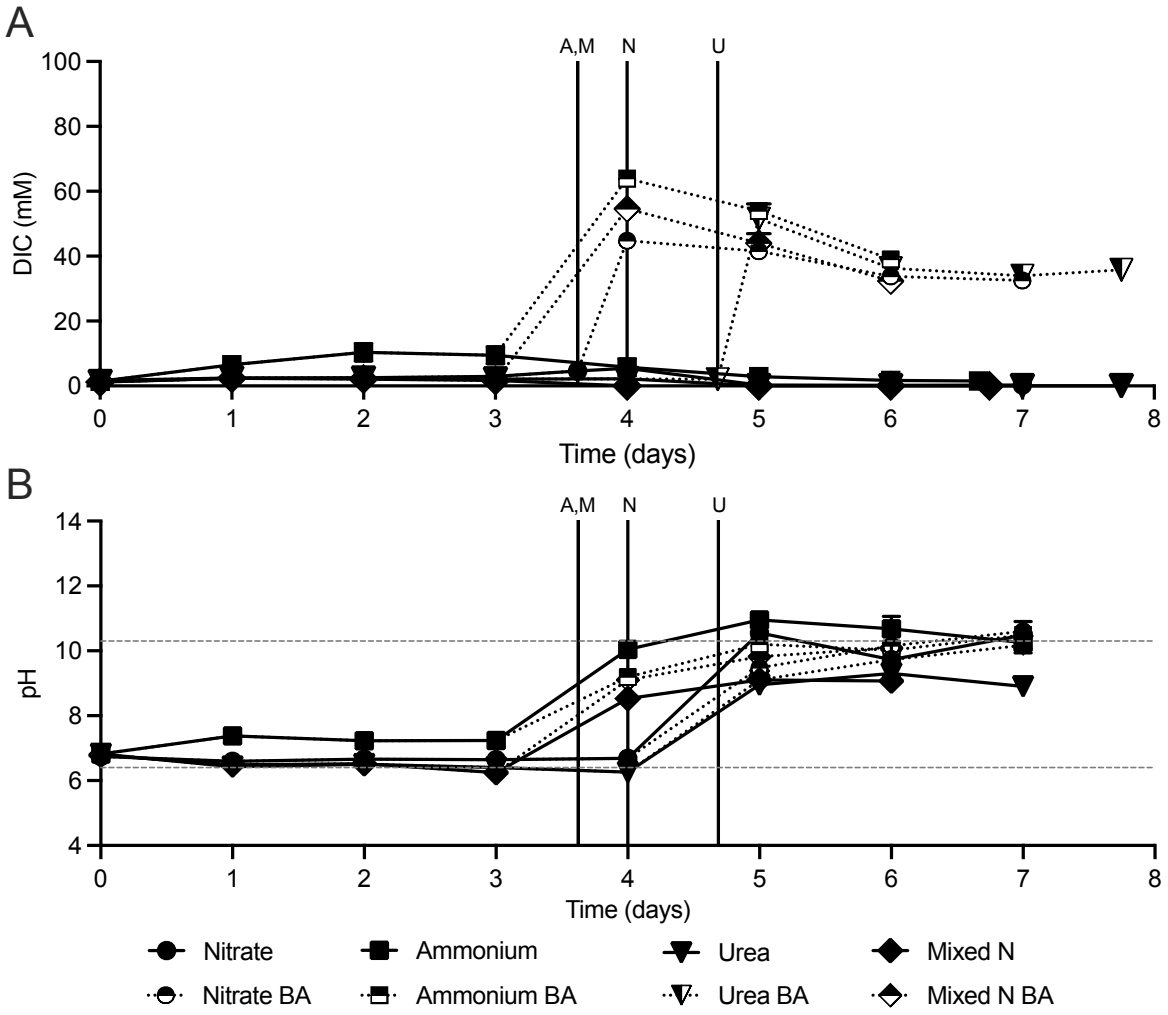

Figure 8: (A) DIC concentration (mM) and (B) pH for the standard nitrogen conditions with and without a bicarbonate amendment (BA) at nitrogen limitation. Vertical lines represent the times of nitrogen limitation for the different conditions as designated by the first letter of each nitrogen condition. Horizonal grey dashed lines show the two equilibrium points for the inorganic carbon system (pH 6.4 for H_2CO_3/HCO_3^- and pH 10.3 for HCO_3^-/CO_3^{2-}). Error bars are one standard deviation of the data. During nitrogen deplete growth the bicarbonate amended conditions exhibited a sharp increase in pH and DIC and maintained DIC concentrations above 35mM for the duration of the nitrogen deplete growth stage. Both the amended and unamended conditions exhibited an increase in pH during nitrogen deplete growth. With the exception of the unamended urea and mixed nitrogen conditions which approached pH 8.3, all conditions approached pH 10.3.

When a 50mM bicarbonate amendment at nitrogen limitation was evaluated, a sharp increase in DIC concentration was observed for all conditions, similar to the standard nitrogen conditions Figure 9A. However, the pH for the bicarbonate amended nitrogen conditions decreased slightly relative to the unamended conditions due to their increased buffering capacity, and the sharp increase in pH observed for the standard nitrogen conditions following nitrogen limitation was not observed Figure 9B. The stabilization of the conditions at a lower pH by the bicarbonate amendment may increase inorganic carbon availability in these systems by shifting HCO_3^-/CO_3^{2-} equilibrium towards HCO_3^-. However, the pH for all high-alkalinity conditions, amended and unamended, remained above pH 10.3, suggesting that the dominant form of inorganic carbon was CO_3^{2-}.

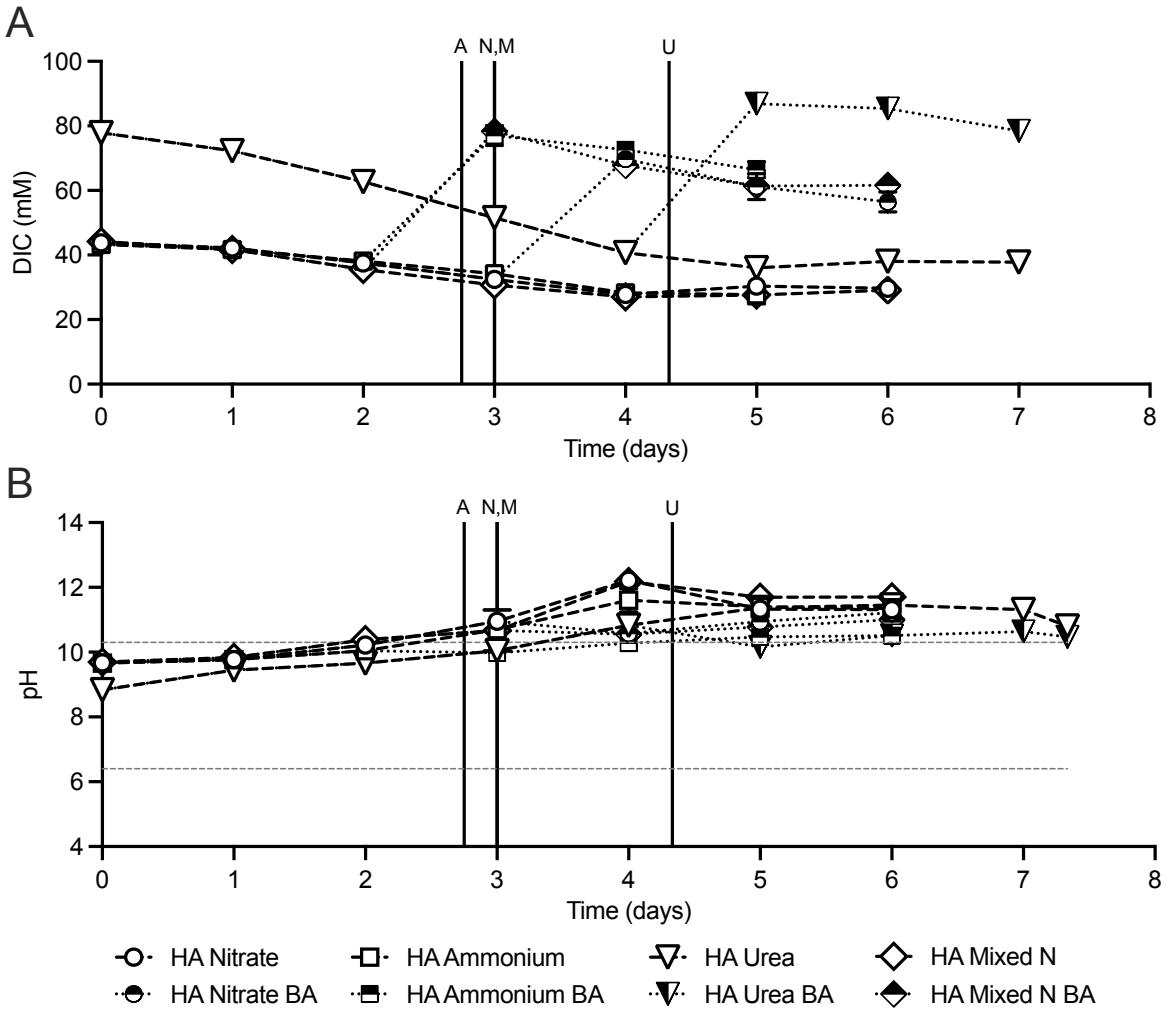

Figure 9: (A) DIC concentration (mM) and (B) pH for the high-alkalinity nitrogen conditions with and without a bicarbonate amendment (BA) at nitrogen limitation. Vertical lines represent the times of nitrogen limitation for the different conditions as designated by the first letter of each nitrogen condition. Horizonal grey dashed lines show the two equilibrium points for the inorganic carbon system (pH 6.4 for H_2CO_3/HCO_3^- and pH 10.3 for HCO_3^-/CO_3^{2-}). Error bars are one standard deviation of the data. Following nitrogen limitation, a sharp increase in DIC concentration was observed for all bicarbonate amended conditions, followed by a gradual decline throughout the nitrogen deplete growth stage. The average pH for all high-alkalinity conditions, amended and unamended, was greater than 10.3 during the nitrogen deplete growth stage (pH=11.6±0.09, 11.3±0.19, 11.2±0.21, and 11.9±0.07 for the unamended high-alkalinity nitrate, ammonium, urea, and mixed nitrogen condition, respectively; pH=10.9±0.16, 10.3±0.03, 10.4±0.12, and 10.8±0.11 for the unamended high-alkalinity nitrate, ammonium, urea, and mixed nitrogen condition, respectively).

Biomass Generation – Bicarbonate Amendment at Nitrogen Limitation

In the standard nitrogen conditions the concentration of biomass generated during nitrogen deplete growth was improved by the addition of the bicarbonate amendment at nitrogen limitation for all conditions except nitrate (0.30±0.02 and 0.62±0.06 $g*L^{-1}$ for the nitrate and bicarbonate amended nitrate conditions, respectively; 0.45±0.02 and 0.66±0.03 $g*L^{-1}$ for the ammonium and bicarbonate amended ammonium conditions, respectively; 0.41±0.10 and 0.73±0.02 $g*L^{-1}$ for the urea and bicarbonate amended urea conditions, respectively; 0.25±0.03 and 0.65±0.06 for the mixed nitrogen and bicarbonate amended mixed nitrogen conditions, respectively; p=0.176, 0.008, 0.034, and 0.003 based on unpaired t-tests comparing standard and high-alkalinity-cultivation for the nitrate, ammonium, urea, and mixed nitrogen conditions, respectively; left side of Figure 10). The amount of biomass generated during the nitrogen deplete growth stage by the bicarbonate amended conditions was similar for all conditions (p=0.199 based on one-way ANOVA using Welch's Test due to unequal variances). The smaller amount of biomass generated for the unamended standard nitrogen conditions is likely a result of insufficient carbon availability without supplementation, however the physiological stress resulting from the sudden shift in alkalinity may also contribute to the resource allocation (*e.g.*, lipids) observed in the bicarbonate amended conditions. In previous research, pH stress has been identified as a stimulus for lipid synthesis, especially when combined with nitrogen stress (Bartley et al., 2014), however the limited availability of inorganic carbon in the standard nitrogen conditions may inhibit the ability to synthesize lipids.

The high-alkalinity nitrate condition also showed an increase in the amount of biomass generated during nitrogen deplete growth with the addition of the 50mM bicarbonate amendment prior to nitrogen limitation (0.32±0.01 and 0.75±0.08 $g*L^{-1}$ for high-alkalinity nitrate and bicarbonate amended high-alkalinity nitrate conditions, respectively; p=0.010 based on unpaired t-test), however no significant change was observed for the other high-alkalinity nitrogen conditions (0.43±0.06 and 0.41±0.03 $g*L^{-1}$ for the high-alkalinity ammonium and bicarbonate amended high-alkalinity ammonium conditions, respectively; 0.54±0.09 and 0.72±0.01 $g*L^{-1}$ for high-alkalinity urea and high-alkalinity urea conditions, respectively; 0.55±0.14 and 0.64±0.10 $g*L^{-1}$ for high-alkalinity mixed nitrogen and bicarbonate amended high-alkalinity mixed nitrogen conditions, respectively; p=0.773, 0.070, and 0.482 based on unpaired t-tests for high-alkalinity ammonium, urea, and mixed nitrogen, respectively). It is noteworthy that addition of the bicarbonate amendment at nitrogen limitation to the high-alkalinity urea condition increased the amount of biomass generated during nitrogen deplete growth when a 90% confidence interval is used. As a result, the impact of the bicarbonate amendment to this condition may have industrial relevance. The lack of response to the bicarbonate amendment observed for the high-alkalinity ammonium condition is suspected to be related to the loss of nitrogen as NH_3 during nitrogen replete growth, however it is not well understood. For the high-alkalinity urea and mixed nitrogen conditions the lack of response is suspected to be a result of the higher biomass concentrations observed for these conditions at the end of nitrogen replete growth, however this observation is also not well understood. It is possible that the improved biomass productivity observed for the bicarbonate amended standard urea and mixed nitrogen conditions is partially a result of pH or alkalinity stress, which was less significant for the high-alkalinity nitrogen

conditions. The efficacy of the bicarbonate amendment for the high-alkalinity nitrate condition, but not the standard nitrate condition is puzzling.

When biomass generated during nitrogen deplete growth for the bicarbonate amended standard and high-alkalinity conditions is compared, the only nitrogen source that responded differently was the bicarbonate amended, high-alkalinity ammonium condition where reduced biomass generation was observed ($p<0.001$ based on ANOVA). This may result from reduced nitrogen availability due to nitrogen losses as NH_3 from this condition, however it is unclear how this would impact the efficacy of the bicarbonate amendment. Other factors that might impact the high-alkalinity ammonium condition include NH_3 toxicity or the ion gradient between the cytoplasm and growth medium. Even at extreme pH algal cells maintain circumneutral pH (Rai et al., 2001). The combination of the bicarbonate amendment with high-alkalinity cultivation does not promote any further increase in the amount of biomass generated relative to the bicarbonate amended standard nitrogen conditions. The significance of differences in biomass concentration generated for the 2-stage growth process for all standard and high-alkalinity nitrogen conditions, with and without bicarbonate amendment at nitrogen limitation are identified by the letters above each bar in Figure 10, where conditions that share a common letter are not significantly different (based on ANOVA). The unamended high-alkalinity urea condition and the amended nitrate, urea, mixed nitrogen, high-alkalinity nitrate, high-alkalinity urea, and high-alkalinity mixed nitrogen conditions generated the highest biomass concentrations.

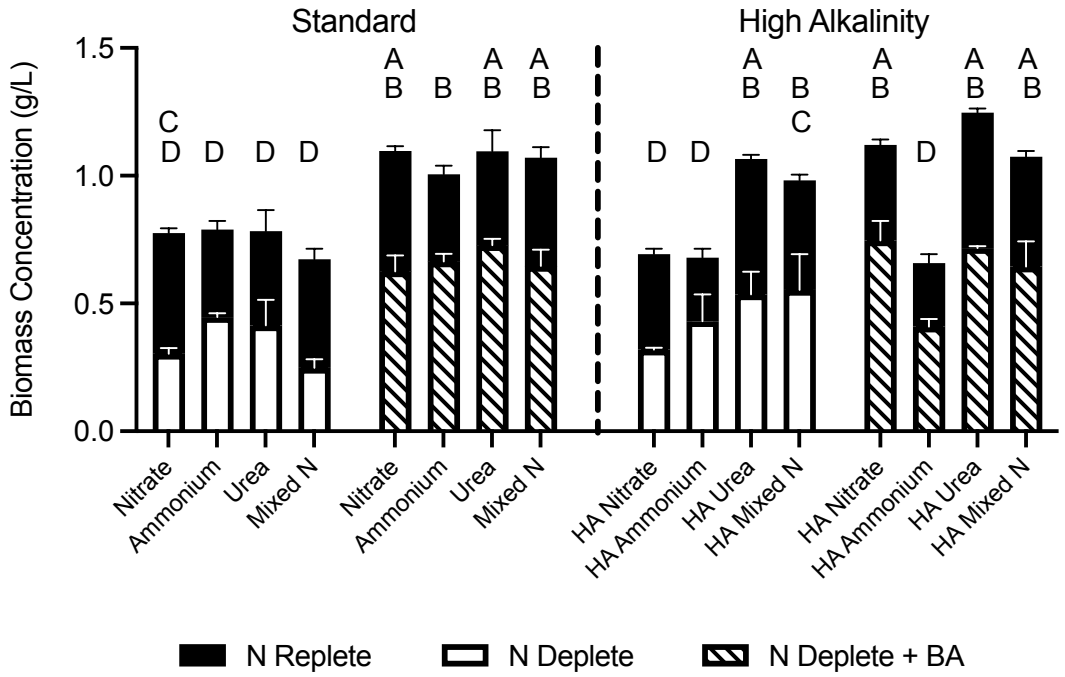

Figure 10: Biomass generated ($g*L^{-1}$) during nitrogen replete (top bars) and nitrogen deplete (bottom bars) growth for the nitrogen (left) and high-alkalinity nitrogen (right) conditions. Biomass generated during nitrogen deplete growth is presented for unamended (white bars) and bicarbonate amended (white bars with black stripes) conditions. Error bars are one standard deviation of data. Shared letters above individual bars identify conditions with biomass concentrations that are not significantly different. The significance presented is for the simultaneous comparison of all standard and high-alkalinity nitrogen conditions during the 2-stage growth process using the Tukey method with 95% confidence following ANOVA. Supplementation with a bicarbonate amendment at nitrogen limitation significantly increased the amount of biomass generated during nitrogen deplete growth for all of the standard nitrogen conditions. For the high-alkalinity nitrogen conditions, increased biomass generation during nitrogen deplete growth as a result of the bicarbonate amendment at nitrogen limitation was only observed for the nitrate condition ($p=0.010$). No impact on the amount of biomass generated was observed for the other high-alkalinity nitrogen conditions.

Biomass productivity during nitrogen deplete growth was proportional to biomass generation during the same growth stage since the length of nitrogen deplete growth was the same for all conditions (3 days). Because nitrogen deplete growth cannot be achieved without a preceding nitrogen replete growth phase it is of interest to consider biomass productivity for the

2-stage growth system with and without a bicarbonate amendment at nitrogen limitation (Figure 11A).

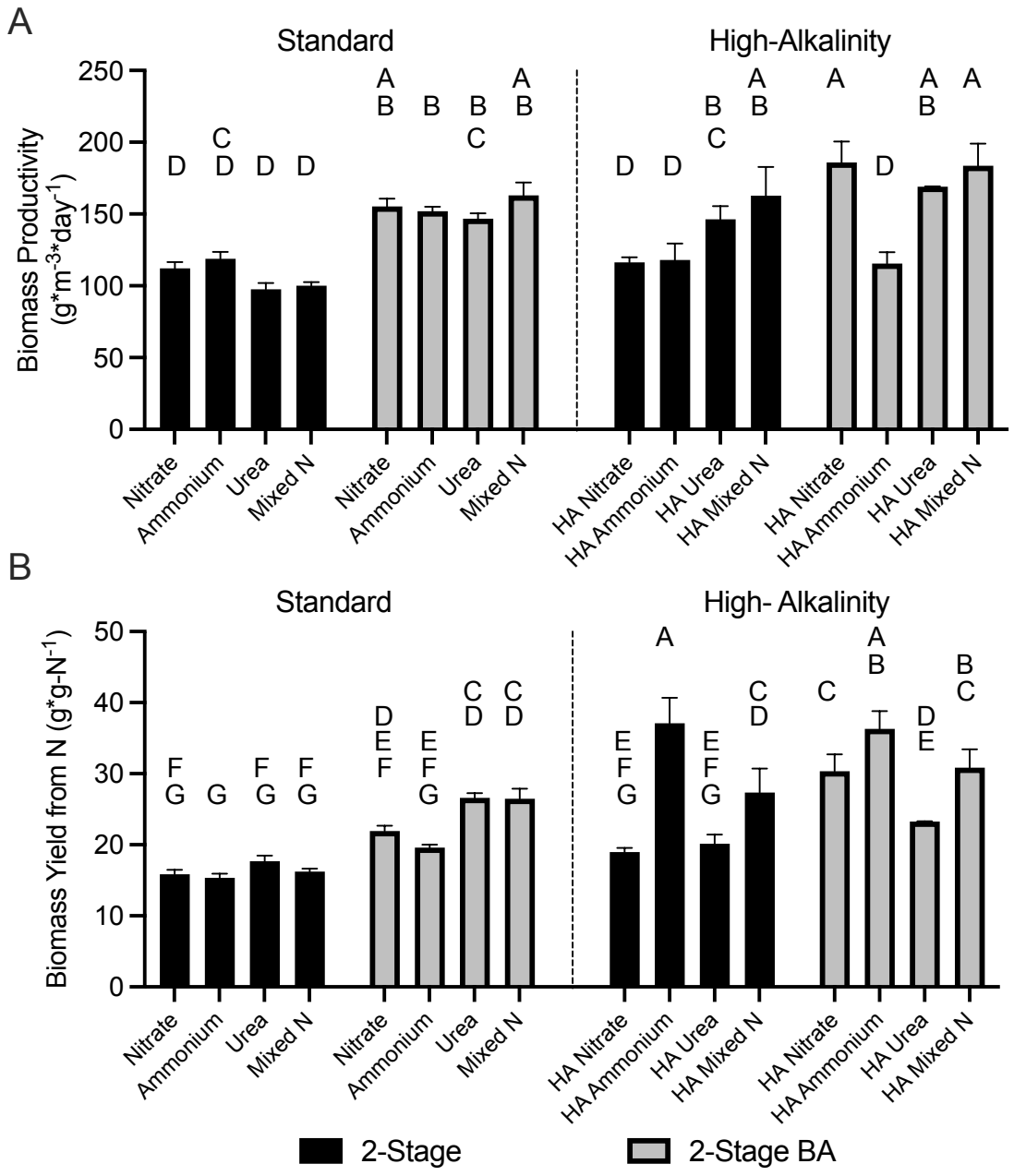

Figure 11: (A) Biomass productivity ($g*m^{-3}*day^{-1}$) and (B) biomass yield from nitrogen ($g_{Biomass}*g_N^{-1}$) for unamended (grey bars) and bicarbonate amended (black bars) conditions during standard (left of vertical dashed line) and high-alkalinity (right of vertical dashed line) cultivation during the 2-stage growth process. Error bars are one standard deviation of the data.

Shared letters above individual bars identify conditions that are not significantly different. The significance presented is for the simultaneous comparison of all standard and high-alkalinity nitrogen conditions during the 2-stage growth process using the Tukey method with 95% confidence following ANOVA. Biomass productivity and yield from nitrogen were improved by the bicarbonate amendment at nitrogen limitation for the high-alkalinity nitrate and all of the standard nitrogen conditions except the nitrate conditions. The impact of the bicarbonate amendment on the standard nitrate condition is significant if a 90% confidence interval is used.

The application of a bicarbonate amendment at nitrogen limitation resulted in a significant increase in biomass productivity during the 2-stage growth process for all standard nitrogen conditions except for the nitrate condition (112±4.34 and 155±5.56 $g*m^{-3}*day^{-1}$ for the unamended and amended nitrate conditions, respectively;119±4.65 and 152±3.12 $g*m^{-3}*day^{-1}$ for the unamended and amended ammonium conditions, respectively; 97.6±4.45 and 147±3.67 $g*m^{-3}*day^{-1}$ for the unamended and amended urea conditions, respectively; 100±2.58 and 163±8.92 $g*m^{-3}*day^{-1}$ for the unamended and amended mixed nitrogen conditions, respectively; p=0.074, 0.002, 0.001, and 0.007 based on unpaired t-tests for nitrate, ammonium, urea, and mixed nitrogen, respectively). It is worth noting that if a 90% confidence interval is used, instead of a 95% confidence interval, the impact of the bicarbonate amendment at nitrogen limitation on the nitrate condition was significant. Biomass yield from nitrogen for the 2-stage process was also improved for the standard ammonium, urea, and mixed nitrogen conditions as a result of receiving the bicarbonate amendment (15.3±0.60 and 19.6±0.40 $g_{biomass}*g_N^{-1}$ for the unamended and amended ammonium conditions, respectively, 17.7±0.81 and 26.6±0.67 $g_{biomass}*g_N^{-1}$ for the unamended and amended urea conditions, respectively; 16.2±0.42 and 26.5±1.45 $g_{biomass}*g_N^{-1}$ for the unamended and amended mixed nitrogen conditions, respectively; p=0.002, 0.001, and 0.007 for the ammonium, urea, and mixed nitrogen conditions, respectively; Figure 11B). No statistically significant change based on the 95% confidence interval criterion was observed in

biomass yield from nitrogen for the nitrate condition (15.8±0.61 and 21.9±0.79 $g_{biomass}*g_N^{-1}$ for the unamended and amended nitrate conditions, respectively; p=0.074 based on unpaired t-test). It should be noted that an increase from ~16 $g_{biomass}*g_N^{-1}$ to ~22 $g_{biomass}*g_N^{-1}$ might be industrially significant though it might not be significant statistically. No differences in biomass productivity were observed between the bicarbonate amended standard nitrogen conditions (p=0.055 based on ANOVA), however the bicarbonate amended urea and mixed nitrogen condition had higher biomass yield from nitrogen than the nitrate and ammonium condition (p=0.004 and <0.001 for the bicarbonate amended urea condition compared to the bicarbonate amended nitrate and ammonium conditions, respectively; p=0.004 and <0.001 for the bicarbonate amended mixed nitrogen condition compared to the bicarbonate amended nitrate and ammonium conditions, respectively). Again, if a 90% confidence interval is used, instead of a 95% confidence interval, the differences in biomass productivity between the bicarbonate amended standard nitrogen conditions is significant, which might be industrially relevant.

For the high-alkalinity conditions, the bicarbonate amendment at nitrogen limitation improved biomass productivity for the nitrate amended condition (116±3.66 and 186±14.6 $g*m^{-3}*day^{-1}$ for the unamended and amended nitrate conditions, respectively; p=0.015 based on unpaired t-test), however no change was observed for the high-alkalinity, ammonium, urea, and mixed nitrogen conditions (118±11.4 and 115±7.97 $g*m^{-3}*day^{-1}$ for the unamended and amended ammonium conditions, respectively; 146±9.30 and 169±0.33 $g*m^{-3}*day^{-1}$ for the unamended and amended urea conditions, respectively; 163±20.1 and 184±15.6 $g*m^{-3}*day^{-1}$ for the unamended and amended mixed nitrogen conditions, respectively; p=0.775, 0.052, and 0.323 based on Welch's test, due to unequal variances, for high-alkalinity ammonium, urea, and mixed

nitrogen, respectively). If a 90% confidence interval is used, biomass productivity was also significantly enhanced by the addition of the bicarbonate amendment to the high-alkalinity urea condition. Biomass yield from nitrogen was also only improved by the bicarbonate amendment for the high-alkalinity nitrate condition (18.5±0.58 and 30.9±1.35 $g_{biomass}*g_N^{-1}$ for the unamended and amended nitrate conditions, respectively; p=0.005 based on Welch's test due to unequal variances). The bicarbonate amended high-alkalinity ammonium, urea, and mixed nitrogen conditions all had similar biomass yields from nitrogen as their unamended counterparts (37.1±3.60 and 37.6±3.19 $g_{biomass}*g_N^{-1}$ for the unamended and amended ammonium conditions, respectively; 20.1±1.28 and 22.1±6.91 $g_{biomass}*g_N^{-1}$ for the unamended and amended urea conditions, respectively; 27.4±3.37 and 34.9±5.24 $g_{biomass}*g_N^{-1}$ for the unamended and amended mixed nitrogen conditions, respectively; p=0.871, 0.680, and 0.322 based on Welch's test, due to unequal variances, for high-alkalinity ammonium, urea, and mixed nitrogen, respectively). During 2-stage growth biomass productivity was similar for the bicarbonate amended high-alkalinity nitrate, urea, and mixed nitrogen conditions (p=0.255 based on ANOVA omitting the high-alkalinity ammonium condition). Biomass productivity in the bicarbonate amended high-alkalinity ammonium condition was significantly lower than in the other bicarbonate amended high-alkalinity nitrogen conditions (p<0.001 based on ANOVA comparing all bicarbonate amended high-alkalinity nitrogen conditions), likely as a result of nitrogen losses due to volatilization of NH_3. Biomass yield from nitrogen was similar for all bicarbonate amended, high-alkalinity nitrogen conditions except when the nitrate and urea conditions are compared, with the bicarbonate amended, high-alkalinity urea condition having a significantly lower yield from nitrogen than the bicarbonate amended, high-alkalinity nitrate condition (p=0.018).

When all eight standard and high alkalinity nitrogen conditions are compared simultaneously, the bicarbonate amended conditions generally exhibited higher biomass productivities than the unamended conditions. The highest productivities were observed in the bicarbonate amended nitrate, bicarbonate amended mixed nitrogen, high-alkalinity mixed nitrogen, bicarbonate amended high-alkalinity nitrate, bicarbonate amended high-alkalinity urea, and bicarbonate amended high-alkalinity mixed nitrogen conditions. Biomass yield from nitrogen was greatest for the high-alkalinity ammonium and bicarbonate amended high-alkalinity ammonium conditions. As discussed above, some of the nitrogen was unaccounted for in the high alkalinity ammonium systems and this is suspected to be due to the volatilization of NH_3. As a result, the nitrogen concentration used to calculate biomass yield from nitrogen for this condition was approximately half the concentration that was observed in the other conditions, which significantly impacted this calculation. When the amended and unamended high-alkalinity ammonium conditions are excluded, the bicarbonate amended urea, bicarbonate amended mixed nitrogen, high-alkalinity mixed nitrogen, bicarbonate amended high-alkalinity nitrate, and bicarbonate amended high-alkalinity mixed nitrogen conditions had the highest biomass yields from nitrogen.

Carbon utilization by SLA-04

Cell Growth and Nitrate Removal – Inorganic Carbon

Cell growth showed extensive variability for the inorganic carbon conditions evaluated (Figure 12A). For the condition where inorganic carbon supplementation was provided via air, an increase in cell growth was first observed one day after inoculation and the maximum specific

cell growth rate was observed between days 1 and 2 (2.36±0.24 day^{-1}). After the third day of the experiment cell growth gradually slowed in the Air condition for the rest of the experiment, but the cell concentration continued to increase. For this condition, nitrogen limitation was not reached until eight days after the beginning of the experiment, however exponential cell growth essentially stopped five days after inoculation and stabilized at 0.5 day^{-1}. At this time the nitrogen concentration was 1.71±0.13 mM and was not suspected to be limiting (Figure 12B). This suggests that carbon availability has a stronger impact on cell growth than nitrogen availability. The average nitrogen removal rate for this condition was 0.44±0.00 $mM*day^{-1}$ and at nitrogen limitation the cell concentration was $9.07*10^7$±$8.08*10^6$ $cells*mL^{-1}$. The average nitrogen removal rate for the Air condition was significantly lower than was observed for all other conditions ($p<0.000$ for comparison with all other carbon conditions). At the end of both growth stages (11 days after inoculation) the final cell concentration was $1.34*10^8$±$8.72*10^6$ $cells*mL^{-1}$.

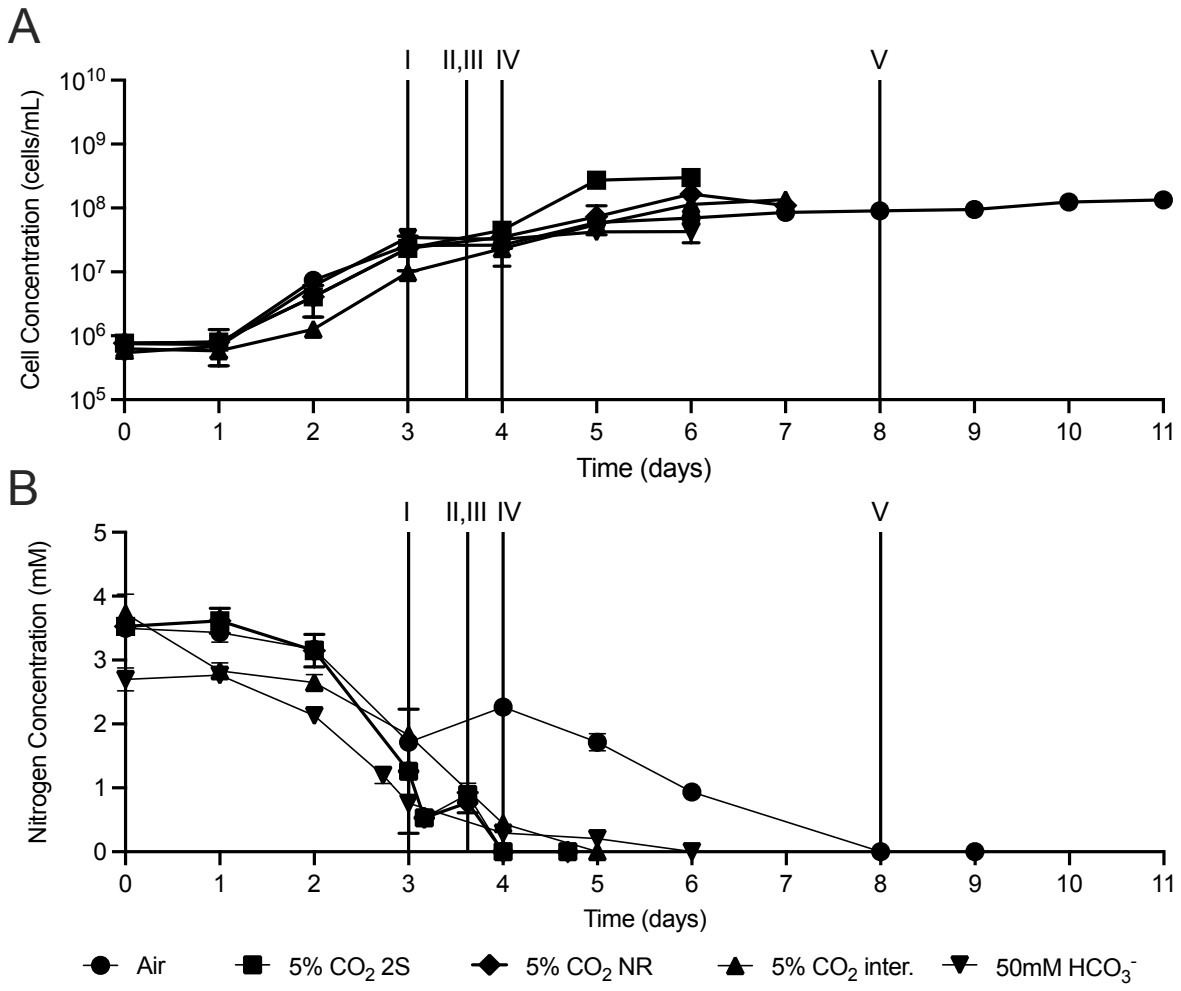

Figure 12: (A) Cell concentration (cells/mL) and (B) nitrogen concentration (mM) as a function of time for the inorganic carbon conditions. Cell concentration is presented on a logarithmic vertical axis. Vertical lines represent the times of nitrogen limitation for the different conditions as designated by (I) 50mM HCO_3^-, (II) 5% CO_2 2S, (III) 5% CO_2 NR, (IV) 5% CO_2 intermittent, and (V) air. Error bars are one standard deviation of the data. A longer lag phase preceding exponential growth was observed for the intermittent CO_2 condition. Higher final cell concentrations were observed for conditions where CO_2 supplementation was provided for the duration of the 2-stage process, with the 5% CO_2 2S condition reaching the highest cell concentration. In the absence of exogenous inorganic carbon supplementation nitrogen removal was markedly slower than in conditions where inorganic carbon availability was supplemented.

During nitrogen replete growth the 5% CO_2 2S and 5% CO_2 NR were the same, and therefore the data for these conditions were analyzed together for the nitrogen replete growth

stage. The maximum specific cell growth rate during nitrogen replete growth was 1.75±0.37 day^{-1}, observed between day two and three after inoculation (Figure 12A). The max growth rate was significantly lower than all other carbon conditions except the 5% CO_2 intermittent condition ($p=0.001$, 0.211, and 0.026 for comparison with the Air, 5% CO_2 intermittent, and 50 mM HCO_3^- conditions, respectively). The average nitrogen removal rate for the 5% CO_2 2S and 5% CO_2 NR conditions was 0.92±0.04 $mM*day^{-1}$ and nitrogen limitation was reached 3.625 days following inoculation (Figure 12B). The average nitrogen removal rate for this condition was significantly faster than was observed for all other conditions ($p<0.000$ for comparison with all other carbon conditions). At nitrogen limitation, cell concentration was $2.34*10^7±1.30*10^7$ $cells*mL^{-1}$. For the 5% CO_2 2S condition, cell growth slowed briefly at nitrogen limitation, likely as a result of metabolic shifts due to nitrogen becoming limited, but then the growth rate increased again. For this condition, the maximum specific cell growth rate was actually reached between days four and five, one day after nitrogen limitation. Five days after inoculation cell growth stopped in the 5% CO_2 2S condition. The final cell concentration at the end of nitrogen deplete growth was $2.99*10^8±2.14*10^7$ $cells*mL^{-1}$. For the condition where the 5% CO_2 supplementation to the air sparge was stopped at nitrogen limitation (5% CO_2 2S), cell growth also continued following nitrogen limitation, however not at as high of a rate as the condition where the 5% CO_2 supplementation was continued through the nitrogen deplete growth stage. The final cell concentration at the end of the nitrogen deplete growth stage for the 5% CO_2 NR condition was $1.10*10^8±6.36*10^6$ $cells*mL^{-1}$.

For the 5% CO_2 intermittent condition, cell growth during nitrogen replete growth exhibited a longer lag period before exponential growth was observed, however the max cell

growth rate, 2.03±0.16 day^{-1}, was not significantly different than the maximum growth rate observed for the other carbon conditions during nitrogen replete growth (p=0.197, 0.211, and 0.790 for comparison with the Air, 5% CO_2 2S and NR, and 50 mM HCO_3^- conditions, respectively; Figure 12A). Nitrogen limitation was reached in the 5% CO_2 intermittent condition only 0.375 days later than for the 5% CO_2 2S and 5% CO_2 NR conditions (4 days after inoculation; Figure 12B). At this time the cell concentration was $2.32*10^7 \pm 1.09*10^7$ cells*mL^{-1}. The nitrogen removal rate of the intermittent 5% CO_2 condition was 0.80±0.02 mM*day^{-1}, significantly slower than the conditions that were provided continuous 5% CO_2 supplementation during the nitrogen replete growth stage ($p<0.001$ based un unpaired t-test), but significantly faster than the Air and 50 mM HCO_3^- conditions ($p<0.000$ for comparison with both conditions). The slower nitrogen removal rate when 5% CO_2 supplementation was inconsistent, relative to when 5% CO_2 supplementation was provided consistently, may be a result of cells attempting to deal with changing pH and DIC availability (speciation and concentration) during the photoperiod. It is suspected that a significant pH increase occurred between the 5 minute pulses of 5% CO_2 supplementation due to off gassing of CO_2 and consumption of inorganic carbon from the system. Following nitrogen limitation, cell growth slowed and stabilized at a final cell concentration of $1.35*10^8 \pm 1.85*10^7$ cells*mL^{-1}.

During nitrogen replete growth, $NaHCO_3^-$ supplementation prior to inoculation (50 mM HCO_3^- condition) resulted in a maximum specific cell growth rate (2.18±0.15 day^{-1}, observed between days 2 and 3 of the experiment) that was similar to all other carbon conditions except 5% CO_2 2S and NR, where a significantly slower max cell growth occurred (p=0.667, 0.026, and 0.790 for comparison with the Air, 5% CO_2 2S and NR, and 5% CO_2 intermittent conditions,

respectively; Figure 12A). The nitrogen removal rate was 0.65±0.03 $mM*day^{-1}$ and nitrogen limitation was reached most rapidly in this condition, three days after inoculation (Figure 12B). The cell concentration at nitrogen limitation was $3.47*10^7±3.76*10^6$ $cells*mL^{-1}$. Following nitrogen limitation, cell growth slowed considerably, with average cell growth during the nitrogen deplete growth stage of 0.03±0.13 day^{-1}. The final cell concentration at the end of the 2-stage growth process was $4.28*10^7±1.43*10^7$ $cells*mL^{-1}$.

Nitrogen removal rates were significantly different for all conditions except the two conditions where 5% CO_2 supplementation to the air sparge was continuous during nitrogen replete growth (5% CO_2 2S and 5% CO_2 NR). As noted, these conditions were the same prior to nitrogen limitation, and their data are pooled for the nitrogen replete growth stage. The fastest nitrogen removal rate was observed for these conditions (0.92±0.04 $mM*day^{-1}$ for both conditions; $p<0.001$ for all comparisons). The slowest nitrogen removal rate was observed for the condition where no exogenous CO_2 supplementation was provided (Air; 0.44±0.00 $mM*day^{-1}$; $p<0.001$ for all comparisons). Despite having the slowest nitrogen removal rate, this condition exhibited the highest cell growth rate (2.36±0.24 day^{-1}), significant only with respect to the condition that received continuous 5% CO_2 supplementation prior to nitrogen limitation (5% CO_2 NR; $p=0.001$). The high cell growth rate for the air condition, where carbon was limited, suggests that cell replication is more dependent on nitrogen availability than carbon availability. Further, it is suspected that increased cell replication may be response for coping with limited carbon availability. For the condition that recieved continuous 5% CO_2 supplementation following nitrogen limitation (5% CO_2 2S) the maximum specific cell growth rate, observed after nitrogen limitation, was not significantly different than the growth rate observed for the

condition that was only provided air as a source of CO_2. The 5% CO_2 2S condition was the only condition where the maximum specific cell growth rate was observed following nitrogen limitation. A comparison of average cell growth rates following nitrogen limitation initially suggests that CO_2 supplementation during nitrogen deplete growth promotes continued cell division, as the highest average growth rates observed during this growth stage were for the conditions that received either continuous or intermittent 5% CO_2 supplementation to the air sparge for the duration of the study (0.79 ± 0.06 day^{-1} and 0.65 ± 0.05 day^{-1} for the 5% CO_2 2S and 5% CO_2 intermittent conditions, respectively). However, when system pH is compared for the different conditions (Figure 13B), it seems likely that the actual cause of the differences observed in cell growth following nitrogen limitation is HCO_3^- becoming more dominant in the conditions that were not supplemented with CO_2 during this growth stage and reducing cell replication, as described by Li et al. (2018). The growth rates for the 5% CO_2 2S and 5% CO_2 intermittent conditions during nitrogen deplete growth were not significantly different from one another ($p=0.214$), but significantly greater than the other inorganic carbon conditions ($p<0.001$, 0.001, and <0.001 for the 5% CO_2 2S condition with respect to the Air, 5% CO_2 NR, and 50mM HCO_3^- conditions, respectively; $p<0.001$, 0.009, and <0.001 for the 5% CO_2 intermittent condition with respect to the Air, 5% CO_2 NR, and 50mM HCO_3^- conditions, respectively).

Media pH and DIC – Inorganic Carbon Supplementation

The direct impact of the evaluated inorganic carbon regimes is best summarized by solution DIC and pH for the different conditions (Figure 13). For the control condition (Air), where no concentrated inorganic carbon supplementation was provided, DIC concentrations increased slightly for the first three days of cultivation, reaching a maximum concentration of

2.88±1.58 mM, before dropping below the quantification limit of the method used (0.100 to 100.0 mM). The initial increase in DIC observed is likely driven by OH^- release during assimilation of nitrogen from NO_3^- causing an increase in system pH, and thus alkalinity. It is suspected that during the first three days of growth, the increase in alkalinity from nitrogen assimilation increased the mass transfer of CO_2 into solution above the rate of inorganic carbon consumption by the algal biomass. After day three, the demand for inorganic carbon from the growing biomass surpassed the ability of the system to regenerate inorganic carbon in solution. After the DIC concentration for this condition dropped below quantification limits, algal growth likely became carbon limited as reflected by the decline in cell growth rates after day three (Figure 12A). The pH for this condition continued to increase for this condition until nitrogen limitation (Figure 13B), reflecting the continued assimilation of nitrogen from NO_3^-. Following nitrogen limitation on day eight of the study, pH stabilized at 11.1±0.11.

The concentration of DIC for the 5% CO_2 intermittent condition increased during nitrogen replete growth (Figure 13A). The rate of increase was similar to the condition that was provided just air until day three of the study. After day three, the DIC concentration continued to increase for one day (until nitrogen limitation) before slowly decreasing. DIC concentrations for the intermittent 5% CO_2 condition remained above the quantification limits (0.100 to 100.0 mM) of the method used for the duration of the study, with an average DIC concentration during nitrogen deplete growth of 3.35±0.15 mM, approximately equal to the concentration of NO_3^- removed from the system. The pH for this condition increased during nitrogen replete growth, and then stabilized at an average pH 8.21±0.16.

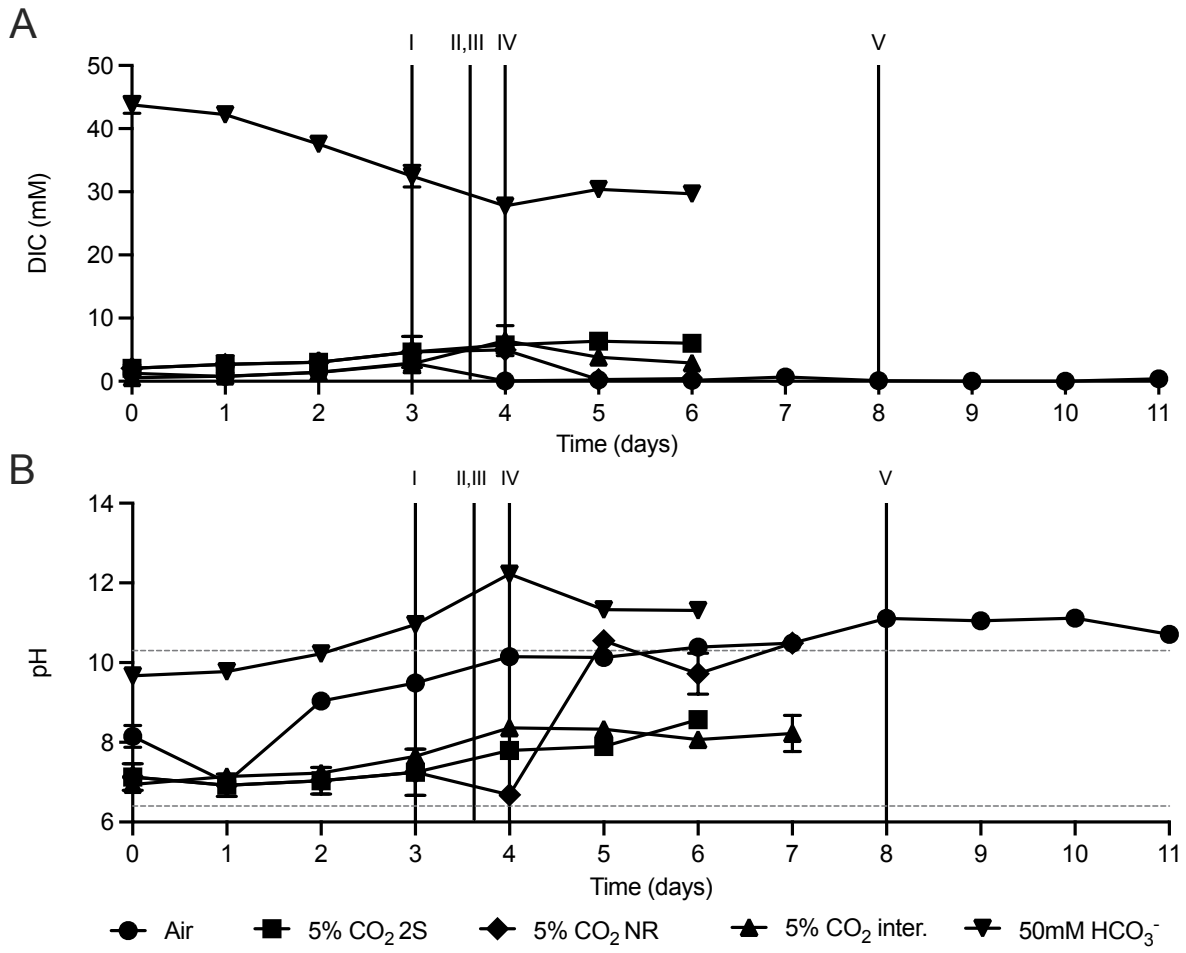

Figure 13: (A) Growth media DIC (mM) and (B) pH for inorganic carbon conditions as a function of time. Vertical lines represent the times of nitrogen limitation for the different conditions as designated by (I) 50mM HCO_3^-, (II) 5% CO_2 2S, (III) 5% CO_2 NR, (IV) 5% CO_2 intermittent, and (V) Air. Horizontal grey dashed lines in the pH plot identify the upper and lower pK_A of HCO_3^- (pH 10.3 and 6.4, respectively). Error bars are one standard deviation of data. During nitrogen replete growth a gradual increase in pH was observed for all conditions. For the 50mM HCO_3^- condition the pH increased far above pH 10.3, suggesting DIC speciation was dominated by CO_3^{2-} during nitrogen deplete growth for this condition. A rapid increase in pH was observed following nitrogen limitation for the condition where 5% CO_2 supplementation was provided to the air sparge only prior to nitrogen limitation (5% CO_2 NR), but was not observed in the other conditions. DIC concentrations gradually rose during nitrogen replete growth in all conditions except the 50mM HCO_3^- condition, where DIC concentrations slowly declined to 30mM at nitrogen limitation, and then leveled off during nitrogen deplete growth. In the conditions without HCO_3^- present a rapid decrease in DIC was observed following nitrogen limitation if there was no CO_2 supplementation provided during the nitrogen deplete growth phase.

For the two conditions that received continuous 5% CO_2 supplementation during nitrogen replete growth (5% CO_2 2S and 5% CO_2 NR), DIC concentrations increased steadily until nitrogen limitation due to the mass transfer of inorganic carbon into solution outpacing carbon consumption by the biomass. The pH in these systems also slowly increased during nitrogen replete growth. Following nitrogen limitation, the 5% CO_2 supplementation was stopped for the 5% CO_2 NR condition, resulting in a rapid decrease in DIC concentration to below quantifiable levels (<0.100 mM). The rapid decrease in DIC observed in this condition suggests that carbon was limited during nitrogen deplete growth. The pH for this condition increased rapidly following nitrogen limitation as mass transfer of CO_2 from the unamended air was no longer enough to compensate for algal consumption of inorganic carbon from solution. The concentration of DIC in the 5% CO_2 2S condition, where 5% CO_2 supplementation was continued for the duration of the nitrogen deplete growth phase, stabilized at 6.06±0.04 mM following nitrogen limitation, and did not decrease. The pH for this condition continued to increase during the nitrogen deplete growth phase, reaching a final pH of 8.57±0.18.

The 50mM HCO_3^- condition exhibited a significantly higher pH and DIC concentration than the other conditions throughout the 2-stage growth process. The pH for this condition increased to around pH 10.3 after 48 hours, then continued to increase to almost pH 12 over the next 48 hours, before leveling off around pH 11.3. As the system pH rose above pH 10.3 CO_3^{2-} became increasingly the more dominant form of inorganic carbon present in the growth medium, potentially limiting carbon that was metabolically available to the culture. The pH increase observed during the first 4 days of growth in this condition is believed to be a result of insufficient inorganic carbon availability in the air sparge to maintain carbonate alkalinity added

as $NaHCO_3$, meet inorganic carbon consumption by the algae, and balance additional OH^- generation during assimilation of nitrogen from NO_3^-.

The presence (and increase) of quantifiable DIC during nitrogen replete growth for all conditions except the control (Air) and 5% CO_2 NR (where CO_2 supplementation was stopped at nitrogen limitation) conditions suggests that sufficient inorganic carbon was available for growth during this stage. During nitrogen deplete growth DIC concentrations in the Air and 5% CO_2 NR conditions are suspected to have become carbon limited due to the observed reduction in DIC below quantifiable levels (<0.100 mM). Since the only driving force for increased alkalinity in these systems was hydroxyl ion generation from nitrogen assimilation, and the initial concentration of nitrogen was similar for all conditions, the mass transfer of inorganic carbon into the growth medium for the Air and 5% CO_2 NR conditions is suspected to be similar after day four, when CO_2 supplementation to the 5% CO_2 NR condition was stopped. As a result, the average pH between day four and seven of the study was similar (pH 10.2±0.09 and 10.3±0.17; p=0.826 based on unpaired t-test).

Biomass Generation – Inorganic Carbon Supplementation

At nitrogen limitation the biomass concentration for the Air condition was significantly greater than for conditions supplemented with inorganic carbon (0.62±0.01, 0.44±0.04, 0.44±0.04, 0.40±0.02, and 0.37±0.02 $g*L^{-1}$ for the Air, 5% CO_2 2S, 5% CO_2 NR, 5% CO_2 intermittent, and 50 mM HCO_3^- conditions, respectively; $p<0.001$ for the Air condition compared to all other inorganic carbon conditions, Figure 14A). This is suspected to be related to the longer duration of the nitrogen replete growth stage for this condition resulting from the slower nitrogen removal, which suggests biomass generation during the nitrogen replete growth phase is more

strongly controlled by cultivation time (which is a function of nitrogen removal rate) than inorganic carbon availability. Inorganic carbon speciation also seems to have influenced biomass generation, with conditions where CO_2 supplementation was provided, rather than $NaHCO_3$, exhibiting higher biomass concentrations at the time of nitrogen limitation.

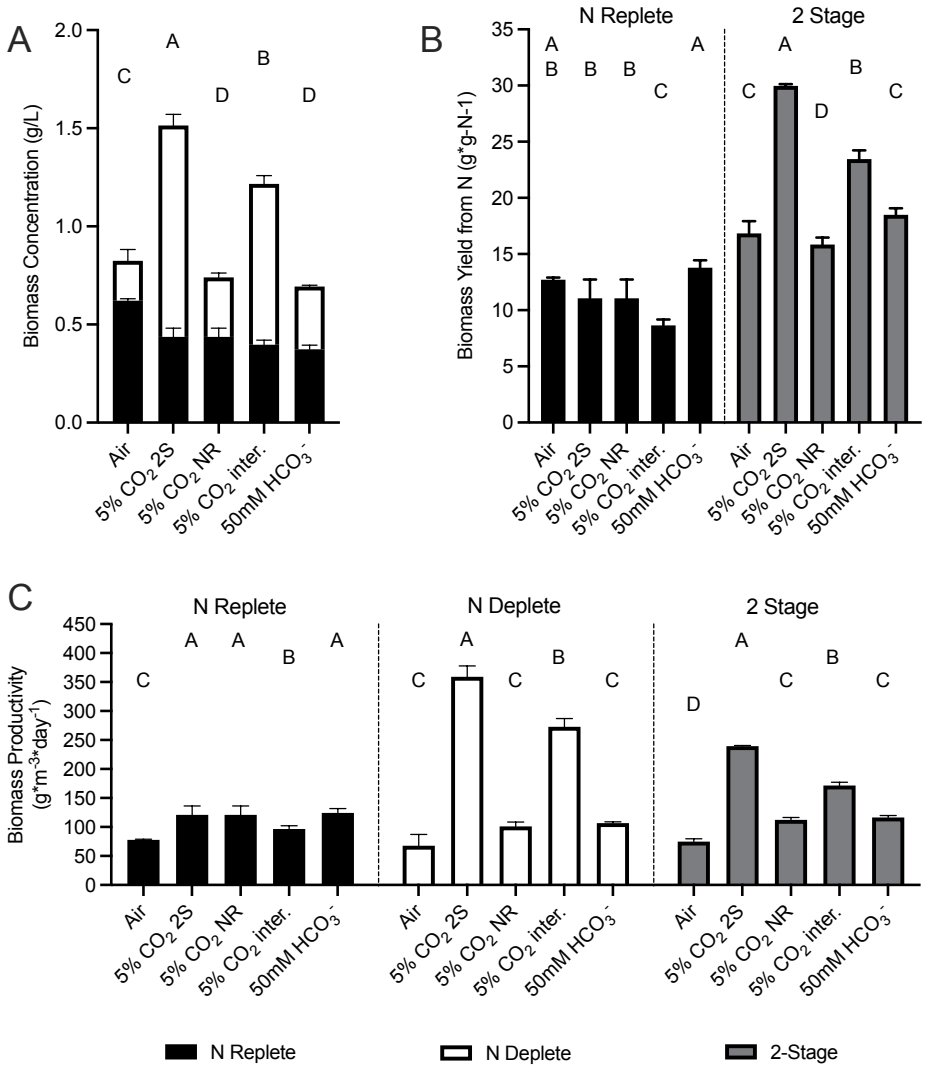

Figure 14: (A) Amount of biomass generated ($g*L^{-1}$), (B) biomass yield from nitrogen as $g_{biomass}*g_{nitrogen}^{-1}$, and (C) biomass productivity as $g*m^{-3}*day^{-1}$ for each of the inorganic carbon conditions. Biomass generated and biomass productivity are presented for nitrogen replete, nitrogen deplete, and 2-stage growth (combination of nitrogen replete and deplete bars for A). Biomass yield is presented for nitrogen replete and 2-stage growth. Error bars are one standard

deviation of data. Conditions with continued inorganic carbon supplementation following nitrogen limitation (5% CO_2 2S and 5% CO_2 intermittent) showed an increase in biomass concentration, biomass productivity, and biomass yield during the nitrogen deplete growth phase.

During nitrogen replete growth, biomass yield from nitrogen was significantly lower for the 5% CO_2 intermittent condition than the other inorganic carbon conditions (12.71±0.21, 11.05±1.68, 11.05±1.68, 8.65±0.52, and 13.78±0.67 $g_{biomass}*g_N^{-1}$ for the Air, 5% CO_2 2S, 5% CO_2 NR, 5% CO_2 intermittent, and 50 mM HCO_3^- conditions, respectively; $p<0.001$, $=0.001$, 0.010, and <0.001 for comparison of the 5% CO_2 intermittent condition with the Air, 5% CO_2 2S, 5% CO_2 NR, and 50 mM HCO_3^- conditions, respectively; Figure 14B). The reduced yield for the intermittent 5% CO_2 condition is suspected to be related to its inconsistent availability of inorganic carbon. The 50mM HCO_3^- condition had the highest biomass yield from nitrogen, significant relative to all conditions except for the Air condition ($p=0.054$, 0.004, 0.004, and <0.001 comparing biomass yield from nitrogen for the 50mM HCO_3^- condition with the Air, 5% CO_2 2S, 5% CO_2 NR, and 5% CO_2 intermittent conditions, respectively). The higher biomass yield for nitrogen observed for the 50 mM HCO_3^- condition, and to a lesser extent the Air condition, is suspected to be driven by inorganic carbon speciation, with HCO_3^- as the dominant inorganic carbon form present. Due to the high pH of the condition supplemented with only air ($pH>8.3$ for all time points after day two), relative to the conditions supplemented with CO_2, the dominant form of inorganic carbon was also HCO_3^-, although at a significantly lower concentration than was present in the 50 mM HCO_3^- condition. The high biomass yield from nitrogen for the Air condition may also be a result of the longer length of the nitrogen replete growth phase for this condition.

Biomass productivity during nitrogen replete growth was strongly influenced by the availability of inorganic carbon, with the HCO_3^- condition and the conditions receiving a continuous supply of CO_2 during nitrogen replete growth having the highest biomass productivities (77.7±1.25, 121±15.35, 96.4±5.64, and 124±7.17 $g*m^{-3}*day^{-1}$ for the Air, 5% CO_2 2S (and 5% CO_2 NR), 5% CO_2 intermittent, and 50 mM HCO_3^- conditions, respectively; $p<0.001$ for comparison of the 50 mM HCO_3^- condition with the Air and 5% CO_2 intermittent conditions; $p<0.001$ and $p=0.004$ for the 5% CO_2 2S condition compared to the Air and 5% CO_2 intermittent conditions, respectively; $p<0.001$ and $p=0.004$ for the 5% CO_2 NR condition compared to the Air and 5% CO_2 intermittent conditions, respectively Figure 14C).

During nitrogen deplete growth the 5% CO_2 2S condition generated significantly more biomass than the other inorganic carbon conditions (0.20±0.06, 1.08±0.06, 0.30±0.02, 0.82±0.04, and 0.32±0.01 $g*L^{-1}$ for the Air, 5% CO_2 2S, 5% CO_2 NR, 5% CO_2 intermittent, and 50 mM HCO_3^- conditions, respectively; $p<0.001$ for the comparison of biomass generation in the 5% CO_2 condition when compared with all other inorganic carbon conditions). The 5% CO_2 intermittent condition also generated more biomass than the other conditions, only being outperformed by the 5% CO_2 2S condition ($p<0.001$ for comparison of the intermittent 5% CO_2 condition with the Air, 5% CO_2 NR, and 50 mM HCO_3^- conditions). There were no significant differences observed for the biomass concentrations generated during nitrogen deplete growth for the Air, 5% CO_2 NR, and 50mM HCO_3^- conditions. The lower biomass generation observed for the Air and 5% CO_2 NR conditions is suspected to be a result of insufficient carbon availability. For the HCO_3^- condition lower biomass concentrations are suspected to be a result of inorganic carbon speciation and/or impact of high pH on cell physiology. Due to the high pH

of the 50 mM HCO_3^- condition during nitrogen deplete growth (pH>10.3), speciation of inorganic carbon in solution is dominated by CO_3^{2-}, followed by HCO_3^-. Biomass productivity during nitrogen deplete growth was proportional to the biomass concentration generated since the duration of nitrogen deplete growth was same for all conditions.

Biomass concentration, biomass productivity, and biomass yield from nitrogen were all greatest for the conditions that were supplemented with 5% CO_2 during nitrogen deplete growth (5% CO_2 2S and 5% CO_2 intermittent), with the continuous supply of 5% CO_2 outperforming the 5% CO_2 intermittent condition for all three parameters. The majority of biomass generated for these conditions was produced during nitrogen deplete growth. Despite having a relatively high concentration of inorganic carbon during nitrogen deplete growth, the 50mM HCO_3^- condition did not exhibit an increase in biomass generated, productivity, or yield from nitrogen during the 2-stage growth process. This may result from the gradual shift in CO_3^{2-}/HCO_3^- equilibrium toward CO_3^{2-} caused by the increase in pH observed for this condition, or the impact of the high pH on cell physiology.

Cell Growth and Nitrogen Removal – Glucose Supplementation

The impacts of glucose supplementation on cell growth (Figure 15A-D) were dependent on inorganic carbon availability (speciation and concentration) and culture pH. Greater cell concentrations were observed at the end of the nitrogen replete growth stage as a result of glucose supplementation for all conditions except the 5% CO_2 2S condition. For conditions where greater cell concentrations were observed at nitrogen limitation, the improvement in growth was largely restricted to the 24 hours preceding nitrogen limitation. This might suggest a relationship between nitrogen availability and organic carbon utilization. For the Air condition,

the greater cell concentration observed at the end of nitrogen replete growth resulting from glucose supplementation only lasted for one day during nitrogen deplete growth before the cell concentration for the Air condition without glucose supplementation was able to catch up. For the 5% CO_2 intermittent and 50 mM HCO_3^- conditions glucose supplementation increased cell growth throughout the 2-stage cultivation process. For the 5% CO_2 2S condition, the addition of glucose did not impact cell growth during the nitrogen replete growth phase but resulted in a notable decrease in cell growth during nitrogen deplete growth. This supports previous research where an inhibitory effect of excess CO_2 (5% CO_2 augmentation to air sparge) on the ability of algae to utilize organic carbon was observed (Sforza et al., 2012). Importantly, Sforza et al. (2012) observed improved growth during mixotrophic cultivation when the air sparge contained atmospheric concentrations of CO_2. During the current study, the different impacts of glucose supplementation on the 5% CO_2 2S and 5% CO_2 intermittent conditions support these previous findings.

Impacts of glucose supplementation on nitrogen removal were limited for conditions where inorganic carbon was supplemented, however more rapid removal was achieved in the condition where the only source of inorganic carbon was the unamended air sparge (air). For conditions with augmented inorganic carbon availability the primary impact of glucose supplementation on nitrogen removal was more rapid removal of trace amounts of nitrogen remaining at the time of nitrogen limitation. This was observed for the 5% CO_2 intermittent and 50 mM HCO_3^- conditions, but not the 5% CO_2 2S condition.

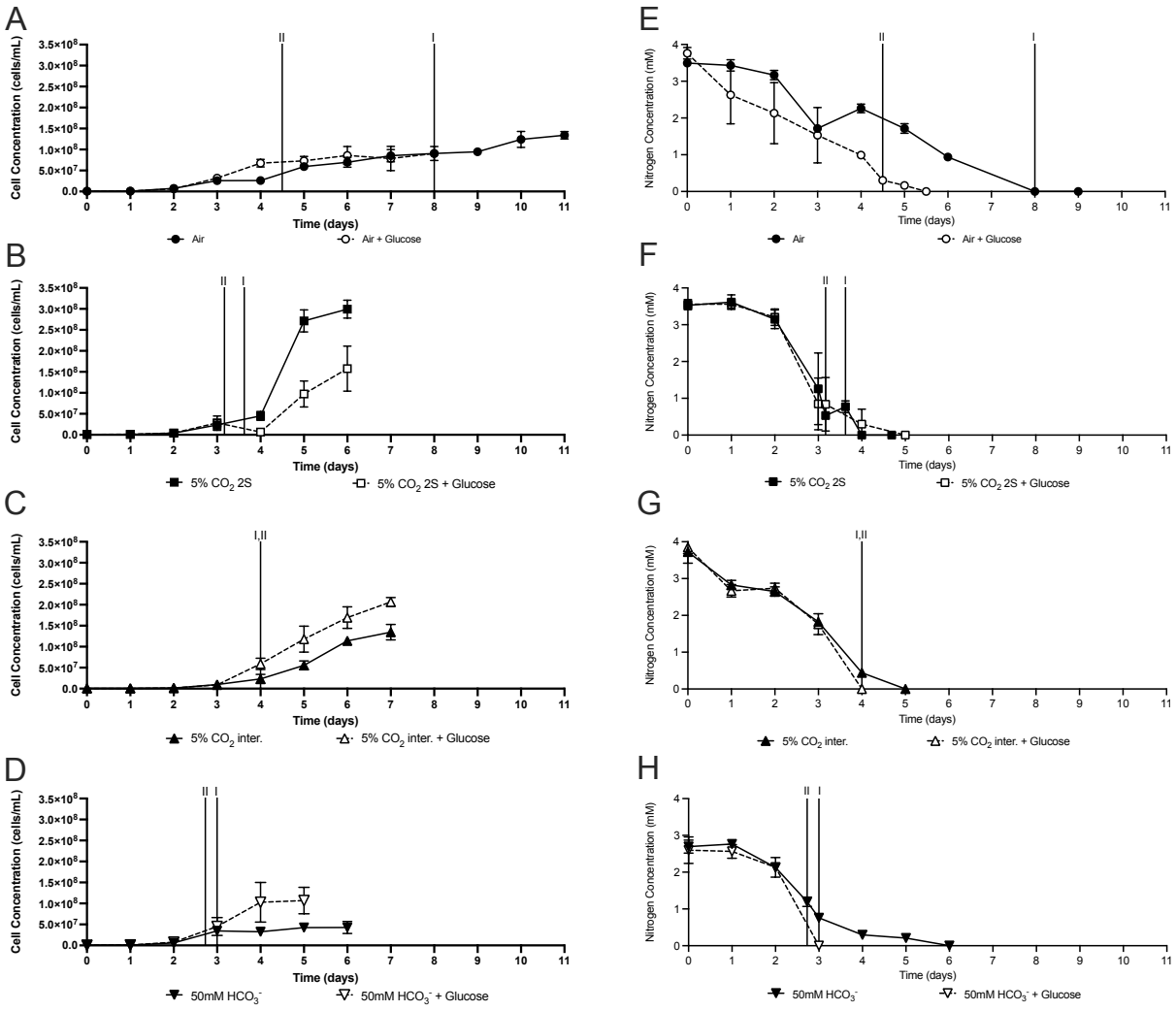

Figure 15: (A-D) Cell and (E-H) nitrogen concentration as a function of time for each of the inorganic carbon conditions with (empty symbols, dashed lines) and without (solid symbols, solid lines) glucose supplementation to the growth medium prior to inoculation. Cell concentration is presented on a linear vertical axis in order to make variations in cell growth resulting from glucose supplementation more apparent. Vertical lines in each plot identify the time of nitrogen limitation for each condition with (II) and without (I) glucose supplementation. Error bars are one standard deviation of data. Glucose supplementation caused a decrease in cell growth for the 5% CO_2 2S condition, particularly during the nitrogen deplete growth phase. In contrast, glucose amended conditions that did not receive CO_2 supplementation (Air + glucose and 50mM HCO_3^- + glucose) or that were only provided CO_2 supplementation intermittently (5% CO_2 inter. + glucose) exhibited an increase in cell growth relative to the same conditions without glucose amendment. For the Air + glucose condition the increase in cell growth was only observed around nitrogen limitation, with final cell concentrations being similar with and without glucose supplementation. Glucose supplementation had little impact on nitrogen removal in conditions where inorganic carbon was supplemented as CO_2 or HCO_3^-, however for the

condition where no exogenous inorganic carbon supplementation occurred (Air), the addition of glucose caused a notable increase in the nitrogen removal rate.

Media pH and DIC – Glucose Supplementation

Generally, glucose supplementation did not impact pH or DIC concentration (Figure 16), however the small changes in pH observed in the Air + glucose and 50mM HCO_3^- + glucose conditions may be significant due to the proximity of the pH for these conditions to the upper pK_A of HCO_3^-. The slight pH shift observed in these conditions may have had a more pronounced impact on HCO_3^-/CO_3^{2-} equilibrium, which in turn impacts the metabolic availability of the inorganic carbon pool present. An increase in DIC concentration for the Air and 50mM HCO_3^- conditions was observed when glucose was added to the growth medium, however it is likely that only the Air condition experienced an increase in DIC availability as a result of the glucose supplementation. The higher DIC concentrations observed for the 50mM HCO_3^- condition when glucose supplementation was performed is suspected to have resulted from excess $NaHCO_3$ being added to the system.

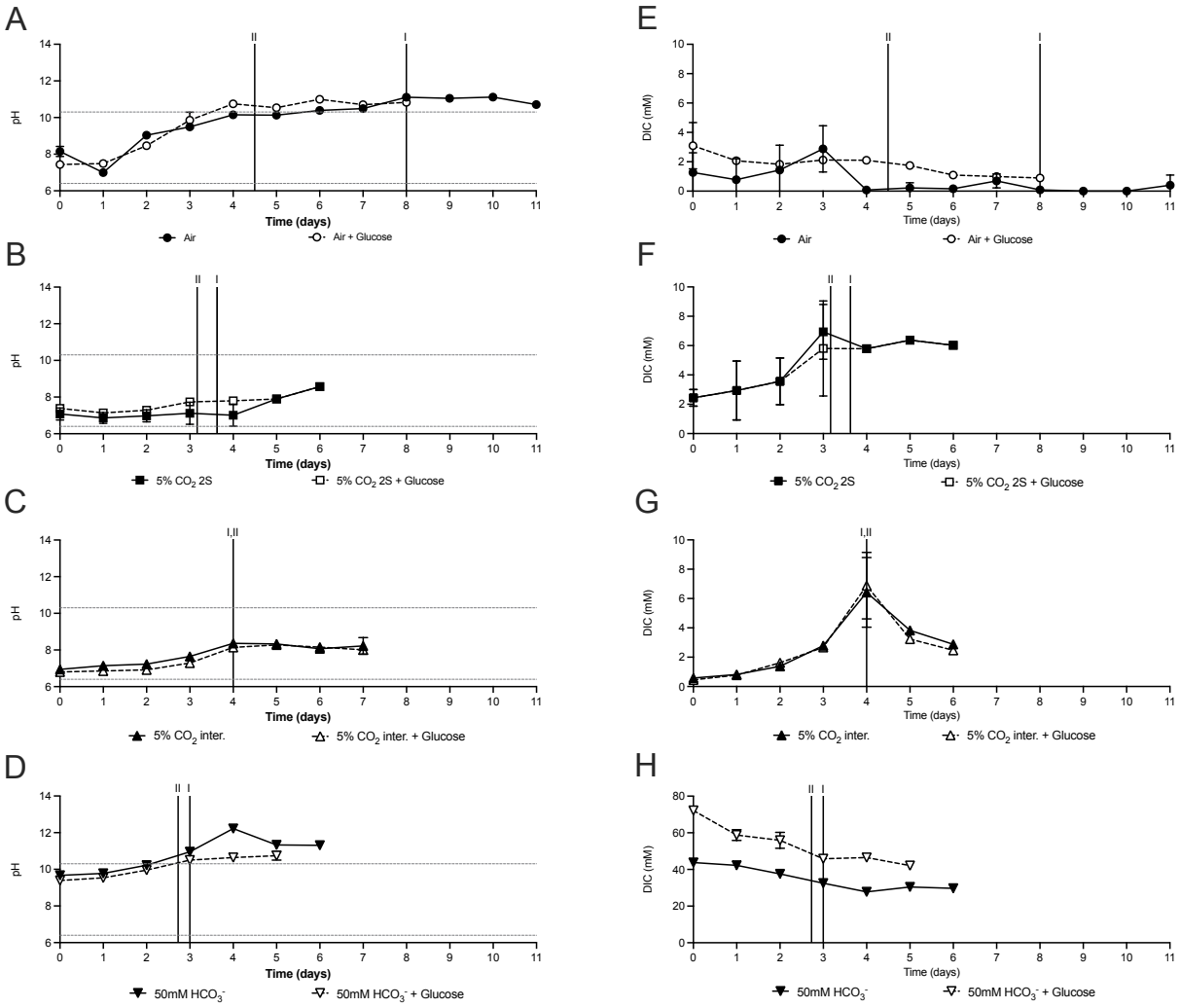

Figure 16: Growth media (A-D) pH and (E-H) DIC for glucose amended (empty symbols, dashed lines) and unamended (Solid symbols, solid lines) inorganic carbon conditions. Vertical lines in each plot identify the time of nitrogen limitation for each condition with (II) and without (I) glucose supplementation. Horizontal grey dashed lines in the pH plots identify the upper and lower pK_A of HCO_3^- (pH 10.3 and 6.4, respectively). DIC concentration for the 50mM HCO_3^- condition is plotted using a vertical axis with a larger range than the other conditions due to the significantly higher DIC concentrations observed. Error bars are one standard deviation of data. Growth medium pH was not significantly impacted by glucose supplementation for any of the inorganic carbon conditions. When glucose supplementation was provided to the condition without exogenous CO_2 inputs (air + glucose) a general increase in DIC was observed, potentially resulting from increased respiration. An increase in DIC was also observed for the 50mM HCO_3^- + glucose condition, however this is a result of excess $NaHCO_3$ added to the growth medium prior to inoculation for this condition relative to its counterpart that was not amended with glucose.

Biomass Generation – Glucose Supplementation

During nitrogen replete growth improved biomass generation was observed for the 5% CO_2 intermittent and 50 mM HCO_3^- conditions when glucose was supplemented (0.40±0.02 and 0.48±0.04 g*L^{-1} for the 5% CO intermittent and 5% CO intermittent + glucose conditions, respectively; 0.37±0.02 and 0.65±0.04 g*L^{-1} for the 50mM HCO_3^- and 50mM HCO_3^- + glucose conditions, respectively; p=0.002 and 0.007 for intermittent 5% CO_2 and 50mM HCO_3^-, respectively, based on unpaired t-tests; Figure 17). The increased biomass concentration observed when glucose supplementation was provided for the 50mM HCO_3^- condition may result from the higher concentration of DIC present in this condition, rather than glucose supplementation. The initial DIC concentrations for the 50 mM HCO_3^- and 50 mM HCO_3^- + glucose conditions were 43.8±1.34 and 72.1±1.58 mM, respectively. The excess DIC was added as $NaHCO_3$, and the error likely occurred during media preparation. For the Air condition, glucose supplementation caused a reduction in the amount of biomass generated during the nitrogen replete growth phase (0.62±0.01 and 0.57±0.01 g*L^{-1} for the Air and Air + glucose conditions, respectively; p=0.015), despite causing an increase in the rates of nitrogen removal and cell growth during the nitrogen replete growth stage. The reduction in biomass might be related to light inhibition of respiration during the photoperiod (da Silva & Fonseca, 2020). During the nitrogen deplete growth stage glucose supplementation had no significant impacts on biomass generated for any of the inorganic carbon conditions. For 2-stage cultivation the 50 mM HCO_3^- condition generated significantly more biomass when glucose was added to the growth medium (0.70±0.02 and 1.01±0.09 g*L^{-1} for the 50mM HCO_3^- and 50mM HCO_3^- + glucose conditions, respectively; p=0.023). When the inorganic carbon conditions, with and without

glucose supplementation, are all compared during 2-stage cultivation, the highest biomass concentrations were observed in the 5% CO_2 2S condition without glucose supplementation and the 5% CO_2 intermittent condition with glucose. The increased biomass generated in the 5% CO_2 intermittent condition with glucose was not significant relative to the 5% CO_2 intermittent condition without glucose ($p=0.651$) or the 5% CO_2 2S condition with glucose ($p=0.122$). Due to the low glucose concentration used during the present study it is possible that glucose was largely removed from the growth medium prior to the nitrogen deplete growth stage, explaining the lack of response during this stage. In previous work with *C. sorokiniana*, strain SLA-04, where high-alkalinity mixotrophic cultivation was evaluated using a glucose concentration of 4 $g*L^{-1}$, a significant increase in biomass production and nitrogen removal rate were observed relative to high-alkalinity phototrophic conditions (Vadlamani et al., 2017). In another study evaluating the impact of mixotrophic cultivation on a freshwater isolate of *C. sorokiniana*, strain CCTCC M209220, biomass production was improved at glucose concentrations between 5 and 25 $g*L^{-1}$, relative to autotrophic cultivation, with the highest biomass concentrations being generated when 15 $g*L^{-1}$ glucose was added to the growth medium (Wan et al., 2011).

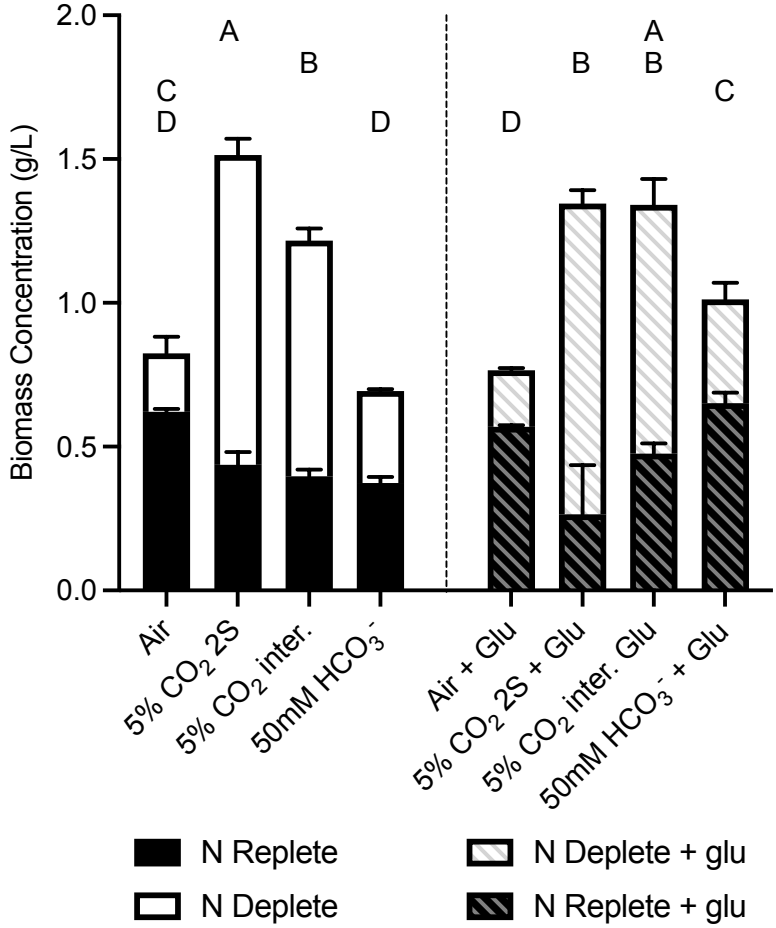

Figure 17: Biomass generated ($g*L^{-1}$) during nitrogen replete (bottom bars) and nitrogen deplete (top bars) growth for the inorganic carbon (left of dashed line) and glucose amended inorganic carbon (right of dashed line) conditions. Total biomass generated during the 2-stage growth process is equal to the combination of biomass generated independently in the two growth stages. Letters above individual bars indicated significance of differences in biomass concentration generated during the 2-stage growth process, with conditions that are not significantly different sharing a common letter. Significance comparisons are for all eight conditions (glucose amended and unamended). Error bars are one standard deviation of data. Glucose supplementation reduced biomass generation for the 5% CO_2 2S condition (primarily during the nitrogen replete growth) but increased it for the 50mM HCO_3^- conditions.

During nitrogen replete growth biomass productivity was improved when glucose was added to the growth medium for the Air, 5% CO_2 intermittent, and 50mM HCO_3^- conditions (77.7±1.25 and 114±1.01 $g*m^{-3}*day^{-1}$ for Air, $p<0.001$; 96.4±5.64 and 191±14.2 $g*m^{-3}*day^{-1}$ for

5% CO_2 intermittent, $p<0.001$; 125±7.17 and 163±9.30 $g*m^{-3}*day^{-1}$ for 50 mM HCO_3^-, $p=0.008$), but not for the 5% CO_2 2S condition (121±15.3 and 83.6±54.0 $g*m^{-3}*day^{-1}$ for Air, $p=0.158$; Figure 18A). The improved productivity observed for the glucose supplemented 50 mM HCO_3^- condition may result from the higher concentration of DIC added to this condition, as discussed previously. When the four glucose supplemented conditions are compared, biomass productivity for the Air + Glucose condition was significantly greater than the other conditions ($p<0.001$, $p=0.015$, and $p=0.046$ when compared with the Air, 5% CO_2 2S, and 50 mM HCO_3^- conditions, respectively). When all eight conditions (glucose amended and unamended condition) are compared, biomass productivity during nitrogen replete growth was greatest for the glucose supplemented intermittent 5% CO_2 and 50 mM HCO_3^- conditions. The improved productivity observed for the glucose supplemented 50 mM HCO_3^- condition may result from the higher DIC concentration present, as discussed previously. During nitrogen deplete growth there was no significant impact on biomass productivity resulting from glucose supplementation for any of the inorganic carbon conditions. This is potentially a result of the complete removal of glucose prior to nitrogen limitation. As discussed previously, the concentration of glucose used during the current study, 0.725 $mg*L^{-1}$, was lower than the concentration used in previous work with SLA-04, 4 $g*L^{-1}$ (Vadlamani et al., 2017), and much lower than the optimal concentration identified for the freshwater C. sorokiniana, strain CCTCC M209220, 15 $g*L^{-1}$ (Wan et al., 2011). Biomass productivity during nitrogen deplete growth was proportional to biomass concentration generated during this growth phase since the duration of the nitrogen deplete growth stage was the same for all conditions (three days).

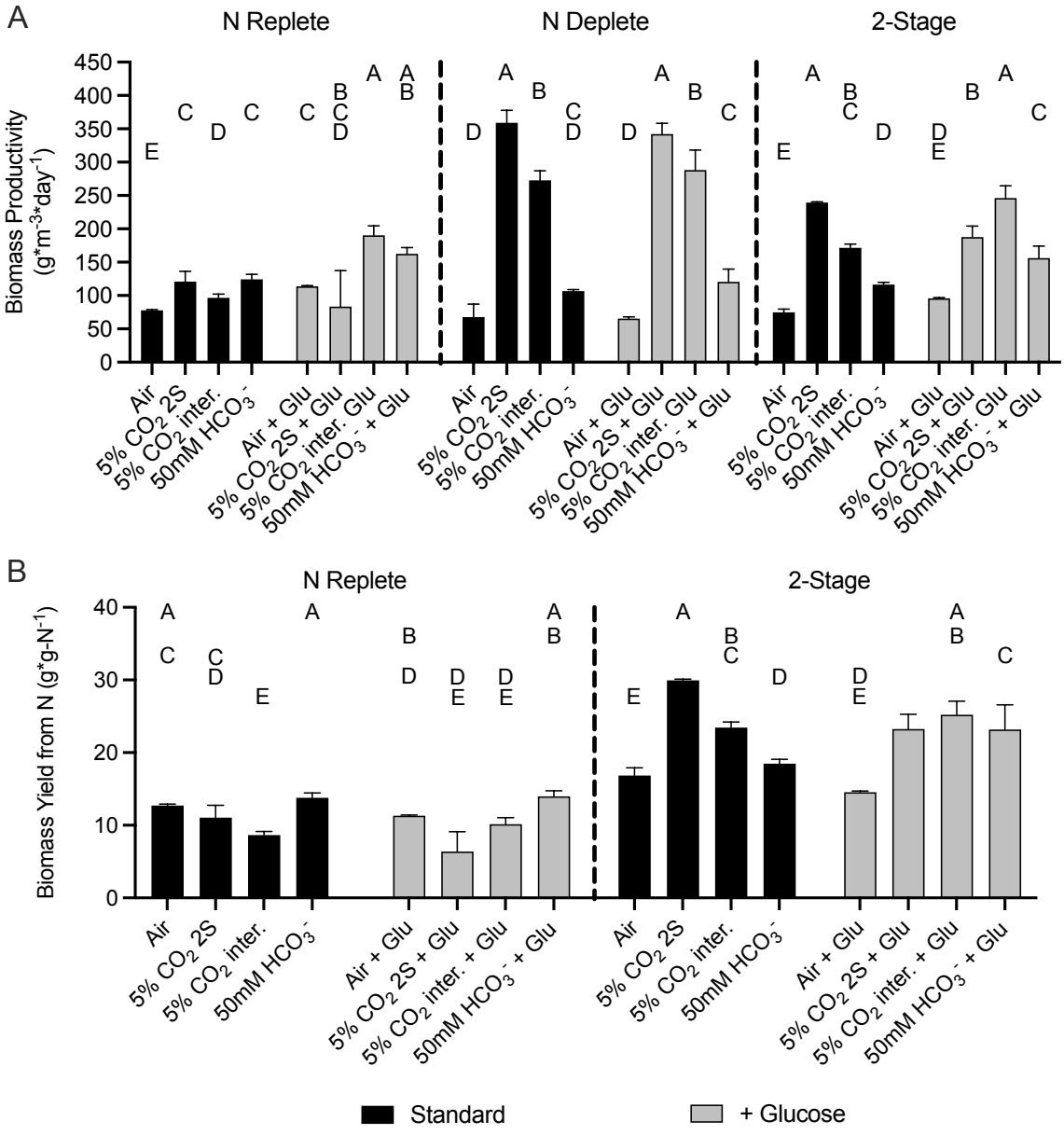

Figure 18: Biomass (A) productivity and (B) yield from nitrogen for each of the inorganic carbon conditions with (grey bars), and without (black bars) glucose supplementation. Biomass productivity is presented for nitrogen replete, nitrogen deplete, and 2-stage cultivation. Error bars are one standard deviation of data. Statistical significance for comparisons for all standard and glucose amended conditions for each growth stage independently are presented using letters above each column. For clarification, significance is not shown for comparisons between growth stages. Conditions that share a common letter are not significantly different. Impacts on biomass productivity from the addition of glucose to the growth medium were primarily observed during nitrogen replete growth, with all conditions except the 5% CO_2 2S condition having a higher productivity when glucose was present. For the 50 mM HCO_3^- + glucose condition the increased

productivity observed may result from the excess $NaHCO_3$ added to this condition prior to inoculation, as discussed previously. For the 5% CO_2 2S condition, the addition of glucose resulted in a decrease in biomass productivity during nitrogen replete growth.

For the 2-stage cultivation process, biomass productivity was improved by glucose supplementation for the Air and 50 mM HCO_3^- conditions (74.9±4.86 and 95.7±1.25 $g*m^{-3}*day^{-1}$ for Air without and with glucose supplementation, respectively; p=0.019; 116±366 and 156±17.9 $g*m^{-3}*day^{-1}$ for 50 mM HCO_3^- without and with glucose supplementation, respectively; p=0.009). Biomass productivity also increased for the 5% CO_2 intermittent condition, however the change was not significant (172±5.61 and 246±18.3 $g*m^{-3}*day^{-1}$ for 5% CO_2 intermittent without and with glucose supplementation, respectively; p=0.112). Biomass productivity was reduced for the 5% CO_2 2S condition during 2-stage cultivation when glucose was supplemented (239±1.29 and 188±16.4 $g*m^{-3}*day^{-1}$ for the 5% CO_2 2S without and with glucose supplementation, respectively; p=0.0.032). The increased productivity observed for the glucose supplemented 50 mM HCO_3^- condition, relative to the 50 mM HCO_3^- condition without glucose supplementation, may be caused by the additional DIC added to this condition, as discussed previously. The highest biomass productivities for the 2-stage cultivation process were observed in the 5% CO_2 2S and glucose supplemented 5% CO_2 intermittent conditions. Continued supplementation with CO_2 during nitrogen deplete growth had a greater impact on biomass productivity than glucose addition to the growth medium. The reduced productivity observed for the 5% CO_2 2S condition when glucose supplementation was provided is suspected to be due to a negative interaction between CO_2 and glucose metabolism, which has been reported previously (Sforza et al., 2012). For the intermittent 5% CO_2 condition, improved productivity relative to the 5% CO_2 2S condition is suspected to be caused by inorganic carbon speciation in the

intermittent condition shifting towards HCO_3^- due to the higher pH observed, and the lack of known interactions between glucose and HCO_3^- metabolism. Alternatively, the improved growth could be a result of the adaptations of SLA-04 to high-alkalinity conditions imposed by its native habitat. The lower pH for the 5% CO_2 2S condition relative to the 5% CO_2 intermittent conditions is a result of the consistent CO_2 supply. Since the 5% CO_2 2S and intermittent conditions are assumed to have similar alkalinities (same growth medium), the lower DIC concentration in the intermittent condition is offset by higher hydroxyl alkalinity and thus pH values.

Glucose supplementation caused a reduction in biomass yield from nitrogen for the Air and 5% CO_2 2S conditions during nitrogen replete growth (12.7±0.21 and 11.3±0.12 $g_{biomass}*g_N^{-1}$ for Air without and with glucose supplementation, respectively; p=0.002; 11.1±1.68 and 6.40±2.72 $g_{biomass}*g_N^{-1}$ $*day^{-1}$ for 5% CO_2 2S without and with glucose supplementation, respectively; p=0.007), however these conditions did not show a significant increase in yield from nitrogen during the nitrogen deplete growth phase. The reduced biomass yield observed for the Air condition is suspected to be a result of the shorter nitrogen replete growth stage, since time seems to play a significant role for growth under nitrogen replete conditions. Glucose supplementation did not impact biomass yield from nitrogen during 2-stage cultivation for the intermittent 5% CO_2 condition (23.5±0.77 and 25.2±1.87 $g_{biomass}*g_N^{-1}$ for 5% CO_2 intermittent without and with glucose supplementation, respectively; p=0.425), but resulted in increased yield for the 50 mM HCO_3^- condition (18.5±0.58 and 23.2±3.42 $g_{biomass}*g_N^{-1}$ for 50 mM HCO_3 without and with glucose supplementation, respectively; p=0.043. For the Air and 5% CO_2 2S conditions biomass yield from nitrogen was reduced due to glucose supplementation during 2-stage

cultivation (16.8±1.09 and 14.5±0.19 $g_{biomass}*g_N^{-1}$ for Air without and with glucose supplementation, respectively; p=0.070; 30.0±0.16 and 23.3±2.03 $g_{biomass}*g_N^{-1}$ for 5% CO_2 2S condition without and with glucose supplementation, respectively; p=0.029). For Air, the reduced yield from nitrogen resulting from glucose supplementation is likely related to the reduced time of the nitrogen replete growth phase. For the 5% CO_2 2S condition the reduced biomass yield from nitrogen resulting from glucose supplementation is likely related to the inorganic carbon speciation in the growth medium (excess CO_2 inhibition of glucose utilization; Sforza et al., 2012).

It is likely that bacterial contamination of reactors occurred during all experiments. Sampling was not performed using aseptic technique. Bacterial contamination is not suspected to have impacted growth for conditions that were not supplemented with glucose, however the availability of organic carbon likely increased promoted bacterial growth when glucose was supplemented. The long-term maintenance of large axenic cultures is not believed to be possible, limiting potential commercial applications.

Media Chemistry – Bicarbonate Amendment at Nitrogen Limitation

The addition of bicarbonate at nitrogen limitation resulted in an instantaneous increase in growth media DIC for all inorganic carbon conditions (Figure 19A). In addition, a shift in pH towards 8.3 (midpoint between pK_A1 and pK_A2 for the $CO_2/HCO_3^-/CO_3^{2-}$ system, where HCO_3^- is the dominant form of inorganic carbon in the system) was observed (Figure 19B). Conditions where supplementation with 5% CO_2 was provided during nitrogen deplete growth (5% CO_2 2S and intermittent conditions) stabilized around pH 8.3 as a result of sufficient CO_2 being supplemented to maintain alkalinity equilibrium. For the 50mM HCO_3 and 5% CO_2 NR

conditions the small concentration of CO_2 present in the air sparge during the nitrogen deplete growth phase was insufficient to replenish carbon used by the culture and the pH was higher than for the 5% CO2 sparged systems (Figure 19B).

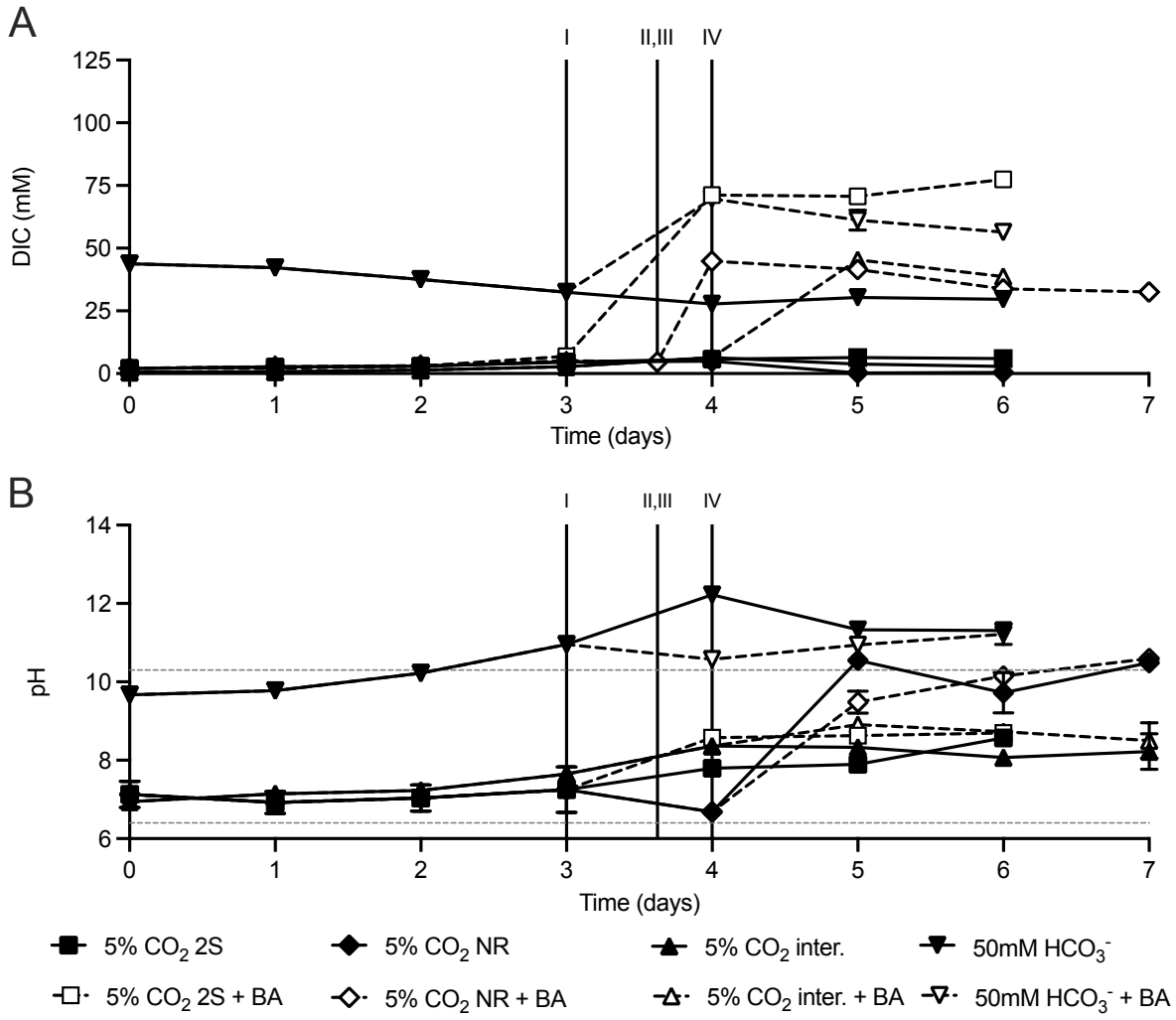

Figure 19: (A) DIC concentration (mM) and (B) pH for the inorganic carbon conditions with and without a bicarbonate amendment (BA) at nitrogen limitation. Vertical lines represent the times of nitrogen limitation for the different conditions as designated by (I) 50mM HCO_3^-, (II) 5% CO_2 2S, (III) 5% CO_2 NR, and (IV) 5% CO_2 intermittent. Horizonal grey dashed lines show the two equilibrium points for the inorganic carbon system (pH 6.4 for H_2CO_3/HCO_3^- and pH 10.3 for HCO_3^-/CO_3^{2-}). Error bars are one standard deviation of the data. The bicarbonate amendment at nitrogen limitation increased DIC concentration for all conditions and shifted pH towards pH 8.3 (midpoint between pK_A1 and pK_A2 for the $CO_2/HCO_3^-/CO_3^{2-}$ system, where HCO_3^- is the

dominant form of inorganic carbon in the system). For conditions where CO_2 was supplied during nitrogen deplete growth (5% CO_2 2S and 5% CO_2 intermittent), HCO_3^- was able to successfully buffer the system around pH 8.3 however, due to insufficient CO_2 provided in the air stream to maintain DIC, the 5% CO_2 NR and 50mM HCO_3^- conditions had a higher pH.

When glucose supplemented conditions were provided with a bicarbonate amendment at nitrogen limitation they responded similarly to the inorganic carbon conditions without glucose supplementation. An instantaneous increase in DIC concentration, along with a shift in pH towards 8.3 were observed (Figure 20). The lower pH observed for the 50 mM HCO_3^- + glucose condition, relative to the 50 mM HCO_3^- condition, is suspected to be related to excess $NaHCO_3$ unintentionally added to the system prior to inoculation.

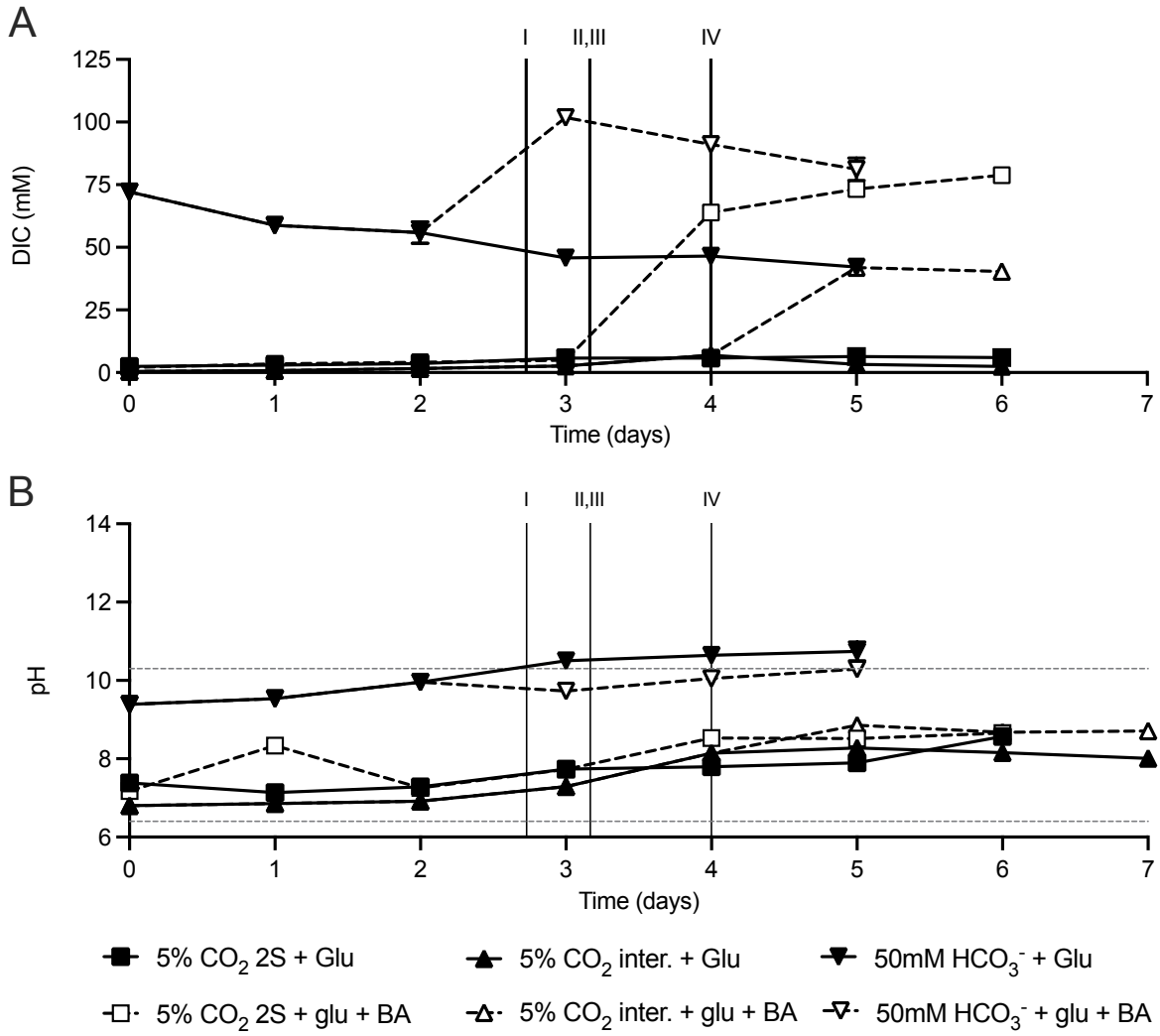

Figure 20: (A) DIC concentration (mM) and (B) pH for the glucose supplemented inorganic carbon conditions with and without a bicarbonate amendment (BA) at nitrogen limitation. Vertical lines represent the times of nitrogen limitation for the different conditions as designated by (I) 50mM HCO_3^-, (II) 5% CO_2 2S, (III) 5% CO_2 NR, and (IV) 5% CO_2 intermittent. Horizonal grey dashed lines show the two equilibrium points for the inorganic carbon system (pH 6.4 for H_2CO_3/HCO_3^- and pH 10.3 for HCO_3^-/CO_3^{2-}). Error bars are one standard deviation of the data. Error bars are smaller than symbol if not visible. Glucose supplementation to the growth medium did not show any apparent impact from the bicarbonate amendment at nitrogen limitation on pH or DIC trends for any of the conditions.

Biomass Generation – Bicarbonate Amendment at Nitrogen Limitation

For the inorganic carbon conditions without glucose supplementation, a bicarbonate amendment at nitrogen limitation resulted in a significant increase in the generation of biomass during nitrogen deplete growth for the 50mM HCO_3^- condition (0.32±0.01 and 0.63±0.15 $g*L^{-1}$ for the 50 mM HCO_3^- unamended and amended conditions, respectively; p=0.003). There was also an increase in the amount of biomass generated during nitrogen deplete growth for the 5% CO_2 NR condition as a result of providing a bicarbonate amendment at nitrogen limitation, however the observed increase was not significant (0.30±0.02 and 0.62±0.06 $g*L^{-1}$ for the unamended and amended 5% CO_2 NR conditions; p=0.091). For the two stage process, the bicarbonate amendment caused a reduction in the amount of biomass generated for the 5% CO_2 intermittent condition (1.22±0.04 and 1.10±0.04 $g*L^{-1}$ for the unamended and amended conditions, respectively; p=0.030). An increase in the amount of biomass generated during 2-stage growth was observed for the intermittent 5% CO_2 condition, however this increase was not significant (0.79±0.03 and 1.09±0.04 $g*L^{-1}$ for the unamended and amended conditions, respectively; p=0.074).

For the glucose supplemented inorganic carbon conditions there were no significant impacts on biomass concentration resulting from providing the bicarbonate amendment at nitrogen limitation. There was an increase in biomass concentration for the glucose supplemented 50mM HCO_3^- condition during 2-stage cultivation when the bicarbonate amendment was provided, but the increase was not significant (1.01±0.08 and 1.16±0.03 $g*L^{-1}$ for the unamended and amended conditions, respectively; p=0.100). In addition, the increased

biomass concentration for this condition may be a result of the higher concentration of DIC added to the glucose supplemented 50 mM HCO_3^- condition, as discussed previously.

When the 14 carbon conditions (inorganic carbon with and without glucose supplementation, with and without a bicarbonate amendment at nitrogen limitation) are compared during 2-stage cultivation (Figure 21) all conditions where 5% CO_2 supplementation was provided during nitrogen deplete growth, except for the intermittent 5% CO_2 condition with a bicarbonate amendment, generated more biomass than the conditions without CO_2 supplementation following nitrogen limitation. Only the bicarbonate amended, glucose supplemented 50 mM HCO_3^- condition generated more biomass than the bicarbonate amended intermittent 5% CO_2 condition, and the difference between these conditions was not significant ($p=0.123$). The 50mM HCO_3^- + glucose and bicarbonate amended 50mM HCO_3^- + glucose conditions both received more than 50mM HCO_3^- prior to inoculation, as discussed previously. The highest biomass concentration generated during 2-stage cultivation was observed for the 5% CO_2 2S condition without glucose supplementation or a bicarbonate amendment at nitrogen limitation 1.48 ± 0.01 $g*L^{-1}$).

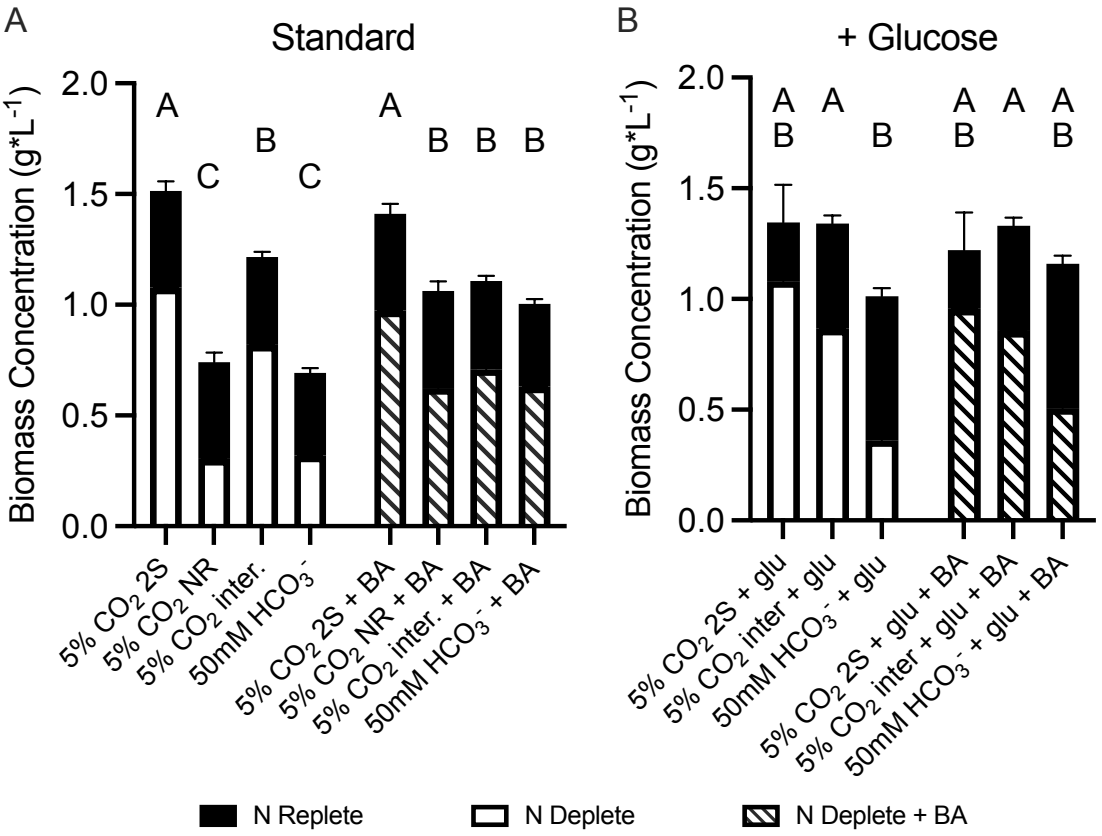

Figure 21: Biomass generated (g*L^{-1}) during nitrogen replete (top bars) and nitrogen deplete (bottom bars) growth for the inorganic carbon conditions with (left side of each plot) and without glucose supplementation (right side of each plot) conditions. Biomass generated during nitrogen deplete growth is presented for unamended (white bars) and bicarbonate amended (white bars with black stripes) conditions. Error bars are one standard deviation of data. Shared letters above individual bars identify conditions with biomass concentrations that are not significantly different. The significance presented is for the simultaneous comparison of all standard and high-alkalinity nitrogen conditions during the 2-stage growth process using the Tukey method with 95% confidence following ANOVA.

For the carbon conditions without glucose supplementation, the addition of a 50 mM bicarbonate amendment at nitrogen limitation resulted in a significant increase in biomass productivity during 2-stage cultivation for the 50 mM HCO_3^- condition (116±3.66 and 171±19.2 g*m^{-3}*day^{-1} for the unamended and amended 50 mM HCO_3^- conditions, respectively; p=0.010; Figure 23A). A decrease in biomass productivity was observed for the 5% CO_2 2S and 5% CO_2

intermittent conditions when a bicarbonate amendment was provided (239±1.29 and 220±4.83 $g*m^{-3}*day^{-1}$ for the unamended and amended 5% CO_2 2S conditions, respectively; p=0.021; 172±5.61 and 154±5.27 $g*m^{-3}*day^{-1}$ for the unamended and amended 5% CO_2 intermittent conditions, respectively; p=0.030). The reduced productivity observed for the 5% CO_2 2S and 5% CO_2 intermittent condition is suspected to result in reduced activity of CCMs. Two acclimation states for CCM activity have been described for eukaryotic algal cells (Spalding, 2008). At CO_2 concentrations at or below atmospheric levels CCM activity is induced, but at elevated CO_2 concentrations no induction is observed. The pH values for the 5% CO_2 2S and 5% CO_2 intermittent conditions both increased above pH 8 during nitrogen deplete growth. At this pH the dominant form of inorganic carbon in solution should be HCO_3^-, and CO_2 should be limited. Due to the supplementation with 5% CO_2 to the air sparge it is possible that CO_2 is present in solution or alternatively, gaseous CO_2 may inhibit induction of CCMs. A higher biomass yield from nitrogen was also observed for the 50 mM HCO_3^- condition when a bicarbonate amendment is provided at nitrogen limitation (18.5±0.58 and 29.6±2.32 $g_{biomass}*g_N^{-1}$ for the unamended and amended 50 mM HCO_3^- conditions, respectively; p=0.015; Figure 23B). A reduction in biomass yield from nitrogen was observed during 2-stage growth when a bicarbonate amendment was provided for the conditions where 5% CO_2 supplementation was provided to the air sparge during the nitrogen deplete growth stage (30.0±0.16 and 27.5±0.61 $g_{biomass}*g_N^{-1}$ for the unamended and amended 5% CO_2 2S conditions, respectively; p=0.021 based on unpaired t-test; 23.5±0.77 and 21.1±0.72 $g_{biomass}*g_N^{-1}$ for the unamended and amended 5% CO_2 intermittent conditions, respectively; p=0.030 based on unpaired t-test). The reduced

yield from nitrogen observed for the conditions where CO_2 supplementation was provided is also suspected to result from no or low induction of CCM mechanisms.

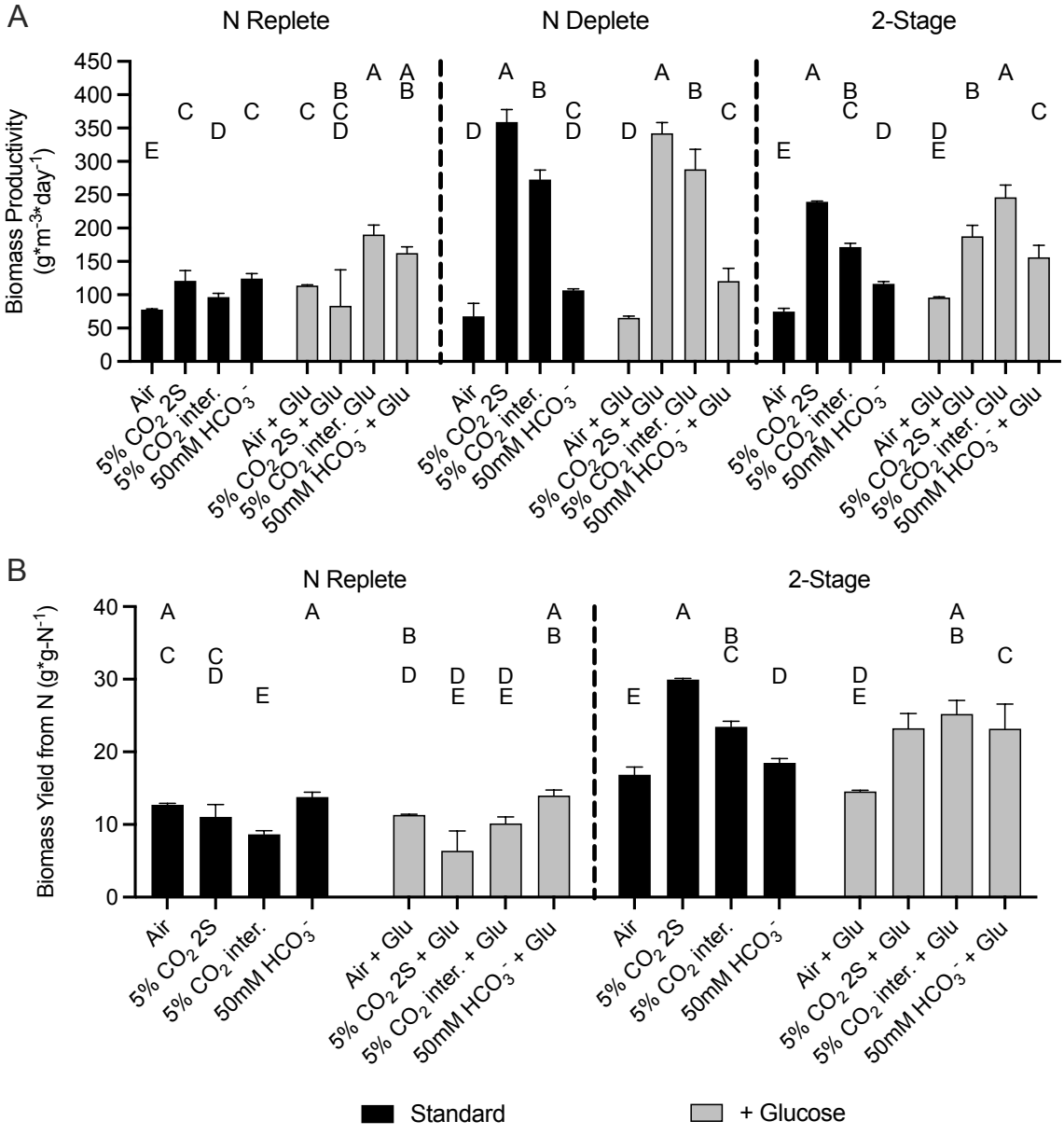

Figure 22: Biomass (A) productivity and (B) yield from nitrogen for each of the inorganic carbon conditions with (grey bars), and without (black bars) glucose supplementation. Biomass productivity is presented for nitrogen replete, nitrogen deplete, and 2-stage cultivation. Error bars are one standard deviation of data. Statistical significance for comparisons for all standard and

glucose amended conditions for each growth stage independently are presented using letters above each column. For clarification, significance is not shown for comparisons between growth stages. Conditions that share a common letter are not significantly different. Impacts on biomass productivity from the addition of glucose to the growth medium were primarily observed during nitrogen replete growth, with all conditions except the 5% CO_2 2S condition having a higher productivity when glucose was present. For the 50 mM HCO_3^- + glucose condition the increased productivity observed may result from the excess $NaHCO_3$ added to this condition prior to inoculation, as discussed previously. For the 5% CO_2 2S condition, the addition of glucose resulted in a decrease in biomass productivity during nitrogen replete growth.

During 2-stage cultivation biomass productivity and yield from nitrogen were both improved for the glucose supplemented 50 mM HCO_3^- condition when a bicarbonate amendment was provided at nitrogen limitation (156±17.9 and 202±5.08 $g*m^{-3}*day^{-1}$ for the unamended and amended 50 mM HCO_3^- conditions with glucose supplementation, respectively; p=0.006; Figure 23C; 23.2±3.42 and 31.9±0.80 $g_{biomass}*g_N^{-1}$ for the unamended and amended 50 mM HCO_3^- conditions, respectively; p=0.002; Figure 23D). For the other glucose supplemented inorganic carbon conditions, the bicarbonate amendment had no significant impacts on biomass productivity or yield from nitrogen.

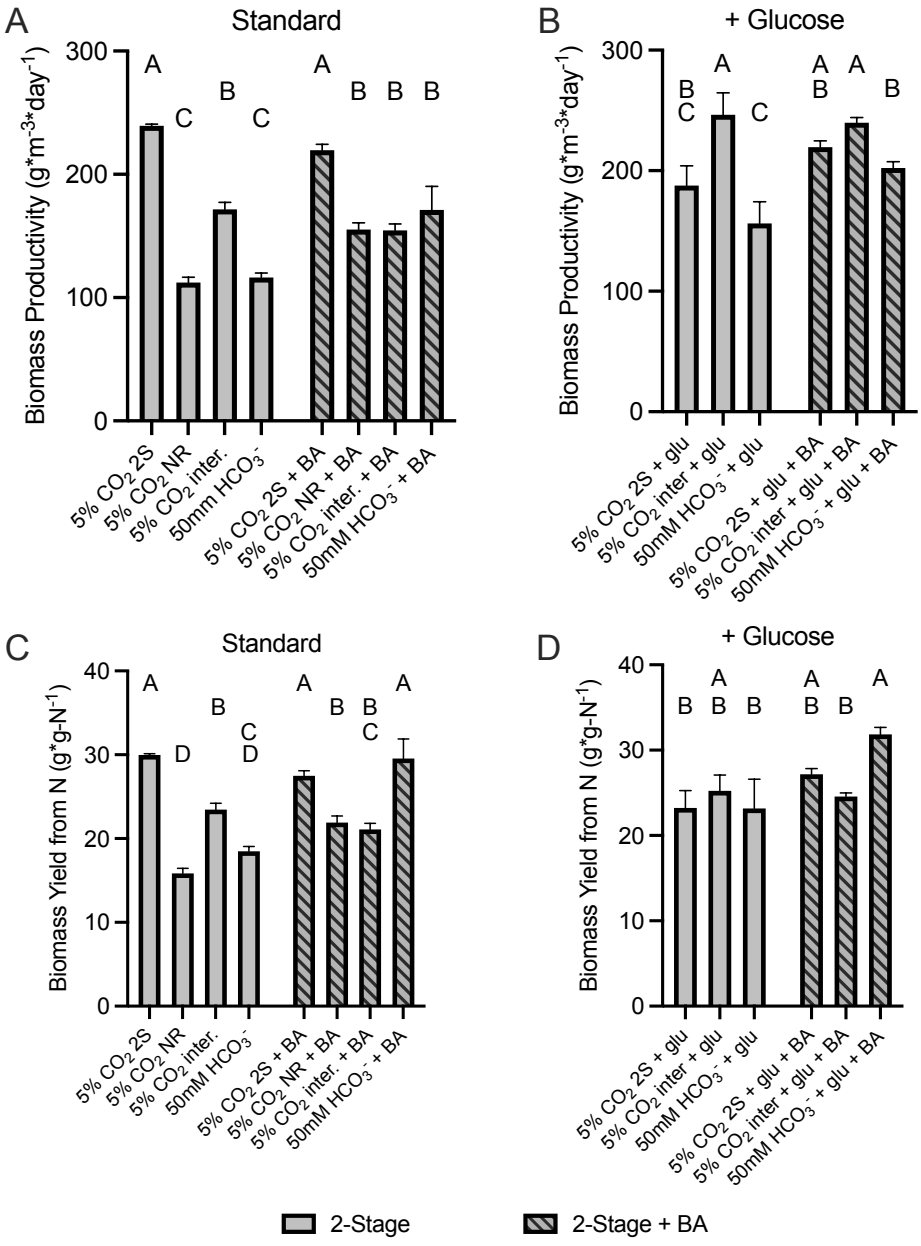

Figure 23: Biomass (A-B) productivity and (C-D) yield from nitrogen during 2-stage cultivation showing the impact of a 50mM bicarbonate amendment added at nitrogen limitation to the inorganic carbon conditions with (bars on left in each figure), and without (bars on right of each figure) glucose supplementation. Error bars are one standard deviation of data. Statistical significance for comparisons for all standard and glucose amended conditions for each growth stage independently are presented using letters above each column. Conditions that share a common letter are not significantly different. Increased biomass productivity resulting from this amendment were observed for inorganic carbon (no glucose added) conditions where no other

inorganic carbon supplementation was observed during nitrogen deplete growth (the 5% CO_2 NR and 50mM HCO_3^- conditions).

In the absence of glucose supplementation, the 5% CO_2 2S condition had the highest biomass productivity, but productivity was not impacted by the addition of a bicarbonate amendment at nitrogen limitation. Biomass yield from nitrogen was also greatest for the 5% CO_2 2S condition, relative to the other inorganic carbon conditions without glucose supplementation, however yield was not significantly different than the bicarbonate amended 50 mM HCO_3^- and bicarbonate amended 5% CO_2 2S conditions. The highest productivity for conditions where glucose supplementation was provided was observed in the 5% CO_2 intermittent condition, but productivities in bicarbonate amended 5% CO_2 2S and 5% CO_2 intermittent conditions were not significantly different ($p=0.296$ and 0.994 for the bicarbonate amended 5% CO_2 2S and bicarbonate amended 5% CO_2 intermittent conditions, respectively). For the glucose supplemented conditions, the highest biomass yield from nitrogen was observed for the bicarbonate amended 50 mM HCO_3^- condition, but yields were similar for the glucose supplemented 5% CO_2 intermittent and bicarbonate amended 5% CO_2 2S conditions ($p=0.059$ and 0.184 for the bicarbonate amended 5% CO_2 2S and bicarbonate amended 5% CO_2 intermittent conditions, respectively).

CHAPTER FOUR

CONCLUSIONS

Nitrogen Sources – Cell growth, Nitrogen removal, and Biomass production

The main conclusion from the evaluation of nitrogen sources using standard BBM and high-alkalinity BBM is that the impact of nitrogen speciation on cell growth, nitrogen uptake, and biomass generation cannot be considered independently from alkalinity (pH and DIC concentration) due to (1) the impact of hydroxyl ion and proton generation during assimilation of nitrogen from NO_3^- and NH_4^+, respectively; (2) the impact of pH on NH_4^+/NH_3 equilibrium; (3) the upregulation of genes associated with carbon fixation and nitrogen assimilation by HCO_3^-. The impacts of HCO_3^- and CO_2 availability on cell physiology are contrary, and their relative contributions to growth in a system where both are available requires further investigation.

Standard Nitrogen Conditions

During cultivation in standard BBM using NO_3^-, NH_4^+, urea, or combination of all three, cell growth appeared to follow one of two distinct trends, either rapid replication resulting in relatively small cells or slower cell replication resulting in larger cells. These two distinct growth modes appeared to result from variations in culture pH and DIC concentration caused by assimilation of nitrogen from the different nitrogen sources. Higher pH and DIC concentrations were observed for the nitrate and ammonium conditions, where slower cell replication was observed; while lower pH and DIC concentrations were observed for the urea and mixed nitrogen conditions, where higher cell replication was observed. The lower cell concentrations observed for the nitrate and ammonium conditions are likely a result of the increased availability

of HCO_3^- in these conditions relative to the urea and mixed nitrogen conditions. Bicarbonate supplementation has been previously linked to suppression of cell replication (Li et al., 2018). The higher pH and DIC concentration for conditions supplemented with NO_3^- were driven by OH^- generation during assimilation of nitrogen from NO_3^-. For the ammonium condition the lower cell concentrations were a result of overcompensation for proton generation during assimilation of nitrogen from NH_4^+ by the pH control system used. The lower pH and DIC observed for the urea and mixed nitrogen conditions were caused by the net neutral impact on system pH of nitrogen assimilation for these conditions. Over-compensation for proton generation by the pH control system used in the ammonium condition highlights the difficulties of precise pH control in actively growing cultures. Improved pH control might be achieved by manual addition of base or by using a buffered growth media.

All of the nitrogen sources evaluated could be utilized by *C. sorokiniana*, strain SLA-04, in standard BBM medium, however urea removal from the growth medium occurred at a significantly slower rate than NO_3^- or NH_4^+. This is likely a result of limited urea carboxylase activity due to the low availability of HCO_3^-, which (1) is required for carboxylation of urea by urea carboxylase, part of the urea amidolyase enzyme complex (Strope et al., 2011; Tu et al., 2018); and (2) has been shown to upregulate gene expression for urea carboxylase (25-34 fold in the alkali-tolerant *Chlorella* sp., strain LPF; Tu et al., 2018). In addition, the higher pH and DIC concentration observed for the nitrate and ammonium conditions may have promoted more rapid assimilation of nitrogen due to HCO_3^- availability, which has previously been linked to increased expression of nitrate reductase (nitrate reduction) and glutamine synthetase, respectively (amino acid synthesis from NH_4^+; Tu et al. 2018). Preferential utilization of nitrogen sources was

observed when all three nitrogen sources were provided together, with NH_4^+ being consumed to very low concentrations before NO_3^- was utilized, and NO_3^- being removed to trace amounts before urea utilization commenced. This is explained by inactivation of nitrate reductase by NH_4^+, a well-established interaction (Fernandez et al., 1989; Hellebust & Ahmad, 1989; Lachmann et al., 2019; Sanz-Luque et al., 2015), as well as NO_3^- inhibition of urea utilization, which is not as well understood (Hellebust & Ahmad, 1989).

During nitrogen replete growth in standard BBM using NO_3^- or a mix of nitrogen sources, biomass productivity was improved relative to the urea and ammonium conditions. The lower biomass productivity observed in the ammonium condition was likely a result of NH_3 toxicity and NH_3 volatilization from the growth medium due to the high pH of this condition (Collos & Harrison, 2014). The lower biomass productivity observed in the urea condition is likely a result of low activity of urea carboxylase caused by the low pH and resulting low concentration of HCO_3^-, which has been shown to upregulate genes related urea carboxylase (Tu et al., 2018). In addition, HCO_3^- is required for carboxylation of urea (Tu et al., 2018), likely further reducing the activity of the urea carboxylase enzyme. Biomass yield from nitrogen during nitrogen replete growth was lowest in the nitrate and ammonium conditions. In these conditions, 5% CO_2 supplementation in the air sparge and a higher HCO_3^- concentration in solution due to the relatively high pH had contrary impacts on metabolism. The availability of CO_2 causes repression of CCM activity, potentially inhibiting the ability to concentrate HCO_3^- near RuBisCO (Gardner, Lohman, et al., 2012; Giordano et al., 2005; Richmond et al., 1982; Spalding, 2008). Simultaneously, high concentration of HCO_3^- in solution has been shown to

upregulate genes associated with nitrogen metabolism (Tu et al., 2018). The relative impact of these co-occurring regulatory mechanisms has not been investigated.

For 2-stage cultivation, biomass yield from nitrogen was only different for the ammonium and urea conditions, with the ammonium condition having the lowest and the urea condition having the highest yields from nitrogen. In the ammonium condition, the reduced yield from nitrogen is likely a result of NH_3 toxicity and NH_3 volatilization (Collos & Harrison, 2014). The increased yield from nitrogen for the urea conditions is not as well understood. During 2-stage cultivation, biomass productivity was higher for conditions that had higher pH values and higher DIC concentrations. Interestingly, biomass productivity during nitrogen deplete growth was highest in the conditions where lower productivity was observed during nitrogen replete growth.

High-Alkalinity Nitrogen Conditions

High-alkalinity cultivation resulted in a shorter lag period before cell growth and nitrogen removal was observed for all of the nitrogen conditions. This is likely a result of upregulation of genes associated with carbon fixation and nitrogen assimilation (Tu et al., 2018). In the ammonium condition a significant loss of nitrogen was observed as a result of NH_3 volatilization (Collos & Harrison, 2014). High-alkalinity cultivation improved biomass productivity during nitrogen replete growth when urea or a mix of nitrogen sources was used for cultivation, but not when NO_3^- or NH_4^+ were used. The lack of impact of high-alkalinity cultivation when NO_3^- was provided as the sole nitrogen source is not well understood, but for the ammonium condition it is suspected to result from loss of NH_3 due to volatilization and NH_3 toxicity (Collos & Harrison, 2014). Increased productivity for the urea and mixed nitrogen conditions is likely caused by

upregulation of genes associated with nitrogen assimilation and carbon fixation due to the presence of HCO_3^-. It is also possible that for the urea condition, the higher initial DIC concentration observed at inoculation played a role in promoting increased biomass productivity, however the most significantly upregulated genes due to bicarbonate supplementation observed by Collos and Harrison (2014) were associated with urea carboxylase (responsible for carboxylation of urea using HCO_3^-; 25-34 fold). In combination with the improved nitrogen removal and cell growth rates observed for the urea condition, it seems likely that the upregulation of genes associated with improved urea metabolism played a part.

Nitrogen Conditions – Bicarbonate Amendment at Nitrogen Limitation

The bicarbonate amendment at nitrogen limitation improved biomass productivity and yield from nitrogen for all standard nitrogen conditions using a 90% confidence interval, however these benefits are not significant for the nitrate condition when a 95% confidence interval is used. When applied to the high-alkalinity nitrogen conditions, HCO_3^- significantly increased biomass productivity and yield from nitrogen for the high-alkalinity nitrate condition only. If a 90% confidence interval is used, instead of a 95% confidence interval, the high-alkalinity urea condition also exhibited higher biomass productivity with the application of a bicarbonate amendment at nitrogen limitation. It is unclear why improved biomass productivity and yield from nitrogen were observed for the high-alkalinity nitrate condition, but not the other high-alkalinity nitrogen conditions, but it may be related to the intracellular generation of hydroxyl ions having an impact on the regulation or performance of transport mechanisms required to move HCO_3^- into the cell.

Carbon Supplementation Strategies

Inorganic Carbon Supplementation

The speciation of inorganic carbon in solution had significant impacts on cell replication and biomass production during the current study. Continuous and intermittent supplementation of 5% CO_2 to the 4 L*min^{-1} air sparge provided a sufficient inorganic carbon pool for algal growth, however due to the low alkalinity of the system, the concentration of HCO_3^- is believed to have remained low relative to conditions where $NaHCO_3$ was added to the growth medium before cultivation. Further, the inorganic carbon pool in CO_2 supplemented conditions is suspected to be dominated by CO_2. Cell concentrations at nitrogen limitation and at the end of the study were higher for these conditions than conditions supplemented with $NaHCO_3$, but the average cell mass was smaller. This is suspected to be caused by the diffusive nature of CO_2, which is not easily concentrated within cells. As a result, the generation of carbon storage compounds (lipids and carbohydrates) is likely less favorable than in conditions where carbon concentrating mechanisms are active. Due to the sufficient carbon availability in these conditions, biomass production was not reduced, but allocation of carbon and energy were directed towards cell synthesis rather than storage compound generation. In addition, HCO_3^- has been shown to hinder cell replication, which may force conditions with high concentrations of HCO_3^- to direct carbon flow towards storage compound generation. For the condition where CO_2 supplementation was limited to atmospheric concentrations present in the air sparge, nitrogen removal was significantly slowed, but cell growth was not notably impacted.

During nitrogen replete growth, biomass productivity was strongly influenced by total inorganic carbon availability, with not significant differences being observed for conditions with HCO_3^- or continuous 5% CO_2 supplementation. Lower biomass productivity was observed during nitrogen replete growth when 5% CO_2 supplementation occurred intermittently (5 min*hour^{-1}), and even lower for the condition supplied with air only. Interestingly, biomass yield from nitrogen during nitrogen replete growth did not correlate well with biomass productivity, but rather seemed to be a function of multiple parameters (pH and DIC speciation, but not DIC concentration). The highest biomass yields from nitrogen were observed for the condition supplemented with $NaHCO_3$ and the condition that was provided only air. During nitrogen replete growth these conditions also exhibited the highest pH values. The high pH of these conditions meant that inorganic carbon speciation in their growth media was dominated by HCO_3^- and to a lesser degree, CO_3^{2-}, with CO_2 being absent or at very low concentrations. The concentration of DIC in the Air condition as the only source of inorganic carbon was very low, and in the condition supplemented with $NaHCO_3$ was very high.

During 2-stage cultivation biomass productivity was most strongly influenced by DIC concentration and speciation during the nitrogen deplete growth stage. The availability of CO_2 during nitrogen deplete growth (5% CO_2 continuous and 5% CO_2 intermittent conditions) promoted significantly higher productivities than the condition that was supplemented with $NaHCO_3$ prior to inoculation. Culture pH during nitrogen deplete growth may also have influenced biomass productivity for the 2-stage process, as the pH in the condition supplemented with $NaHCO_3$ was greater than 11 during this growth stage. This high pH likely inhibits photosynthesis and other cellular functions. Conditions without CO_2 supplementation during

nitrogen deplete growth and without $NaHCO_3$ addition exhibited lower biomass productivities, and the condition that did not receive any inorganic carbon supplementation had the lowest biomass productivity observed. Biomass yield from nitrogen during the 2-stage growth process was promoted similarly to biomass productivity, with conditions that received continued CO_2 supplementation during the nitrogen deplete growth stage having the highest observed yields, followed by the bicarbonate amended condition. The lowest biomass yields from nitrogen during 2-stage cultivation were observed for the conditions that did not contain HCO_3^- and were not supplemented with CO_2 during nitrogen deplete growth.

Organic Carbon Supplementation

Glucose supplementation promoted more rapid nitrogen removal for conditions that were not provided CO_2 supplementation (Air and 50 mM HCO_3^- conditions) but did not impact nitrogen removal when CO_2 was supplemented. A decrease in cell growth was observed when glucose supplementation was combined with continuous 5% CO_2, however an increase in cell growth was observed when CO_2 supplementation was intermittent. Previous work has found that CO_2 inhibits glucose metabolism in green algae (Sforza et al., 2012). The improved growth achieved through glucose supplementation to the intermittent 5% CO_2 condition, relative to the continuous 5% CO_2 condition, is suspected to be a result of glucose metabolism being possible during periods when 5% CO_2 supplementation was not provided. Increased cell growth was also observed for the glucose supplemented bicarbonate condition, relative to the 50 mM HCO_3^- condition without glucose supplementation, although this condition also received a greater amount of $NaHCO_3$ than the 50 mM HCO_3^- without glucose supplementation. Due to repression of cell replication by HCO_3^- (Gardner, Cooksey, et al., 2012; Gardner, Lohman, et al., 2012), it is

suspected that the increased cell growth was a result of glucose rather than the excess addition of $NaHCO_3$.

For 2-stage cultivation, improved biomass productivity was observed for the 50mM HCO_3^- and Air conditions, when glucose was supplemented, however productivities were still significantly lower than were observed in the conditions where 5% CO_2 supplementation was provided during the nitrogen deplete growth phase (5% CO_2 continuous and 5% CO_2 intermittent conditions), with or without glucose supplementation. Glucose supplementation did impact the conditions that received 5% CO_2 during nitrogen deplete growth though, with productivity being improved with glucose supplementation to the intermittent 5% CO_2 condition and reduced productivity with glucose supplementation to the continuous 5% CO_2 condition. This is likely a result of glucose metabolism being possible during periods when CO_2 supplementation was not active in the intermittent 5% CO_2 condition, but being inhibited in the condition where supplementation was continuous (Sforza et al., 2012). The reduction in productivity was not expected for the 5% CO_2 condition, which may suggest that physiological responses to combined glucose and CO_2 availability are more than just inhibition of glucose metabolism. Biomass yield from nitrogen for different inorganic carbon regimes during 2-stage cultivation was also impacted by glucose supplementation, with impacts mirroring the impacts observed for biomass productivity.

Carbon Conditions – Bicarbonate Amendment at Nitrogen Limitation

A bicarbonate amendment at nitrogen limitation promoted increased biomass productivity and yield from nitrogen for the inorganic carbon conditions (without glucose supplementation) that were not supplemented with 5% CO_2 during nitrogen deplete growth (5%

CO_2 NR and 50 mM HCO_3^-). For the 5% CO_2 intermittent and 5% CO_2 2S conditions, the addition of a bicarbonate amendment at nitrogen limitation did not impact biomass productivity or yield from nitrogen. For conditions supplemented with glucose, only the 50 mM HCO_3^- condition exhibited increased biomass productivity and yield from nitrogen as a result of providing the bicarbonate amendment. The other glucose supplemented conditions were not impacted by the bicarbonate amendment with respect to biomass production.

Summary of Findings

The 5% CO_2 intermittent with and without glucose supplementation, as well as the 5% CO_2 2S without glucose supplementation conditions exhibited the highest biomass productivities observed, with the bicarbonate amendment at nitrogen limitation having no effect on these conditions. The addition of the bicarbonate amendment to the glucose supplemented 5% CO_2 condition improved biomass productivity, making its productivity comparable to the conditions mentioned above. The use of 5% CO_2 supplementation is associated with high demand of CO_2 and therefore does not address issues associated with the environmental impacts of production and transportation of CO_2 or losses of CO_2 due to off-gassing from the culture medium. Biomass yield from nitrogen was more similar between conditions than biomass productivity, suggesting an optimal C/N ratio exists for SLA-04 that can be achieved with different combinations of N and C sources. During nitrogen replete growth, the average C/N ratio for SLA-04, for all carbon and nitrogen conditions evaluated, was 6.09±0.59. During nitrogen deplete growth an increase in the C/N ratio to 7.21±0.77 was observed as a result of continued carbon fixation in the absence of nitrogen. Variations in biomass yield from nitrogen were more significant for the different

nitrogen conditions than for the carbon conditions, with the high-alkalinity ammonium condition having the highest yield from nitrogen observed. As noted previously, the off gassing of NH_3 due to the high pH of this condition likely made the biomass yield from nitrogen for this condition inaccurate. For optimization of resource utilization, the high-alkalinity urea condition without bicarbonate amendment at nitrogen limitation was most promising due to its use of $NaHCO_3$ as an inorganic carbon supply, rather than CO_2, and the use of urea which is cheaper and more abundant than nitrate or ammonium fertilizers.

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