Title: A preliminary implementation of metabolic-based pH control to reduce CO₂ usage in outdoor flat-panel photobioreactor cultivation of *Nannochloropsis oceanica* microalgae

Authors & Affiliations: Jun Wang¹, Theresa Rosov², Pierre Wensel², John McGowen², Wayne R. Curtis^{1*}

¹ Department of Chemical Engineering, The Pennsylvania State University, University Park, PA 16802

² Arizona Center for Algae Technology and Innovation, Arizona State University, Mesa, AZ 85212

* Correspondence author, wrc2@psu.edu, 814.863.4805

ABSTRACT

A crucial challenge associated with high-density, commercial-scale, outdoor microalgal cultivation is maintaining pH stability without excessive use of CO₂ buffering. This includes a media-dependent, intracellular metabolic proton imbalance leading to the alkalization or acidification of growth media that results when algae consume, respectively, nitrate or ammonium ions. Feeding these two nitrogen sources can theoretically achieve balanced proton metabolism as well as pH control that is economically more favorable as compared to CO₂ buffering or acid/base addition. To accomplish this, a fed-batch nutrient feeding strategy must be adopted as a component of a model-based pH control system, particularly in the case of ammonium preference. This work represents a preliminary study of the challenges of implementing a nitrogen metabolism based open-loop pH control strategy in the challenging environment of an outdoor photobioreactor at the DOE-ATP³ testbed facility in Mesa, Arizona during the high solar insolation period of summer 2015. The approach was limited to twice daily fed-batch addition while accounting for ammonium-N preference in a background of nitrate-based algae growth media. Despite these limitations, growth achieved for a photobioreactor operated based on predicted metabolic nitrogen demand (PND) was comparable to ‘CO₂-on-demand’ (CoD) for pH control. PND reduced CO₂ usage to less than 10% of CoD control, where the reduced buffering resulted in much greater pH fluctuations due to daily variations in light availability, and a much lower and more consistent media alkalinity (5.73 - 5.79 mEq/L) as compared to the CoD control (6.51-8.49 mEq/L). While this effort illustrates the utility of feed-forward model-based control, it further illustrates the need for far more sophisticated ‘real time’ monitoring and modeling to accommodate

the dynamic outdoor conditions. A need for improved analytics for accurate closure of nitrogen mass balance is also indicated.

Keywords: pH control, alkalinity, algae cultivation, *Nannochloropsis oceanic*, photobioreactor, nitrogen assimilation, stoichiometry, carbon yield.

INTRODUCTION:

Concerns of continued use of transportation fuels and other fossil-fuel derivatives in relation to national security, global economics, environment, and rural economic development have led to increased interest in renewable fuels and products from biomass feedstocks like oleaginous microalgae. Nevertheless, commercialization of the algal industry is constrained by high operating costs associated with growth control in large, outdoor raceway open ponds or photobioreactors (PBRs) (Slade & Bauen 2013). Culture pH has the potential to influence a wide range of parameters within an alga production process; extracellular pH influences trans-thylakoidal membrane ionic gradients and electromotive forces in photosynthesis, the assimilation of nutrients including inorganic carbon, and even the surface charge / zeta-potential that mediate subsequent flocculation-mediated harvesting (Liu et al. 2013). Because carbon is typically provided by CO₂ (or dissolved wastewater carbon), the major inorganic nutrient (and associated process cost) is nitrogen. Nitrogen sources can be provided in a range of states of reduction (e.g. ammonium, nitrate, urea), where the resulting nitrogen metabolism can have dramatic effects on the media pH through secretion or uptake of protons (Wang & Curtis 2015).

Small-scale culturing typically obscures the buffering role of CO₂ because the amount of gas supplied is in great excess of stoichiometric requirements, such that the CO₂ approaches an equilibrium condition that is amenable for algae growth (Wang 2015). In outdoor operation, pH control is often implemented by sparging CO₂ on demand (CoD) when the pH goes above a specified upper bound, and the resulting carbonate equilibrium acidifies the medium (Berenguel et al. 2004).

Because CO₂ suffers from poor solubility of gasses in warm water, the majority of the CoD may not transfer into the culture for consumption, thereby representing a significant process cost as well as greenhouse gas emissions.

Attempting to retain gas while allowing light penetration rapidly results in rapid heating of the culture and/or significant evaporative losses. Rather than utilizing CO₂ buffering, acids or bases can be added via feedback control based on pH sensor measurements. This approach is hindered, however, by the undesirable accumulation of inhibitory counter-ions and costs of acids—which are nearly as great as the nitrogen sources (Khan et al. 2014). The conceptually simpler and more economical approach of formulating balanced growth-media by combining ammonium and nitrate nitrogen sources is limited due to the significant and preferential assimilation of ammonium before nitrate by algae (Scherholz & Curtis 2013), which lowers pH to the point of cell death.

In a circumstance where the algae cannot regulate its own nitrogen utilization to maintain pH, the alternative is to supply different nitrogen sources based on the metabolism that will result in the desired pH control. Metabolic modeling-based pH control faces many challenges that are even greater than control issues in a typical bioreactor due to the dynamic condition of outdoor culture; one must consider both predictable and unpredictable components of light, temperature, rainfall, and evaporation rates—all operating on different time scales. While incorporating sophisticated feed-forward modeling is a long-term goal that combines both the biology, physics, and environment, this initial manual implementation was focused on translating laboratory experience to outdoor conditions—including brutal temperatures exceeding 40°C to gain further insights into the overall challenge. The DOE's ATP³ (Algae Testbed Public-Private Partnership) facility at Arizona State University's (ASU) Mesa campus' AzCATI (Arizona Center for Algae Technology and Innovation) provided an opportunity for access to a large number of analytics and PBRs designed specifically for established algal cultivation methods. The transition from indoor experimental studies (Wang 2015) to outdoor operation in this work represents a useful characterization of what challenges to anticipate in the next phase of moving the proposed metabolic pH control strategy from pilot to large scale systems.

In this work, a simple stoichiometric-based metabolic model for open-loop pH control was implemented based on daily predicted nitrogen demand (PND) and ammonium addition into nitrate-based algae medium in an outdoor pilot-scale, flat-panel PBR at AzCATI. The existing method of CO₂ on demand (CoD) was operated as the control. The observations and impact of this approach on operation and performance of these two different pH control operational strategies are discussed.

MATERIALS AND METHODS

Algae Cultivation Methods:

Microalga strain. The marine microalga *Nannochloropsis oceanica* sp. 0209 was obtained from the National Center for Marine Algae and Microbiota, Bigelow, Maine (formally known as the National Center for Culture of Marine Phytoplankton (CCMP). This algae was chosen as the model marine, lipid accumulating eukaryotic microalgae species for the comprehensive multi-site evaluation of algae performance for the U.S. Department of Energy ATP³ program (www.atp3.org).

Growth media. Cultures were maintained in f/2 salt-water medium. Modified f/2 medium consisted of (per 1 L): 0.754g NaNO₃, 0.067g NaH₂PO₄, 0.0063g FeCl₃•6H₂O, 0.002 g Na₂EDTA•2 H₂O, 1.8x10⁻⁴ g MnCl₂•4H₂O), 6.3x10⁻⁶ g Na₂MoO₄•2H₂O, 9.8x10⁻⁶ g CuSO₄•5H₂O), 1.0x10⁻⁵ g CoCl₂•6H₂O), 2.2x10⁻⁵ g ZnSO₄•7H₂O), and 35 g Oceanic™ Sea Salt Mix (Detroit, MI, etc.). A stock solution of 500 g/L NH₄Cl was prepared for pH adjustment for outdoor PBRs. Culture media were prepared by adding stock solutions to tap water inside the bubble-columns and PBRs.

Inoculation and cultivation scale-up. Microalgae were pre-cultured indoors and scaled up sequentially for inoculation of 60 L flat-panel PBRs. Microalgae were initially cultured in bubble-columns first at 0.1 L and then 0.8 L working volume. They were then inoculated at a minimum OD₇₅₀ of 0.3 and cultured in logarithmic phase at 10-15 L working volume in 15 L flat-panel PBR with dimensions of 4'x 4'x0.05'. All indoor cultures were cultivated under continuous light at 200~300 µE m⁻² s⁻¹ light intensity supplied by polychromatic fluorescent light, while receiving 2% (v/v) CO₂ gas. Outdoor PBRs (**Figure 1**) were then inoculated at a minimum OD₇₅₀ of 1.0 (to avoid photo-bleaching) at 50 L working volume in flat panel PBRs with dimensions of 48"x72"x4" supplied by gas bubbling from the bottom via 1/16"-diameter

holes drilled on the side of a PVC pipe. Prior to inoculation, the PBRs were thoroughly cleaned with a water jet, then filled with a 0.03% NaClO bleach solutions for at least 30 min contact time, and rinsed 3 times with water to remove residual chlorine levels as confirmed using test strips Pooltime™ 6-way test strips.

INSERT FIGURE 1: Flat panel photobioreactor used for outdoor experimentation at the Arizona Center for Algae Technology and Innovation (AzCATI).

The temperature range for indoor bubble columns and PBRs was 22-28°C based on laboratory room thermostat. For outdoor PBRs, culture temperature ranges of 24-29°C were maintained by cycling 25°C chilled water from a nearby Glacier® pool evaporative cooler model #GPC-250 through 1 cm ID stainless steel cooling coil with a 6 meters of contact length per PBR. Temperature and pH of outdoor PBR cultures were measured using a handheld pH100 ExStick® meter and probe. The pH probe was calibrated with temperature compensation using standard pH 4, 7, and 10 buffer solutions and was recalibrated every night to adjust for signal drift during cultivation. Data was acquired by YSI 5200A multiparameter monitor, wirelessly transmitted, and recorded to a PC computer running AquaManager software version 6.0 Build 5001 housed in a nearby control room. A web browser-based virtual control panel is also available through the on site wireless network using a smart phone.

Photobioreactor Sampling and Analysis:

Approximately 1 L of culture was sampled daily at 12:00 noon from the outdoor PBRs and analyzed for (a) dry weight (DW), (b) ash-free dry weight (AFDW), (c) fatty acid methyl ester (FAME) content; (d) substrate concentration of ammonium and nitrate in supernatant (e) optical density at wavelength 750 nm (OD_{750}) and 680 nm (OD_{680}). Also, approximately 20 mL of culture was sampled from the outdoor PBRs immediately before, 10 min after, and 2-5 hrs after dosing NH_4Cl and analyzed for (a) OD_{750} and OD_{680} and (b) substrate concentration for ammonium and nitrate.

Optical density, dry weight and ash free dry weight. OD_{750} and OD_{680} were measured using the Hach® DR 2800 v.1.11 bench-top spectrophotometer with non-linear calibration and 1 mm quartz/plastic cuvette. Algal culture samples were blanked with f/2 medium, diluted to an OD of 0.2-0.8, and read in

triplicate at both OD₇₅₀ and OD₆₈₀, which further served to quickly indicate culture stress levels. Total dry algae biomass and dry ash were determined in triplicate using a gravimetric filtration and oven drying. A measured volume of sample visually determined to contain at least 5 mg of dry biomass was added to pre-ashed and pre-weighed filters in manifolds while applying a vacuum pressure. The filter with algae cake was then rinsed with 1 M ammonium formate solution to remove salt dried in an oven for 12 hrs at 105 °C, cooled in a desiccator for 1 hr, and then re-weighed to determine combined total dry algae biomass and ash. The filter was then ashed in a muffle furnace at 500°C, cooled in a desiccator for 3 hrs, and re-weighed to determine total ash.

Light availability as assessed by integration of Photosynthetically Active Radiation (PAR). Due to outdoor growth, light levels vary throughout the day. While a rigorous assessment of light flux to the reactor requires a projection of the solar beam onto the photobioreactor geometry, we present a simpler approach to reasonably assessing light availability by simply integrating the light normal to the earth as measured by a field-mounted LICOR Biosciences LI-250A light meter with quantum Q41000 PAR sensor (μE/m²/s).

$$\text{Cumulative PAR} = \int_{\text{inoculation}}^{\text{processtime}} \text{PAR}(t) \cdot dt$$

In this manner, the differences in light of a given day can be captured while still accommodating for lack of light at night and effectively replacing time as the dependent variable with a quantitative index of light availability. This approach to presenting the data also provides for comparisons of growth in artificial constant light environments or with defined variable photoperiods. In these outdoor studies, the vertical line of midnight encompasses the entire dark culture period.

Substrate concentration of nitrate and ammonium. Substrate concentration of nitrate, ammonium, and orthophosphate in culture samples were determined using QuickChem® methods with references for nitrate + nitrite (Wendt 1995) and for ammonium (Diamond 1995). Samples are flow-injected in triplicate into a Lachat Instruments QuikChem® 8500 Automated Ion Analyzer equipped with Omnion 3.0 software for analysis.

Elemental analysis of biomass. Elemental composition for carbon and nitrogen in algal biomass was measured with a CE Instruments Elemental Analyzer EA 1110 (Thermo Electron Corp). For this, about 2~5 mg of biomass was lyophilized, homogenized, and instantly combusted into CO₂ and NO_x, gaseous products which were then collected and analyzed to estimate mass composition of carbon and nitrogen.

Alkalinity. Total alkalinity is the acid equivalents (equ) required to neutralize dissolved media species to a final pH of 3.5. 50 mL of algae culture supernatant was sampled and stored at 4°C prior to measurements. The sample was incrementally titrated with 0.02 N H₂SO₄ solution while monitoring the pH from the initial condition to a final pH of 3.5 (corresponding to a complete removal of media bicarbonate).

FAME lipid analysis. Analysis of Fatty Acid Methyl Ester (FAME) content is performed with Agilent Technologies GC analytical instrumentation following standard basic transesterification procedure developed in NREL for algae biomass (Laurens et al. 2012).

Photobioreactor pH Control Strategies:

Feedback pH Control using CO₂ sparging. CoD based pH control utilized automatic CO₂ sparging to reduce the pH. The upper pH setpoint was programmed as 7.9 (lower bound = 7.8, upper bound = 8.0), where a normally-closed solenoid would open to deliver CO₂ at a flow rate of 12.5 L min⁻¹ (LPM) (or 24.6 g min⁻¹) until pH reached the lower bound, though the preset sample interval of 15 min often resulted in the pH well below the lower bound.

Feed-forward pH control using metabolic assimilation of nitrogen. PND based pH control utilized a sparge of CO₂ just sufficient for inorganic carbon demand such that pH could be controlled by the proton imbalance associated with nitrogen consumption. The constant supply flow rate of 0.25 CO₂ LPM was empirically determined to provide growth rate-dependent proton imbalance, determined as a moderately fluctuating pH as described in more detail below. Growth stoichiometry provides a well-defined quantity of protons according to:

where $(\psi/\phi)_{\text{NH}_4} \approx 0.92$ and $(\psi/\phi)_{\text{NO}_3} \approx -1.12$. Therefore, there is a direct correlation between the amount of growth and the anticipated secretion or uptake of protons as developed previously (Wang & Curtis 2015).

$$\Delta H^+_{\text{metabolism},i} = (\psi/\phi)_i \cdot \xi_{\lambda}(\Delta OD_{\lambda}) \quad (\text{Eq. 2})$$

where ξ_{λ} is the wavelength (λ)-dependent conversion factor between optical density (OD) and cell concentration (X) (e.g. $OD_{750} = \xi_{750} \cdot X$) (Myers et al. 2013). A nitrate-based basal media was used for cultivation, and NH_4Cl was dosed at appropriate concentrations and time intervals as described below.

The constant supply flow rate of 0.25 CO_2 LPM was iteratively determined to be the CO_2 supply rate needed to sustain growth at a relatively constant rate while minimizing pH fluctuation (± 0.01 pH change per 15 min). A feed-forward manual dosing for NH_4Cl was calculated based on the anticipated growth for the next culture period utilizing the stoichiometry represented in Equation 2. Predicting the amount of NH_4Cl to add for the next day was straight-forward due to linear growth, a result of light limitation and minimal light disruption in the summer Arizona climate. Note that although any growth kinetic model could be utilized to calculate the predicted increase in optical density ($\Delta OD_{750, \text{predicted}}$), we utilized an experimentally determined average dry weight conversion factor ($\xi_{750} = 0.3$) under the initial assumption of a typical algae biomass composition of 10% N dry weight.

$$\Delta (NH_4Cl)_{\text{predicted}} = (\psi)_{NH_4Cl} \cdot \xi_{750} \Delta OD_{750, \text{predicted}} \quad (\text{Eq. 3})$$

Note that these initial approximations for conversion factors are readily updated and refined based on experimental yield and experimentally determined *Nannochloropsis* biomass composition. An important consideration for experimental execution is how large a daily NH_4Cl addition will be, and into how many increments the feed must be divided to provide for a reasonably small change in pH as a result of proton secretion during metabolism to amino acids. Based on preliminary experiments, the anticipated growth capability in the PBR corresponds to an $\Delta OD_{750} \leq 2$ with a corresponding biomass accumulation of 0.5 gDW/L. Based on 10% nitrogen content by weight, this corresponds to a maximum nitrogen assimilation of 0.05 g N/L (3.6 mM NH_4-N). Near neutral bioreactor pH, this level of secretion of protons would correspond to a capacity for unbuffered pH change of 4.6 pH units ($\Delta pH_{\text{unbuffered}} = -4.6$), therefore, to maintain a pH change of a pH unit due to secreted protons would require 4-5 incremental media additions per day. It is important to note, however, that in the case of a salt-water based media, the pH change due to alkalinity is significantly buffered near neutrality and is on the order of 0.5 ΔpH units / mN-equivalents H^+ (as will be confirmed from an alkalinity titration curve, **Figure 5**). A secretion of 3.6 mM H^+ would

therefore correspond to a pH change of 1.8 pH units ($\Delta\text{pH}_{\text{sea-water}} = -1.5$), suggesting that two media additions should be sufficient to maintain the ammonia-metabolism based pH change within a given day to less than a single pH unit.

In the actual experiment, based on the end of day sample from previous day, the growth predictions (OD_{750}) are: $\Delta\text{OD}_{750,\text{day}3} = 2.06$, $\Delta\text{OD}_{750,\text{day}4} = 1.61$; $\Delta\text{OD}_{750,\text{day}5} = 1.38$, and calculated NH_4Cl dosages (mmol) are 4.40, 3.45, and 2.95, respectively. The preceding calculations provide a basis for experimental design as well as insight into the fundamental principles that govern pH control (i.e. considerable dependence upon the dynamics of nitrogen utilization, CO_2 supply). Due to inherent constraints of ATP³ outdoor configurations, CO_2 sparge rate could not be readily varied to minimize use and match stoichiometric carbon demand.

In this study, the PBR was therefore initially operated at constant CO_2 supply rate without NH_4Cl dosing for the first two days to generate a baseline CO_2 supply and associated pH fluctuation as well as provide culture growth rates from which to predict the subsequent NH_4Cl supplements. The daily dosage calculated by Equation 3 was split evenly into two doses administered at 9:00 (a time in the day when culture was expected to have already photo-adapted) and 15:00 (a time in the day to allow for the assimilation of the majority of the first dose and provide ammonium for the second half of the day during maximum PAR).

Replication and Statistical Analysis:

Biomass composition CHN analysis was measured in triplicate for all samples with a consistent standard deviation of 0.06 %N and 0.09 %N respectively for CoD and PND photobioreactors. Media nitrate and media ammonium ion concentrations were performed in triplicate for all samples with an average analytical standard deviation of 0.06 ppm for nitrate and 0.08 ppm for ammonium (corresponding to error bars smaller than figure datapoints). The absolute variation duplicate total FAME measurements was on average 0.37% FAME for CoD photobioreactor, and 0.25% FAME for PND photobioreactor (smaller than figure datapoints); the relative reproducibility for overall FAME measurements is a standard deviation of 2.6% of the measured values. Optical density was taken as single values due to insignificant differences for replicate measurements. Dry weight and ash free dry weight for each sample was taken as single

measurements to provide sufficient material for accurate gravimetric measurement. The accuracy of continuous monitored data such as pH and PAR relied on daily calibration for accuracy. An ANOVA confirmed the obvious linearity of the dry weight and cumulative PAR time courses, and a Student T-test compared the linear slopes for bioreactor correlations between dry weight, ash-free dry weight and cumulative PAR and found no statistical difference at a 99.9% confidence level. The standard deviations of the slopes were calculated from matrix operations in Excel. Although a piecewise linear model could be used for ANOVA comparison of provided and summed analytical nitrogen measurements, the large differences obviate the utility of a statistical assessment.

RESULTS AND DISCUSSION

Growth for alternative pH Control strategies:

Both flat-panel photobioreactors operated using the different pH control strategies, respectively PND (metabolic pH control using predicted nitrogen demand) and CoD (CO₂ on-demand pH control), displayed similar growth (**Figure 2**). PND and CoD both displayed an OD₇₅₀ which increased from 2.5 to 9 with only minimal biomass loss during the unlighted night-time hours. The appearance of the PND suggested greener culture which is reflected in the optical density (OD) ratio of OD₆₈₀ / OD₇₅₀ which correlates to the chlorophyll-a content to dry weight. This ratio has also been suggested as an indicator of the level of stress within a culture (Chekanov & Solovchenko 2015); in this case however, since the metabolic pH control is mediated by increased levels of nitrogen addition in the form of ammonium, this likely reflects the impact of that additional nitrogen on chlorophyll content (Myers et al. 2013) and not a specific physiological stress.

INSERT FIGURE 2: Growth of flat panel PBRs grown with alternative pH control strategies of CO₂ addition on demand (CoD) and fixed minimal CO₂ addition with pH control based on predicted nitrogen metabolic demand (PND). **A**) growth as indicated by OD₇₅₀ (light scattering regime) and **B**) an indicator of chlorophyll pigmentation from the ratio of absorbance max for chlorophyll-a (680 nm) to the biomass level (750 nm). Timepoints indicated correspond to midnight.

Additional indicators of comparable growth are shown as more direct measurements of dry weight and associated ash content. In **Figure 3**, the daily measurements of dry weight, %-ash and associated ash-free dry weight (AFDW) are shown with respect to the integrated photosynthetically active radiation (PAR) as described in the methods. In this particular outdoor study, since the solar insolation is quite constant (within 5% for the 5 day period, data not shown), the relatively consistent sampling time and light conditions for these biomass measurements results in very little need for correction to PAR relative to process time. We feel that it is important, however, to present data in the context of solar insolation as it provides a more accurate basis for comparing data not only across different growth periods, climates, and sites, but also to indoor growth conditions, which are often operated with continuous light. The observation of a nearly perfectly linear increase in biomass over these five days strongly suggests that the growth under both PBR conditions is light-limited and the slightly increased chlorophyll content was not significant in terms of improving biomass accumulation. These correlations were found to be linear by ANOVA ($p < 0.006$) and the slopes of the two bioreactors were not statistically different ($\alpha < 0.001$) for a ash free dry weight yield of 7.0 ± 0.2 mg/L per PAR (mole photons per aerial square meter). There is a small but consistent variation in %-ash that could reflect small changes during the increase in biomass loading, but is more likely a systematic error in the measurement of these quantities.

INSERT FIGURE 3: Biomass dry weight and ash content as a function of cumulative photosynthetically active radiation (PAR) for *Nannochloropsis* grown in flat panel PBRs with alternative pH control strategies of CO₂ addition on demand (CoD) and predicted metabolic nitrogen demand (PND). **A)** Dry weight and ash free dry weight (AFDW) and **B)** %-ash content over a 5-day outdoor growth at AzCATI site.

Culture pH and Alkalinity:

The two alternative pH control methodologies resulted in very different pH profiles with the CoD fluctuating rapidly between a pH of 8 and 6.5 and the PND displaying relatively smooth variations from 7.5 to as high as 9 (**Figure 4A,B**).

INSERT FIGURE 4: Time courses of pH, temperature and solar insolation (PAR) for the alternative pH control strategies. **A)** pH fluctuations during metabolic demand for nitrogen assimilation (PND). **B)** pH

fluctuations during CO₂ sparging on demand (CoD) C) Temperature in both reactors, and D) solar insulation measured as photosynthetically active respiration (PAR).

Comparable growth despite these large pH changes corroborates the general observation that many algae have a rather broad pH growth range (Khatoon et al. 2014) which provides flexibility in implementing pH control while trying to minimize nutrient resources. The outdoor pilot scale setting presented a considerable challenge to maintaining pH control for both methods as neither approached the optimal pH control achievable in small indoor systems. An ammonium addition twice each day provides minimal control relative to prior work with multiple small additions under constant indoor conditions (Scherholz & Curtis 2013). Similarly, the 0.25 VVM sparge rate of the CO₂ pH control line was observed to be excessive by the formation of large bubbles, achieving poor CO₂ transfer. In combination with a slow sampling rate (i.e. 15 min. to accommodate highly multiplexed logging), this resulted in comparatively large pH fluctuations relative to what can be achieved with fine bubble dispersion, rapid sampling, and more precise gas flow control.

Ambient daytime temperatures exceeding 40°C presented a physical challenge to personnel, and resulted in bioreactor temperatures that varied from 20 to 30°C throughout the day and night despite the internal PBR heat exchangers. These fluctuations substantially alter gas solubility as well as pH probe response despite daily efforts to maintain calibration with off-line measurements and temperature compensation.

The comparable rates of growth observed for these highly variable conditions are a testament to the resilient nature of algae, which is further reflected by its growth kinetics in the light-limited growth condition, where the effects of other factors are attenuated by comparison. The fluctuations in temperature result from the daily dynamics of light flux (**Figure 4D**), where it should be noted that at ‘solar noon’, a vertical flat panel will have a period of reduced light due to the sun’s rays predominantly impinging on the narrow top, rather than the sides of the photobioreactor. This results in the ‘bimodal’ response of pH and corroborates the choice for sufficient but not excessive gas flows. During the reduced photon flux of ‘solar noon’, the reduced CO₂ consumption rate is reflected in a temporary drop in pH, wherein CO₂ instead favors dissolution into and subsequent acidification of the media, until the light starts to enter the other side of the flat panel reactor

where higher CO₂ consumption rates resume and increase the pH. The baseline flow rates of 0.005 vvm CO₂ utilized during the PBR operation resulted in an estimate of 25.8% efficiency for carbon utilization based on the experimental average 45% carbon dry weight basis. Although this reflects a relatively low efficiency of gas-liquid mass transfer, it is an order of magnitude higher than what is typically observed in heterotrophic bioreactors for oxygen. A poor efficiency of gas use is typical for bioreactors where for example O₂ use in heterotrophic microbial culture also utilizes less than 5% of the supplied gas. By reducing gas flow and not relying on CO₂ for pH control the supply can be much closer to the demand.

Alkalinity encompasses a measure of the media capacity to neutralize acid, which was measured on samples at noon the day after a previous afternoon inoculation where the media alkalinity of both flat panel PBRs were comparable (CoD, 6.51 mEq/L; PND, 5.79 mEq/L) with a slight elevation in alkalinity for photobioreactor that was more heavily sparged with CO₂. The alkalinity for metabolic control (PND) was found to drop slightly by the end of culture to 5.73 mEq/L in contrast to the CO₂ sparge approach which increased substantially to 8.49 mEq/L over the 5 day culture period (**Figure 5A**).

INSERT FIGURE 5: Alkalinity ranges during growth of *Nannochloropsis* in flat panel photobioreactors with alternative pH control strategies of CO₂ addition on demand (CoD) and predicted nitrogen metabolic demand (PND). The lower panel presents the alkalinity titration curve which provides a direct illustration of the effects of alkalinity on process control because the slope of the alkalinity is a measure of buffering capacity and attenuates the change in pH for a give secretion or uptake of protons due to nitrogen metabolism. The alkalinity titration curves measured at the end of culture are provided in **Figure 5**. As expected, the use of CO₂ sparging as the basis of suppressing pH results in an increase in alkalinity relative to metabolic pH control with reduced CO₂ buffering. Although increased alkalinity enhances pH buffering capacity, which functions as passive control, it makes active model predictive control (MPC) of pH response to metabolic proton flux more difficult. To quantify this behavior, the derivative slope of the alkalinity curves can be compared at pH=6. The more highly buffered CoD bioreactor displays a derivative of -2.0 mM H⁺/ΔpH as compared to -4.44 mM H⁺/ΔpH, which are both dramatically smaller than an unbuffered water system (-2.33 × 10⁶ mM H⁺/ΔpH). Implementation of model-predictive pH control would require this information either as empirical experimental input or a more complex prediction of the buffering based on media species.

It is noteworthy that the metabolic control (PND) treatment did not only accumulate acid-neutralizing species, but also demonstrated minimal reduction in the alkalinity, suggesting that any contribution of ammonium metabolism to the neutralization of media, was minor relative to buffering provided by the saltwater and the baseline CO₂ sparge rate. As noted in the background, the ability to achieve metabolic pH control is dependent upon the growth stoichiometry providing sufficient protons from ammonium assimilation. The total ammonium-N fed to the PND was 130 mg N/L (9.3 mM NH₄-N/L), which corresponds roughly to the same acid equivalents based on growth stoichiometry. Combined with the observed slope of the titration curves in the relevant operational range of pH ~6.0 of 0.5 ΔpH-units / H⁺ mEq the capacity for pH change in this experimental implementation was 5 pH units. Since the ammonium nitrogen is comparable to the initial nitrate ammonium (8.9 mM NO₃-N/L) there is comparable potential for pH control based on secreted and assimilated protons. Noting that the total growth of the two PBRs was comparable, it is unclear how much of the nitrogen was assimilated which emphasizes the critical nature of verifying the overall nitrogen mass balance.

In this particular implementation, the overall biomass yield on nitrogen for the PND was reduced due to starting with the same media nitrate content in both photobioreactors. An improved strategy for the nitrogen assimilation pH control method would start with much lower nitrate levels with fed-batch addition to minimize total nitrogen fed and allowing for controlled nitrogen limitation. Since the methodologies of nitrogen measurement have undergone considerable standardization at the AzCATI site, it is informative to examine the nitrogen mass balance by adding up the total of the various measurements including nitrogen supplied, inside the cells, and within the media. As shown in **Figure 6**, the media nitrate levels (measured at noon) had declined to essentially zero for both bioreactor configurations. The ammonium levels were functionally zero throughout the entire run, even in the case of PND where NH₄Cl was added roughly three hours before the mid-day sample point. The biomass nitrogen composition measured between 5 and 7.5% by weight, declined after nitrate depletion as expected for the CoD bioreactor and remained between 6 and 7% for the CoD case that included ammonium feed (**Figure 6A**).

INSERT FIGURE 6: Assessments of nitrogen mass balance during operation of the flat plate photobioreactors using ‘standard’ methods for media and cellular nitrogen content. **A)** Biomass nitrogen

content (% of dry weight), **B**) Media nitrate levels, **C**) Media ammonium levels (near zero), and **D**) a comparison of predicted and summed total media and cell content of nitrogen reflecting a 'loss' of considerable nitrogen due to sum of the individual analytics.

The lower panel (**Figure 6D**) represents a predicted total nitrogen mass balance as compared to the sum of the various analytically measured components multiplied by the magnitude of that nitrogen pool (total cells and total media). The sum of the analytical methods is considerably lower than predicted by mass balance, suggesting there is either an unaccounted medium nitrogen-containing species or a systematic error associated with the measurements and/or the associated calculations. The daily ammonia volatilization rate was measured to be around 3 mg/L/day (~0.5% per day of f/2 media N) from a separate experiment utilizing an un-inoculated PBR, suggesting that this is not a significant contributor to nitrogen loss. While secretion of amino acids or proteins remains a possibility, the levels of unaccounted nitrogen are quite large and seems unlikely. Noting that the inorganic nitrogen media components contribute a declining and eventually negligible amount to the overall mass balance, the associated errors must come from the elemental analysis and dry weights. The batch nitrogen addition of the CoD is more easily interpreted, since the total nitrogen (corrected for media and cell sampling) should remain constant. The constantly declining summed total nitrogen indicates that the biomass composition analysis or its associated dry weight determination is likely systematically underestimating the actual nitrogen levels. This is consistent with a cumulative loss of calculated nitrogen as well, since successive samplings would be removing more nitrogen than calculated in a systematic cumulative error in the overall mass balance. The deviation of the PND which includes nitrogen addition twice a day does display the trend of a calculated increase in total system nitrogen, but that amount falls considerably short of what is predicted by mass balance. There are several important implications to this observation. First, the tendency to utilize data as absolute quantities and even conversion factors can result in considerable error. Second, the goal of model predictive control is undermined by systematic inaccuracies in the associated nitrogen mass balance. Successful implementation of the proposed strategy requires a greater focus on validating the overall mass balance of nutrients and avoiding the typical focus on reproducibility that can miss large systematic errors.

Lipid Productivity:

Significant differences in fatty acid profiles were observed for the alternative pH control strategies (Figure 7).

INSERT FIGURE 7: Fatty Acid Methyl-Esters (FAME) lipid profiles for the two outdoor flat panel PBRs (m = metabolic controlled PND, c = feedback CO₂ controlled, CoD) give the fatty acid compositions throughout the 5-day growth period for *Nannochloropsis*.

The CoD culture had final lipid content that was 8% higher lipid than that of PND. This was most likely caused by the depletion of nitrogen in CoD which did not occur in PND because it was periodically dosed with an ammonium that effectively doubled the total N content of the bioreactor. The increased lipid content was comprised primarily of C16:0 (palmitic acid), C16:1n7 (palmitoleic acid), and C18:1n9 (oleic acid), the major fatty-acid constituents of *Nannochloropsis oceanica* lipid fractions (Chini Zittelli et al. 1999; Crowe et al. 2012). The content of high-value ω -3 fatty acid C20:5 (eicosapentaenoic acid [EPA]), however, remained constant, as previously observed in literature (Pal et al. 2011). Therefore, in this specific case, nitrogen starving might not have been a determinant for EPA productivity.

CONCLUSIONS

Utilizing CO₂ to both provide carbon and media buffering results in poor biomass yield on CO₂ and a large loss of the provided CO₂ into the environment. Choosing a low baseline flow of CO₂ that allows pH fluctuations combined with ammonium additions to introduce proton secretion provides a means to decrease CO₂ usage and still maintain pH in a range for growth.

The alkalinity presented by sea-water and increased by CO₂ represents a significant challenge to implementing nitrogen-metabolism based pH control due to the reduction in pH response caused by alkalinity. Nonetheless, the slope of titration curves in the range of desired operating conditions combined with the stoichiometrically released protons due to growth gives a pH response that was sufficient to implement control. Achieving such a control will require models of growth that can more precisely predict the nitrogen

mass balance as well as growth in response to the dynamic conditions of outdoor growth. The largely manual implementation of sampling, nitrogen demand prediction and feedings is not sufficient to achieve the desired control, and will require more sophisticated instrumentation and control algorithms. Most importantly, the nature of high density algae growth which is inherently light-limited, constrains the response of the system to be surprisingly predictable despite the large fluctuations in light and pH observed in these outdoor studies. This insensitivity of growth response should be an asset towards implementing a pH control that is more precise, and includes manipulation of CO₂ sparge rates for example to further reduce the overall CO₂ use during algae photobioreactor operation. This type of bioreactor control work requires conducting such studies in the challenging environment of outdoor algae culture systems.

Contributions:

All authors approved the final version of the submitted manuscript. JM provided funding/facilities for this work. JW designed the study, acquired and analyzed data, and drafted the article. TR and PW provided technical support and acquisition of data. WRC conceptualized the study and provided critical analysis/interpretation of the data in manuscript revisions critical for important intellectual content.

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FIGURE 1: Flat panel photobioreactor used for outdoor experimentation at the Arizona Center for Algae Technology and Innovation (AzCATI).

FIGURE 2: Growth of flat panel PBRs grown with alternative pH control strategies of CO₂ addition on demand (CoD) and fixed minimal CO₂ addition with pH control based on predicted nitrogen metabolic demand (PND). **A)** growth as indicated by OD₇₅₀ (light scattering regime) and **B)** an indicator of chlorophyll pigmentation from the ratio of absorbance max for chlorophyll-a (680 nm) to the biomass level (750 nm). Timepoints indicated correspond to midnight.

FIGURE 3: Biomass dry weight and ash content as a function of cumulative photosynthetically active radiation (PAR) for *Nannochloropsis* grown in flat panel PBRs with alternative pH control strategies of CO₂ addition on demand (CoD) and predicted metabolic nitrogen demand (PND). **A)** Dry weight and ash free dry weight (AFDW) and **B)** %-ash content over a 5-day outdoor growth at AzCATI site.

FIGURE 4: Time courses of pH, temperature and solar insulation (PAR) for the alternative pH control strategies. **A)** pH fluctuations during metabolic demand for nitrogen assimilation (PND). **B)** pH fluctuations during CO₂ sparging on demand (CoD) **C)** Temperature in both reactors, and **D)** solar insulation measured as photosynthetically active respiration (PAR).

FIGURE 5: Alkalinity ranges during growth of *Nannochloropsis* in flat panel photobioreactors with alternative pH control strategies of CO_2 addition on demand (CoD) and predicted nitrogen metabolic demand (PND). The lower panel presents the alkalinity titration curve which provides a direct illustration of the effects of alkalinity on process control because the slope of the alkalinity is a measure of buffering capacity and attenuates the change in pH for a give secretion or uptake of protons due to nitrogen metabolism.

FIGURE 6: Assessments of nitrogen mass balance during operation of the flat plate photobioreactors using ‘standard’ methods for media and cellular nitrogen content. **A)** Biomass nitrogen content (% of dry weight), **B)** Media nitrate levels, **C)** Media ammonium levels (near zero), and **D)** a comparison of predicted and summed total media and cell content of nitrogen reflecting a loss of considerable nitrogen due to analytics.

FIGURE 7: Fatty Acid Methyl-esters (FAME) lipid profiles for the two outdoor flat panel PBRs (m = metabolic controlled PND, c = feedback CO₂ controlled, CoD) give the fatty acid compositions throughout the 5-day growth period for *Nannochloropsis*.