The Pennsylvania State University

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Department of Chemical Engineering

QUANTITATIVE STUDY OF STOICHIOMETRIC PROTON IMBALANCE IN PHOTOTROPHIC ALGAL GROWTH

A Thesis in

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by

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ABSTRACT

The high production of algae based biofuel requires high density of algae culture. One of the important issues associated with high density algae cultivation is pH instability, which is due to inherent proton imbalance in algae metabolism. Among different nitrogen sources in typical algae culture media, nitrate and ammonium lead to alkalinization and acidification of the media, respectively, as they are consumed. Compared to other pH control strategies such as buffering or acid-base addition, the metabolic control of pH based on feeding different nitrogen sources provides a potential pH control strategy that is technically feasible in large scale outdoor biofuel production. To accomplish this, a fed-batch nutrient feeding strategy would need to be adopted in a model-based pH control system. Due to the current lack of understanding of the magnitude of the stoichiometric pH imbalance under different nitrogen sources, this MS thesis presents a quantitative assessment of the proton imbalance for micro algae (*Chlorella vulgaris*) grown on different nitrogen sources. Our results give tentative experimental values for the pH imbalance associated with nitrate and ammonium media, which can be used as initial parameters in a pH control system based on stoichiometric utilization of nitrogen during algae growth. This thesis also presents a preliminary interpretation of these results as they relate to the biology of nitrogen uptake and metabolism as well as the chemistry associated with media buffering.

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I would like to give special thanks to Nymul Khan, who is my labmate, classmate, and roommate. He has helped me the most on many aspects of my lab work.

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Finally, I would like to thank Dr. Wayne Curtis and all former “Team Algae” members, who established the basis upon which I could start my research. This thesis is a work that directly follows their work on high density algal cultivation and pH control.

Chapter 1

Background

Over the past decades, algae based biofuel have become a more and more popular potential renewable and sustainable fuel source. It provides many advantages over other bio-based energy sources. These include high productivity, more sustainability, low investment and operating cost, high speed, and no competition with human food (Chisti 2007). Algae have the ability to grow on saltwater (Pulz et al. 1998), which significantly increases the available space for such a system. Also, under certain conditions, algae are reported to have a up to 85% lipid content of its dry weight (Borowitzka 1988). Additionally, with respect to environmental benefit, algae also decrease the amount of waste carbon dioxide in the environment by utilizing CO_2 as a carbon source (Keffer and Kleinheinz 2002). Unlike heterotrophic growth production systems, algae use photoautotrophic growth and therefore require much lower power input and no sugar (Eriksen 2008). Based on the above features, research and development of various algae-based production systems have been ongoing to evaluate the economic feasibility, which will be the key to success at large scale. One of the major problems in large scale production of algae is the need for ultra high density culturing to increase the productivity of the fuel. This also has an impact on the operating cost, as well as the difficulty of post-processes, such as the extraction of oil from oil culture, pumping, and dewatering.

A typical early stage commercial algae production system uses an outdoor open pond as reactor that harvests sun light as the energy source. These systems show productivities that range from 5 to 65 $g/m^2/day$ (James and Alkhars 1990), but often lead to significant water loss due to evaporation, which is mainly due to the maintenance of temperature and low efficiency of

photosynthesis. Limiting the radiation to photosynthesis active range could decrease the water loss, but would make the system much more complex. Besides evaporation, pond systems also suffer from low mass transfer, which leads to poor nutrient mixing and CO_2/O_2 distribution as well as low light harvesting efficiency. It is also a difficult task to deal with the contamination from other organisms and post-process cell harvesting (Suh and Lee 2003, Richmond 1987).

Because of the above drawbacks, there is a lot of ongoing research on closed reactors to gain better process control, increase mass transfer, and reduced contamination. However, the challenges such as the distribution of light, fluid dynamics, and carbon dioxide mixing can be even greater in these high density cultures.

Inherent pH instability in photosynthetic algae growth

So far, the pH instability has become one of the most important issues with regards to high density algae culture (Scherholz and Curtis 2013). High density cultures require defined media that can avoid accumulation of inhibitory levels of counter-ions while providing sufficient amount of inorganic salts. Various methods have been used to optimize algae cultivation media, but little has been done to couple the nitrogen mass balance with photosynthetic energy balance.

Overall mass balance of phototrophic growth

As the power source of renewal biofuel, photosynthetic growth presents a significant challenge to stoichiometry. In a simplified case, considering only carbon and nitrogen mass balance, the stoichiometry of photosynthetic algae growth can be written as (use carbon as stoichiometric unit):

$$CO_2 + \psi N_i + \delta H_2O \xrightarrow{\text{hv}} CH_xN_yO_z + \lambda O_2 \qquad (1\text{-}1)$$

There are four elements and six unknown. However, noting that carbon has already been set to one, only three elements are left to establish a mass balance equation. When the biomass composition is specified, the above equation is uniquely determined.

Unbalanced proton from photo reaction

Unlike heterotrophic growth, one important aspect of photosynthetic growth is that photosynthesis provides all energy as well as reducing power for mass balance. Therefore, energy balance is directly coupled with mass balance. The photosynthetic energy can be expressed in the amount of water being split as:

$$2H_2O \xrightarrow{\text{hv}} O_2 + 4H^+ + 4e^- \qquad (1\text{-}2)$$

Here, the four free electrons will be acquired by NADP+ to generate NADPH and protons available for the carbon reduction reaction.

Since the splitting of water provides the hydrogen and energy in final biomass composition, part of above water split has to be a sub-process of total mass balance of photosynthetic growth. All proton and electrons generated by splitting water in the photosynthetic reaction has to be consumed to maintain a constant biomass. The carbohydrate generation in the dark reaction of photosynthesis is the first major acceptor of these imbalanced proton and electrons. These imbalanced charges are countered by external nitrogen source, which is the next major component biomass. Note that though biomass composition for microalgae does vary to some extent, for a specific organism under a specific growth condition, the biomass composition is generally fixed. Therefore, the imbalanced proton part has to be written as following:

$$CO_2 + \psi N_i + \delta H_2O \xrightarrow{\text{hv}} CH_xN_yO_z + \lambda O_2 + \phi \mathrm{H}^+ \qquad (1\text{-}3)$$

Undoubtedly, charge also has to be balanced in an overall biochemical process. This is hidden in the change of nitrogen chemical valence. Note that the letter N in above equation is only a symbol that represents the same mole number of nitrogen in various forms, which typically could be charged ions. Details will be addressed in the later part of this paper.

Qualitative study has been done on the proton imbalance issue. In the case where ammonium based media is used, the experimental value of ϕ for micro algae is positive, and the ambient pH decreases as algae grows. For nitrate based media, ϕ is negative and ambient pH will increase as algae grow. Our major goal in this paper is to quantitatively measure the value of ϕ for growth on different nitrogen source, as well as discuss other about related issues.

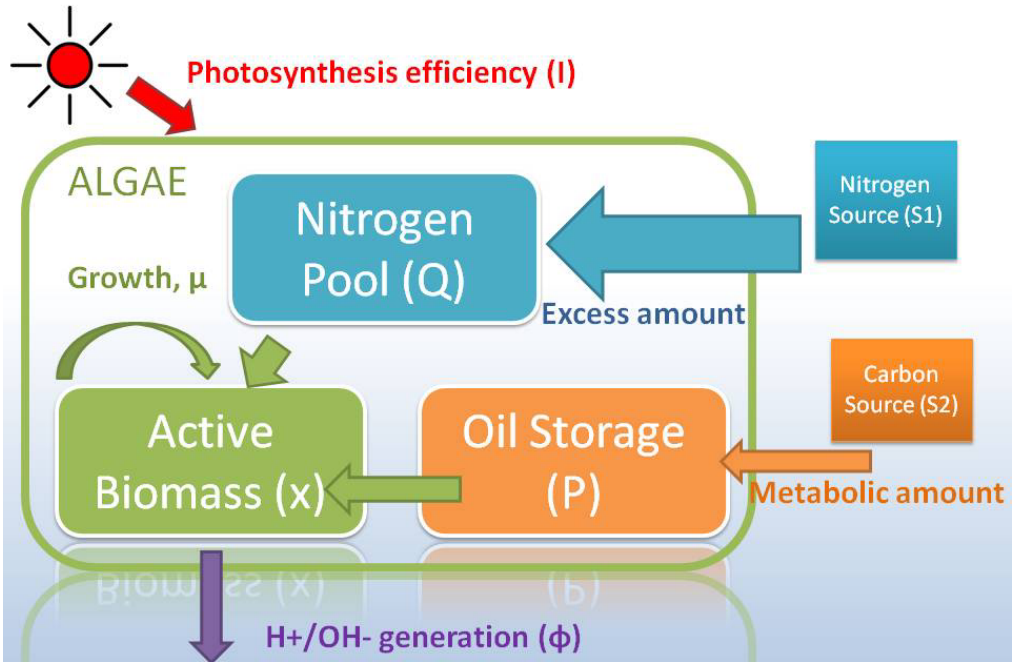


Figure 1-1. The Droop model of algal growth.

Nitrogen assimilation bias and problem with stoichiometric balance media

Though the effect on pH by ammonium and nitrate based media is known, it is still not feasible to make stoichiometrically balanced media. This is because algae have a strong preference for ammonium over nitrate in terms of nitrogen assimilation. Unlike higher plants (Tischner 2000), that are able to selectively assimilate nitrogen sources, in the presence of both ammonium and nitrate, algae will only assimilate the ammonium, nitrate assimilation is blocked. Biologically, it makes sense because nitrogen in ammonium is more close to the form of that in the biomass and requires less energy to integrate into biomass due to its similar oxidation state as those in biomass. Therefore, ammonium can directly join the synthesis of glutamate without additional modification while nitrate has to be reduced before it can be assimilated. This preference will be kept even if nitrate is necessary to mitigate ambient pH. One suitable explanation is that ammonium rich media rarely happens in nature. In nature, ammonium is not as abundant as nitrate due to the existence of competing microbes that converts ammonium into more oxidized forms, and therefore is usually present in small concentration. Besides, natural algae rarely grow up to ultra high density. This makes the change of ambient pH due to nitrogen assimilation not as significant as in lab conditions. As a result, unicellular algae will preferentially assimilate turn to assimilate the nitrogen in ammonium due to its simpler assimilation process and the lack of evolutionary pressure to maintain ambient pH. Consequently, it appears that nitrate is not used as long as ammonium is present. This would lead to high pH drop before ammonium will be used up, and typical algae cannot grow at such low ambient pH. Therefore, a fed-batch nitrogen feeding strategy has to be used and different nitrogen sources have to be added simultaneously in small amount as algae grow. This calls for the quantitative knowledge about the proton imbalance through the algae's metabolism.

Effect of Carbon on pH and nitrogen assimilation

The growth conditions also largely affect the pH behavior of algae culture. When carbon is sufficient and algae are under nitrogen limited condition, such as under 5% v/v CO_2 gas concentration, the pH dynamics follows above mentioned rules. However, when carbon is limited, which could typically happen in air-grown culture, pH dynamics become unpredictable. When carbon is limiting, RuBisCo cannot reach its maximal turnover rate, feedback regulation will influence nitrogen assimilation capability. This adds complexity to pH control so that detailed knowledge of carbon transportation, nitrogen assimilation, and metabolic fluxes is required to achieve a reliable dynamic control system. However, the stoichiometric relation from mass balance aspect should not be different as long as the general metabolism does not change, though dynamics control will be more difficult (Raven 1968, Giraldez, Aparicio, and Quinones 2000, Nimer, IglesiasRodriguez, and Merrett 1997, Thielmann et al. 1990).

Chapter 2

Method

This chapter illustrates the instruments and operation procedures of the experiment.

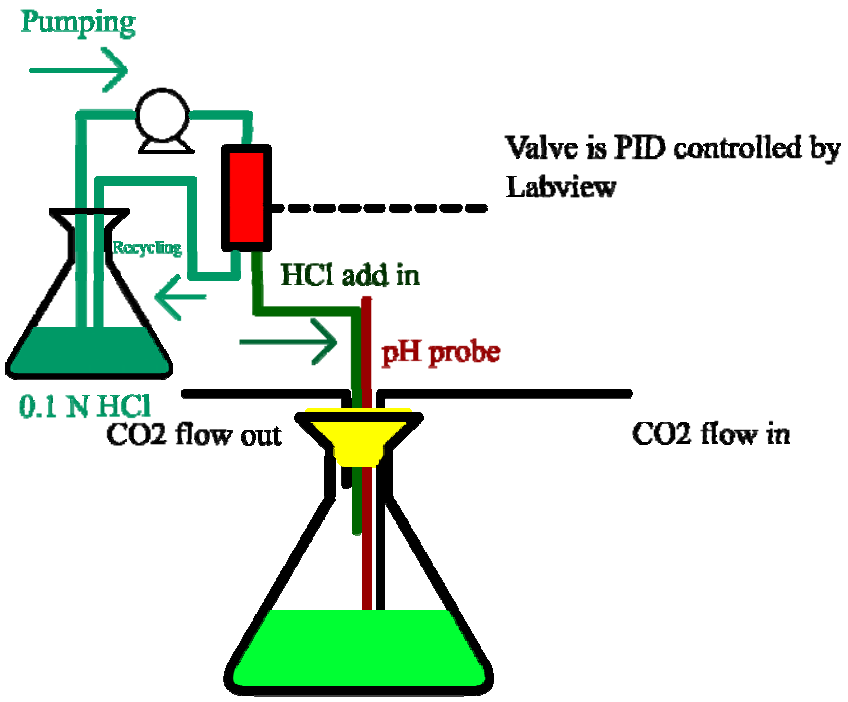


Figure 2-1. Schematic of ϕ measurement experiment setup.

The major instruments of this experiment includes two parts: a controlled cultivation flask where algae are cultivated, and an acid or base delivery system is used to adjust and maintain the pH in the cultivation flask.

The cultivation flask is carefully chosen so that mass transfer of CO_2 is adequate for algal growth. The flask's cultivation conditions, such as light intensity, headspace CO_2 concentration and flow rate, shaking rate, and temperature are controlled to maintain stable output.

The chemical delivery system is consisted of a chemical reservoir and flow control system. Diluted acid or base are stored in the reservoir and get pumped to the culture flask when necessary. The flow control system uses a pump to move the chemicals and a valve to delivery them into cultivation flask in order to maintain a constant pH environment. The flow control system is programmed in LabVIEW.

Experiments start with fresh inoculation of a micro algae organism: *Chlorella vulgaris*. Cultivation is maintained in a constant pH by dosing acid or base from the chemical delivery system. Algae samples for cell density, as well as the weight of consumed chemicals are periodically recorded to analyze the relation between proton imbalance and the accumulation of biomass. Experiments were performed for different nitrogen sources and under different CO_2 gas concentration for comparison. Details of the experiment settings are given in the following sections.

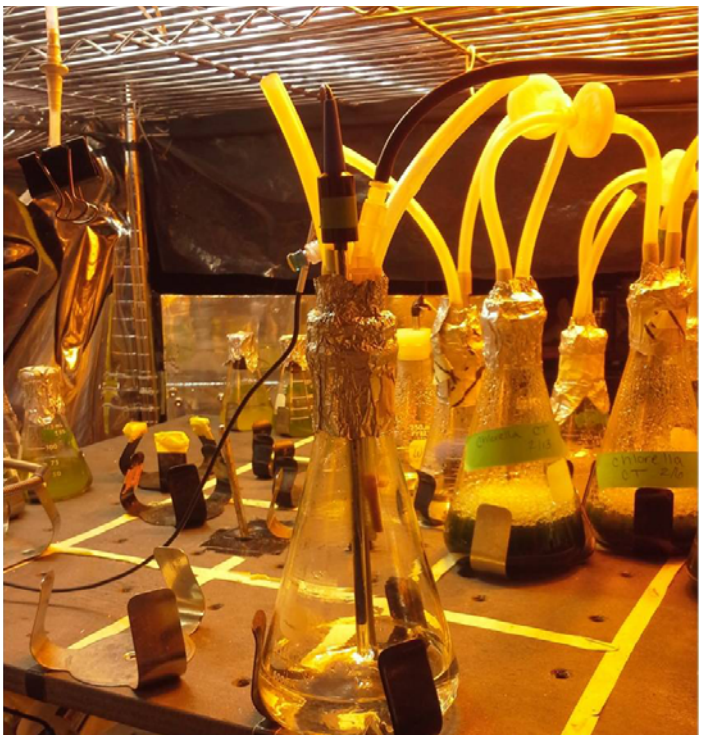


Figure 2-2. ϕ Value Measurement Flask with pH probe, acid/base dosing tubing and sampling septum.

Figure 2-3. Controlled acid/base delivery system.

Cultivation Settings

Algae culture

The algae strain used in experiment is *Chlorella vulgaris*, with culture number 2714 from UTEX culture collection.

Culture media

The media used is named WFAMC in our lab. This stands for Wayne's Freshwater Algal Medium for Chlorella. A complete list of chemical components is given in appendix D. It was originally designed for culturing *Botryococcus braunii*, but also contains enough nutrient for *Chlorella vulgaris*.

Reactor Settings

Mass transfer coefficient measurement

Unsteady-state sulfate addition method was carried out to evaluate CO_2 mass transfer coefficient. The mass transfer coefficient of oxygen was first measured by using the redox reaction between oxygen and sulfate. Then, the CO_2 mass transfer coefficient was evaluated using the relation between the O_2 and CO_2 mass transfer coefficients as given in the following equation (Gulliver 2007):

$$(k_L a)_{CO_2} = (k_L a)_{O_2} \sqrt{\frac{D_{CO_2}}{D_{O_2}}} \qquad (2\text{-}1)$$

Dissolved oxygen concentration measurements were taken with a Mettler Toledo InLab ® 605 probe. Baffled Bellco flasks were used to improve mass transfer and a k_La value of around 50 1/hr was reached.

Daisy-Chain culture flask

Algae were cultured in 500ml baffled Bellco flasks, which were shaken at 120 RPM, by a New Brunswick Scientific G-10 gyratory platform shaker. These flasks were sealed with silicone stoppers containing several holes for pH probe, acid dosing, gas inlet and outlet. The culture flasks were connected one after another in series to form a chained gas line. Each flask had a 0.2 µM daisy chain filter on the inlet tubing and loosely stuffed with cotton in the outlet tubing in order to minimize external and cross contamination between flasks.

Chamber light and temperature

High intensity light was generated using Philips 400W metal-halide and Philips 400W high pressure sodium vapor lamps. To imitate natural lighting condition, the lights were turned on for 16 hours and off for 8 hours each day. The photosynthetic active radiation was measured to be around 300 µE/s/m^2.

The temperature was controlled between 28 to 34 Celsius degree by using household portable air condition and a heater.

Gas delivery and humidification system

Pure CO_2 was provided from a 800~1000 psi CO_2 cylinder, and regulated to 12 psi. The flow of CO_2 was controlled by a solenoid valve that followed the same on/off cycle as the lights. Compressed air was obtained from the common facility, regulated to 12 psi and mixed with pure CO_2 to obtain the desired CO_2 percentage. Gas flow rate was regulated by Sho-Rate rotameters (R-2-15 AAA, with Spherical Glass Float, 1355E Rib Guided Tube). Each rotameter's volumetric flow rate was calibrated using bubble-O-meter and linear regression.

After the mixing point, the gas with the desired CO_2 concentration was bubble through a series of water flasks using gas spargers to maintain a high level of humidity. Humidity of the mixed gas had to be kept in a high level otherwise the culture flasks connected to this gas line could have dried over the long experimental period.

The final CO_2 concentrations used were 0.6% v/v (low CO_2) and 5% v/v (high CO_2). The high CO_2 is commonly used in lab cultivation of algae while low CO_2 is a balance between providing sufficient CO_2 and minimizing buffer effects. The concentration of low CO_2 was estimated based on the growth rate of previous experiments under 5% v/v CO_2 concentrations, and over 10% extra is given to reach this number.

Optical density (OD) measurement

Optical density was measured using 1cm path length cuvettes in Beckman Coulter DU 520 spectrophotometer. Algae samples were read at 550 nm to provide optimal estimation of the culture density (Myers, Curtis, and Curtis 2013). Tap water was used as reference.

pH measurement

An online Cole-Parmer pH electrode probe were inserted into each culture flask through a hole drilled in the silicone stopper. The pH probe was connected to a LI-COR LI-1400 datalogger via BNC connectors.

Standard titrant

0.1 mol/L hydrochloric acid and sodium hydroxide were chosen as the standard acid and base to dose for pH control.

Biomass Composition Measurement

Biomass composition was measured through using a CE Instruments (Thermo Electron Corp) Elemental Analyzer EA 1110 with thermal conductivity detector (TCD). Elemental carbon, hydrogen, nitrogen were completely combusted and the amount of their oxidized products were separated by a chromatographic column and detected by thermal conductivity detector. Average values of biomass composition for cultures grown under the same conditions were used for analysis.

On/off pH Control System

Acid and base standard solutions were kept in 500ml flask containers, with two holed silicone stoppers for output and recycling lines. A peristaltic pump is used to deliver the acid or base, and small tubing is used to obtain a slow flow rate. The standard titrants are passed through a valve controlled by a computer. The computer controlled whether the acid or base was delivered

to the culture flask or was recycled back to the original source flask. Finally, the tubing with titrant was connected to a glass fitting which pass through a hole on the stopper of the culture flasks. The opening of that glass fitting was then connected to a short piece of tubing with a small diameter to prevent the titrant from forming large dosing drops.

Since the pH change is unidirectional for a given media, only one of standard titrant (acid or base) was used in a single experiment. For the case of nitrate media cultivation, hydrochloric acid was used as the standard titrant to bring down the increasing pH caused by algae growth. For ammonium media, sodium hydroxide was used.

At the beginning of each experiment, a pH set point of 6.00 was determined. In the case of nitrate media, if pH became higher than 6, acid titrant was dosed to bring it down to maintain the pH. Physically, the pump turned on with the solenoid switched to recycle for 30 seconds before dosing. This ensures that the pump is fully running.

To avoid possible overdosing caused by the pH probe delay, each titrant dose is discrete and has fixed valve opening time. A one minute interval was given for mixing after each dosing of titrant. The total valve opening time was calibrated and the volumetric flow rate was calibrated using a graduate cylinder and stop watch. Every valve opening time, as well as the pH was recorded during the experiment.

Determination of ϕ value

The Stoichiometric Coefficient for H^+

ϕ value was obtained by measuring the number of molecules of acid or base dosed to maintain culture pH, and the number of carbon molecules converted into biomass during that time interval. The amount of consumed acid/base molecule was obtained from the change of total

weight of acid/base container, which was then converted into moles. The total amount of carbon accumulated in the biomass was indirectly measured from the change in optical density of the culture. The optical density was used to calculate total biomass. The carbon mass was then calculated from the total biomass using known biomass composition.

The phototrophic growth of algae is represented as:

$$CO_2 + \delta H_2O + \psi[N] \rightarrow CH_xN_yO_z + \phi H^+ + \lambda O_2$$

Where x, y, z are biomass composition, which is typically a constant for a certain organism and already known. Besides, note that the [N] in above equation only represents the mole number of nitrogen atoms, and the concrete form of nitrogen could be different. Therefore the whole equation is not rigorously mass balanced.

Since the only carbon source is CO2, the coefficient of carbon is set to 1 and biomass composition is also then based on the relative amount of carbon. So the value of ϕ will represents the amount of imbalanced proton per unit of fixed carbon.

The amount of imbalanced proton is measured by the amount of counter chemical (acid or base) used to keep pH constant. The amount of fixed carbon is measured by the change of biomass, which is usually measured from the optical density of culture suspension.

The amount of counter chemical is measured through the change of weight of the counter chemical's container. This is calculated as:

$$n_{H^+} = \frac{\Delta m}{\rho} \times M$$

Where Δm is the change of weight in gram, ρ is the density in g/L, and M is the molarity in mol/L. The amount of imbalanced protons, n_{H^+}, is then calculated in mol unit.

The amount of fixed carbon is then calculated through the optical density (OD) through the OD to dry weight biomass (DW) relation. This relation varies between different spectrometers.

Our lab's instrument has a DW/OD conversion factor of 0.471 gram dry weight (gDW) of Chlorella per OD per mL. Therefore, the mole number of assimilated carbon, n_C, is given as:

$$n_C = \Delta OD \times f \times V \times \frac{\mathrm{MW_{Carbon}}}{\mathrm{MW_{biomass}}}$$

Where f is the conversion factor from OD to dry weight per volume, V is the total liquid volume of cultivation flask, $\mathrm{MW_{Carbon}}$ and $\mathrm{MW_{biomass}}$ are the molecule weight of carbon and the dry biomass (equivalent value based on carbon), respectively. The ratio $\mathrm{MW_{Carbon}/MW_{biomass}}$ is directly obtained from the biomass composition measurement.

The value ϕ is therefore calculated as:

$$\phi = \frac{n_{H^+}}{n_C}$$

Another important way to describe this relation is through the imbalanced proton to nitrogen consumption ratio π, which is calculated by the nitrogen composition, y, in the biomass.

Likewise, the moles of assimilated nitrogen, n_N, is given as:

$$n_N = \Delta OD \times f \times V \times y \times \frac{\mathrm{MW_{Nitrogen}}}{\mathrm{MW_{biomass}}}$$

Where nitrogen composition y is used to determine the amount of nitrogen.

Then, the proton to nitrogen ratio is:

$$\pi = \frac{n_{H^+}}{n_N}$$

A typical nitrogen composition in biomass is around 10% of the total dry weight and carbon is close to 50%, therefore the π value is typically 5 folds great than the ϕ value.

Chapter 3

Result & Discussion

The Φ value experiment was performed under two concentrations: 5% CO_2 and 0.6% CO_2. 5% CO_2 is typically used to culture algae with high density. It provides high concentration of dissolved CO_2 in the culture flask as well as higher buffering effect. This buffering effect is a good protection against pH fluctuation due to various reasons, but will affect the measured pH value by masking the changes. Besides, the higher concentration would also affect the growth kinetics of algae, and could enhance its growth.

On the other side, gas with low CO_2 concentration (0.6% v/v), was also used in this experiment to compare the affect of different CO_2 levels. The lower 0.6 % CO_2 will have a lower buffering capacity, allowing the substrate's pH to have higher fluctuations. Though equilibrated CO_2 buffer should not change the stoichiometric relation, it is a good comparison to study how the buffering effect would affect this phenomenon since CO_2 buffering is widely used in many algae cultivation to handle the pH instability.

The algae culture growth was stopped after it reached a certain density, or a correlating OD of 2.5. This OD is below 1/3 of the maximal OD observed under the same growth conditions. This procedure was done in order to guarantee that the algae never reached nitrogen limited conditions, which might potentially produce inappropriate data.

An initial sample of the culture is taken right after the culture flask is set up and inoculated, but is discarded in the calculations since certain amount of acid/base will be used to counter the CO_2 buffer before pH could reach the equilibrium at 6. All the data use the first sample after a certain amount of stable growth.

A summary of our measured π value and ϕ value are listed in table 3-1. Basically two different methods are used to analyze the result: end point comparison and regression analysis. These two methods are used based on difference considerations.

Summary and Analysis Methods

Table 3-1. Summary of ϕ and π value of different media under different CO_2 concentration.

Methods	Properties	Media			
		NH4 (positive)		NO3 (negative)	
		5% CO2	0.6% CO2	5% CO2	0.6% CO2
End Point Difference	H^+/N (π)	0.95	0.93	-1.02	-0.96
	H^+/C (ϕ)	0.18	0.18	-0.20	-0.18
Regression Analysis	H^+/N (π)	1.02±0.21	0.92±0.13	-0.93±0.18	-1.06±0.17
	H^+/C (ϕ)	0.19±0.04	0.18±0.02	-0.18±0.03	-0.20±0.03
	Relative Standard Error	21%	14%	19%	16%
	Coefficient of Determination (R^2)	93.8%	97.3%	95.0%	95.3%

End point comparison is used here based on the conservation principle that the difference between start and end point is the cumulative contribution of the whole growth. Due the cumulative contribution, the measurement error at end points has much smaller impact on

analysis than those at single sample point. As a result, the difference of OD between start and end point is used to calculate the π and ϕ value with the amount of used acid/base.

Regression analysis is used based on the fact that the choosing of end point is arbitrary and might affect the result. Therefore, the slope from a regressed line is more reliable.

One could tell from the table that the π value for ammonium based media is quite close to 1 for both CO_2 concentration and analysis methods. On the other side, nitrate based media has π value close to -1, also for both CO_2 concentration and methods of analysis, though the regression result closer to -1 for higher CO_2 cultivation. The ϕ values are close to 0.18 or -0.18 in all cases, due to quantitative relation between the elemental composition of carbon and nitrogen in biomass. This also explains the similarity in π value.

Higher CO_2 concentration apparently led to higher variance as seen in the standard error and R^2 values of the regression analysis. This is not only due to the buffering effect, but also likely due to asynchronization between nitrogen assimilation and cell growth, which will be discussed later in this chapter.

Besides, one should note that there are other sources which also contribute to the instability of the substrate pH, such as the secretion of lactic acid. Though not a dominant factor compared to nitrogen metabolism, these factors would still affect the value of our measurement to a certain extent.

Growth under 5% (high) CO_2 concentration

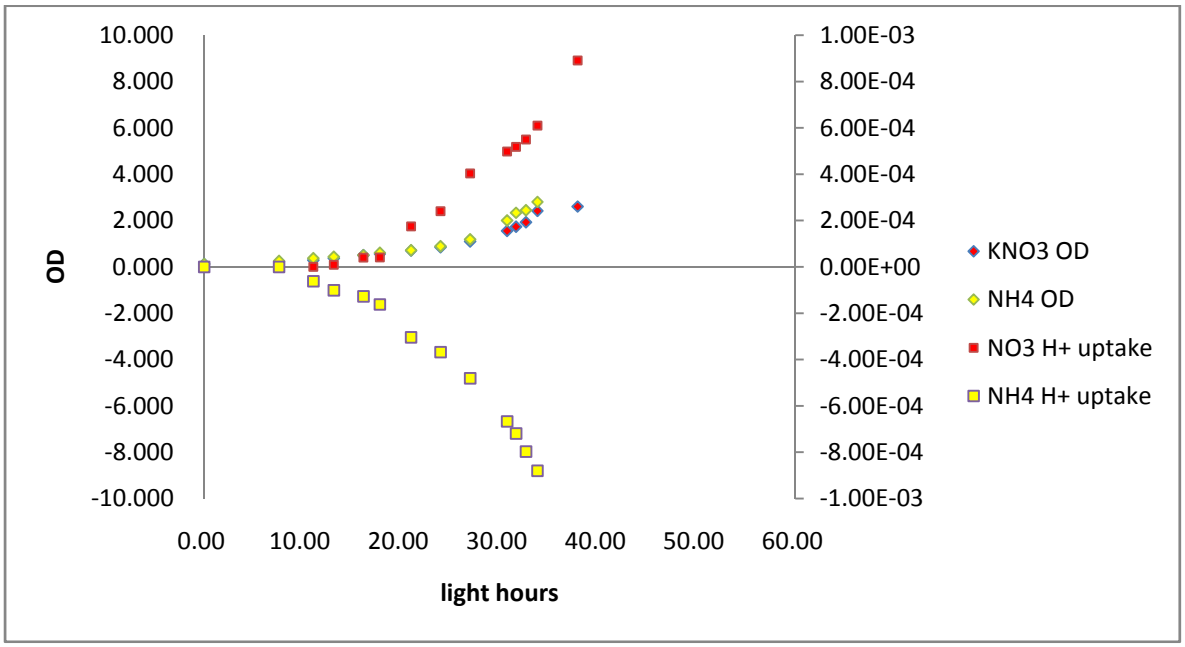


Figure 3-1.OD (diamond) and imbalanced proton (square, right hand side of Y axis, in mole) of acid/base VS light hours for KNO_3 (Red) and NH_4Cl (Yellow) media, under 5% CO_2 gas.

Figure 3-1 shows the optical density data versus growth time under light, as well as the mole amount of imbalanced proton, for both potassium nitrate and ammonium chloride. From the plot, both the H^+ uptaking curves of nitrate and ammonium show similar shape in their growth curve (OD data) except the signs are different. The growth on nitrate based media has a positive proton uptaking while the one on ammonium based media has a negative value. The positive sign indicates that growth of algae under phototrophic growth will have a net uptake of the proton from substrate, therefore make the environment more alkalic, while on the other side, the growth on ammonium base media will have a net secretion of protons, which consequently let the environment become more acidic.

The shapes of algae's growth curves show typical exponential growth behavior, while their corresponding imbalanced proton curves also give such results. The similar curve trend

shows a strong relation between the growths of algae and the amount of imbalanced proton. However, detailed information of their relation has to be read from other following figures.

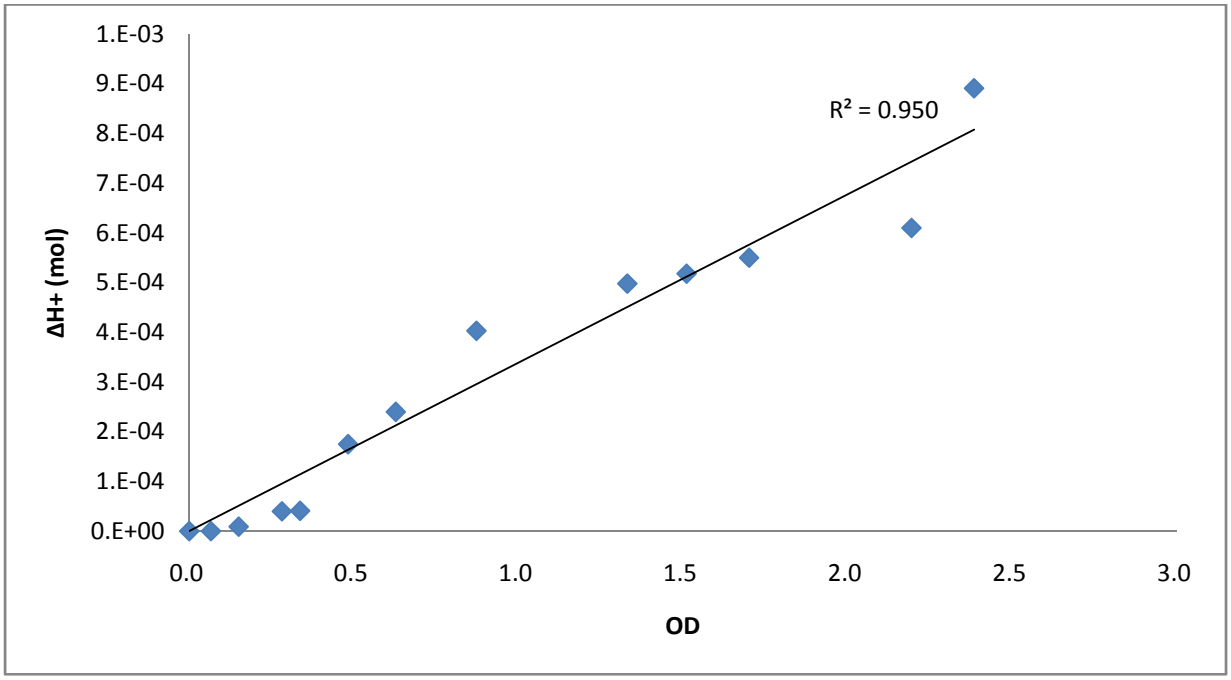


Figure 3-2.Mole number of consumed H^+ vs optical density for growth on KNO_3 media, under 5% CO_2 gas.

Plot 3-2 shows more detailed relation between imbalanced protons versus the growth of algae. The amounts of imbalanced protons are measured through the amount of used counter chemical used to titrate the media back to the pH set point, i.e., HCl for nitrate media or NaOH for ammonium media. From the result shown in Figure 3-2, one could see a generally linear relation, though certain fluctuation exists. The curve shows a wave like fluctuation around the linear fit. This gives a relative standard error of estimate of 19%.

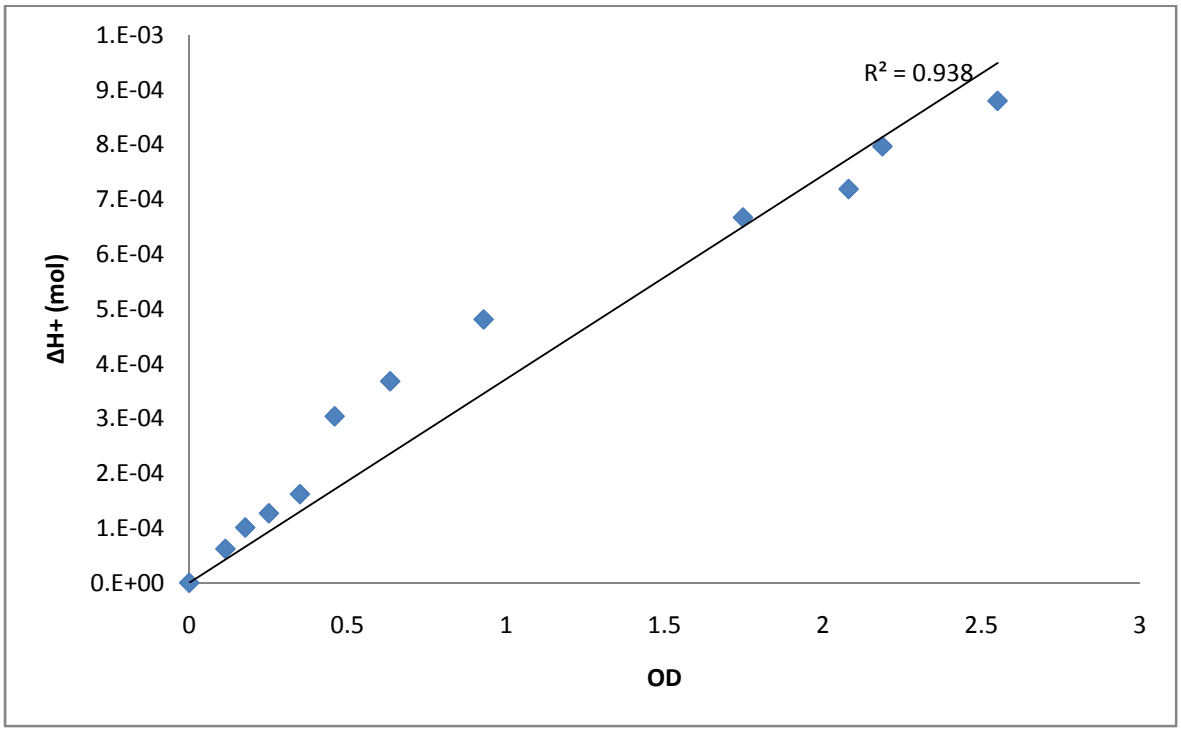


Figure 3-3.Mole number of H^+ released vs optical density for growth on NH_4Cl media, under 5% CO_2 gas.

Plot 3-3 shows the amount of imbalanced H^+ versus the growth of algae for ammonium based media. The sparse data at high OD is due to the faster growth at those periods. The data gives a π value of 1.02 and ϕ value of 0.19 from regression, and a 21% relative standard error, which is the highest among all the experiments.

Growth under 0.6% (low) CO_2 concentration

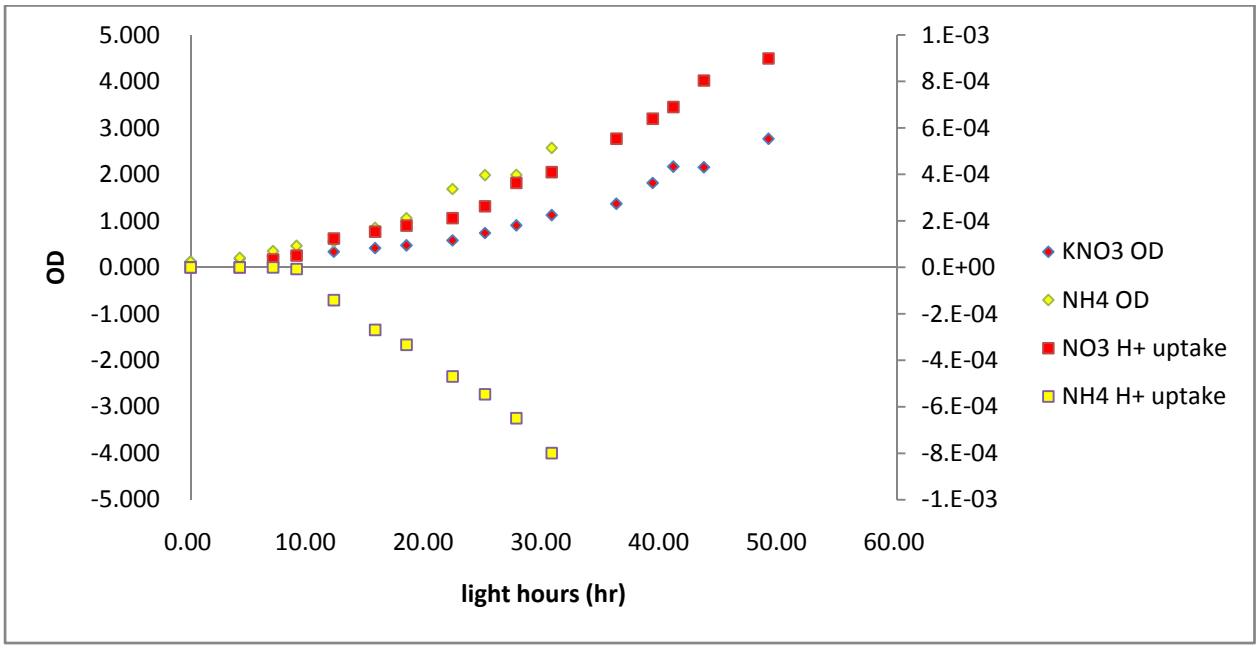


Figure 3-4.OD (diamond) and imbalanced proton (square) of acid/base vs light hours for KNO_3 (Red) and NH_4Cl (Yellow) media, under 0.6% CO_2 gas.

Plot 3-4 summaries the amount of imbalanced proton versus algae growth. The growth on nitrate based media is slower than the ammonium based media, and therefore spent longer time to reach the same cell density. One can also tell the growth on two media generate or consume similar amount of imbalanced proton when reaching the same OD.

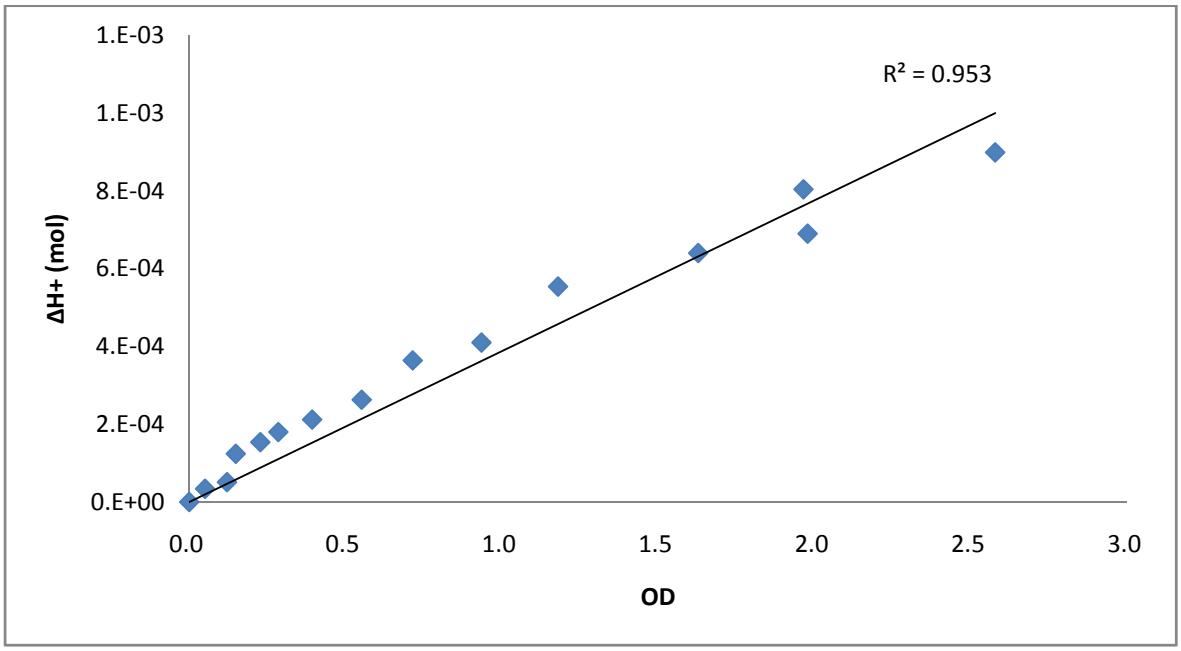


Figure 3-5. Mole number of HCl used vs optical density for growth on KNO_3 media, under 0.6% CO_2 gas.

Figure 3-5 shows the relation between the consumption of H^+ and the change of optical density in a cultivation using nitrate based media under 0.6% CO_2 gas. The fluctuation of data is smaller and the linear regression has a smaller relative standard error (16%) compared with the one in 5% CO2 (19%).

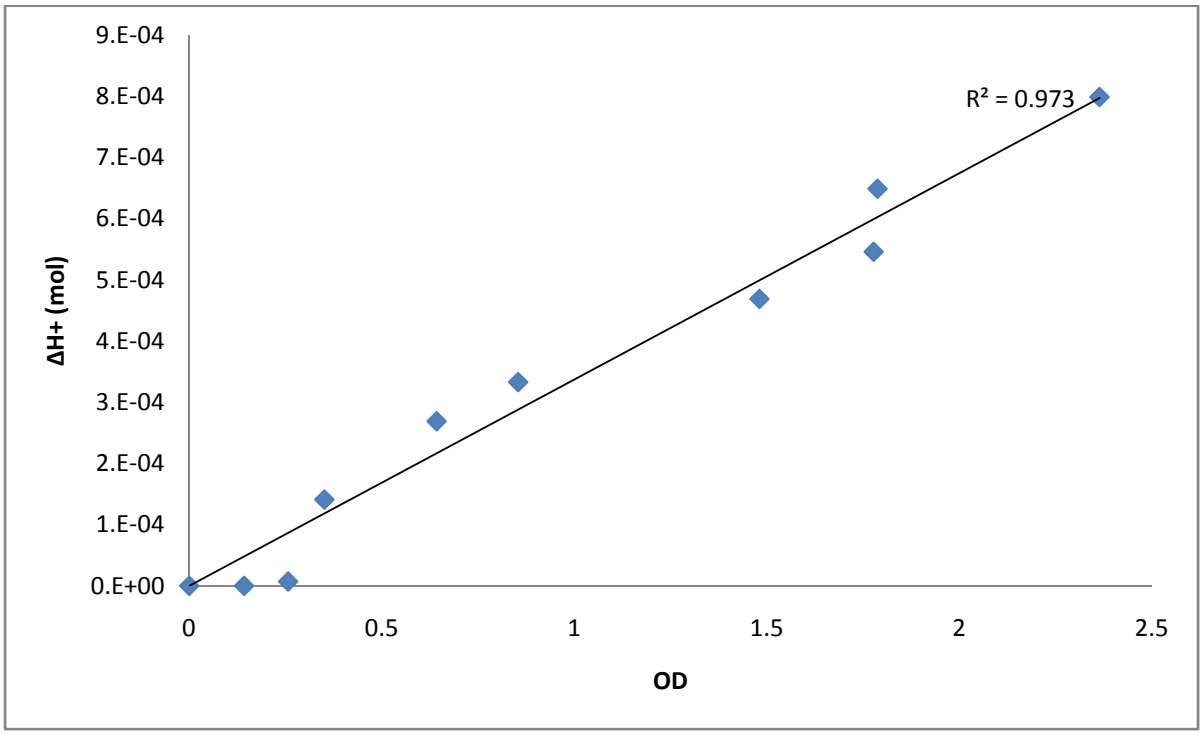


Figure 3-6. Mole number of NaOH used vs optical density for growth on NH_4Cl media, under 0.6% CO_2 gas.

Figure 3-6 shows the relation between the release of H^+ and the change of optical density in a cultivation using ammonium based media. Similarly, this data has smaller relative standard error (14%) compared with the corresponding 5% CO_2 (21%). A wave like fluctuation also appears, but is not significant.

Discussion of the Result

Value of ϕ and π

In general, all experiment shows similar π value and ϕ value, independent from the CO_2 concentration in the gas. For the cultivation on nitrate based media, the assimilation of one nitrate

ion will approximately consume one proton. For the cultivation on ammonium based media, the assimilation of one ammonium ion will generate approximately one net proton.

That is:

$$NO_3^- \xrightarrow{\text{algae}} \text{OH}^- \tag{4-3}$$

$$NH_4^+ \xrightarrow{\text{algae}} \text{H}^+ \tag{4-3}$$

A detailed explanation of experiment results is given in next chapter. For the ammonium case, the amination reaction that fixes the amino group onto the carbon skeleton of oxo-glutarate will introduce a free ionizable proton to the system, therefore causing an imbalanced proton.

For nitrate, since nitrate will be reduced into ammonium before assimilated into algae, the net outcome of imbalanced proton is the summation of the nitrate reduction and the assimilation of ammonium. As discussed in early chapter, the reduction of nitrate into ammonium will cause the generation of two hydroxides. Considering the generation of one proton during ammonium assimilation, the net result of nitrate assimilation will then produce one hydroxide.

Besides, if the above is expressed in the full equation of phototrophic growth:

For ammonium,

$$CO_2 + yNH_4^+ + \delta H_2O \xrightarrow{\text{hv}} CH_xN_yO_z + \lambda O_2 + \text{yH}^+ \tag{4-3}$$

For nitrate,

$$CO_2 + yNO_3^- + \delta H_2O \xrightarrow{\text{hv}} CH_xN_yO_z + \lambda O_2 - \text{yH}^+ \tag{4-4}$$

The Φ value then equals to y for the case of ammonium, and –y for the case of nitrate.

Data Variance

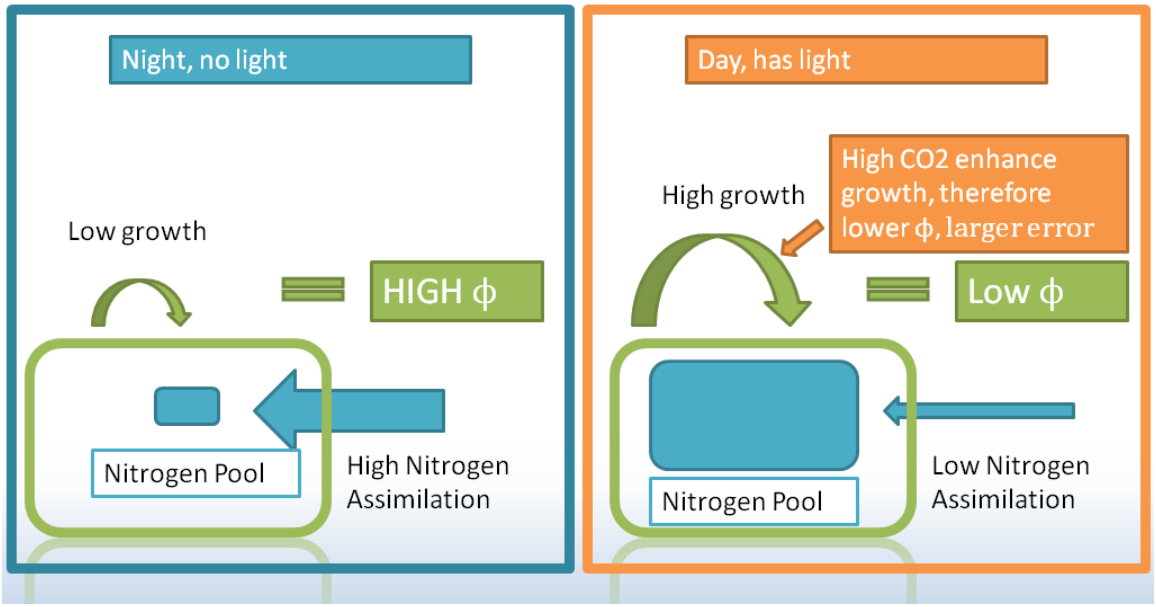


Figure 3-7. Illustration of day-night light cycle, the cause of measurement fluctuation, and difference between experiments under 0.6% CO_2 and 5% CO_2gas concentration.

Besides, the growth under different CO_2 concentration attributes to some of the data variance. However, another explanation can attribute this variance to the asynchronization between nitrogen assimilation and cell growth. This is also the basic assumption of the classical Droop model of algae growth.

Figure 3-7 shows the basic idea of how this fluctuation happens. The Droop model assume algae has a nitrogen pool that could store the nitrogen from the substrate, without assimilating them into active biomass. It is reasonable since bio-available nitrogen is more precious than carbon dioxide in nature. This would make the assimilation of nitrogen relatively independent from biomass growth, and could happen before the real growth of biomass.

To imitate the natural growth condition, our light, as well as CO_2 gas were both turned off for eight hours at night. Nitrogen could still be assimilated at night. After light is turned back on, algae need a period of time to get adapted with the light before photosynthesis could be fully

performed. However, assimilation of nitrogen could still be going on, this could lead to low growth vs high nitrogen assimilation. After the adaption, algae growth would increase significantly. During this period, high concentration of CO_2 would stimulate the growth more than the lower one, therefore algae could grow quickly with the nitrogen already assimilated and consequently show a high growth vs low nitrogen assimilation. Those two phases would cause the fluctuation of measured ϕ value. Theoretically, a lower CO_2 concentration would not make this day time data deviate too much from the stoichiometric relation, therefore a lower measurement fluctuation.

Chapter 4

Imbalanced Proton during Nitrogen Assimilation of Algae

This chapter discusses one explanation of the observed experimental result from the aspect of biochemistry and chemistry. In general, the whole process includes:

1. The assimilation of ammonium into biomass generates an extra proton.
2. The assimilation of nitrate is consisted of two major steps: the nitrate reduction to ammonium and the ammonium assimilation into biomass. The net result is the generation of one hydroxide.

The nitrate reduction is discussed at the beginning since it is relatively straightforward. It is then followed by the ammonium assimilation. In addition, two other nitrogen sources, nitrite and urea, are also discussed due to their theoretical importance.

Proton Imbalance during Nitrate Reduction to Ammonium

When nitrate ion is converted into ammonia, nitrogen has to be reduced from +5 into -3 chemical valence while, this requires 8 electrons per molecule (Aparicio et al. 1994, Ullrich et al. 1998, Azuara and Aparicio 1983, Eisele and Ullrich 1975):

$$N(+5) + 8\mathrm{e}^- = N(-3) \tag{4-1}$$

Additional hydrogen ions are also required to provide the hydrogen in ammonia ion as well as forming water with the oxygen in nitrate:

$$NO_3^- + 8\mathrm{e}^- + 10H^+ = NH_4^+ + 3\mathrm{H_2O} \tag{4-2}$$

Where 6 hydrogen ions are used to form water with the 3 oxygen from nitrate, the other 4 hydrogen ions are used to form the ammonia ion, 8 electrons are used to bring +5 nitrogen into -3 nitrogen.

Now rewrite the 10 hydrogen ions into 8 and 2 ions, the above equation becomes:

$$NO_3^- + 8\mathrm{e}^- + 8H^+ + 2H^+ = NH_4^+ + 3\mathrm{H_2O} \tag{4-3}$$

Then add 2 oxygen molecules on both sides:

$$NO_3^- + 8\mathrm{e}^- + 8H^+ + 2O_2 + 2H^+ = NH_4^+ + 3\mathrm{H_2O} + 2O_2 \tag{4-4}$$

As it is known, the net photoreaction of splitting water can be written in such form:

$$2\mathrm{H_2O} = 4H^+ + 4\mathrm{e}^- + O_2 \tag{4-5}$$

Multiply equation (4-5) by 2, then add with equation (4-4),

$$NO_3^- + 8\mathrm{e}^- + 8H^+ + 2O_2 + 2H^+ + 4\mathrm{H_2O} = NH_4^+ + 3\mathrm{H_2O} + 2O_2 + 8H^+ + 8\mathrm{e}^- + 2O_2 \tag{4-6}$$

The part "$8\mathrm{e}^- + 8H^+ + 2O_2$" appears on both sides will cancel with each other, above reduced to:

$$NO_3^- + 2H^+ + 4\mathrm{H_2O} = NH_4^+ + 3\mathrm{H_2O} + 2O_2 \tag{4-7}$$

By adding 2 hydroxyl groups on both sides, it becomes:

$$NO_3^- + 2H^+ + 4\mathrm{H_2O} + 2\mathrm{OH}^- = NH_4^+ + 3\mathrm{H_2O} + 2\mathrm{OH}^- + 2O_2 \tag{4-8}$$

When combined with the 2 protons on the left hand side, the 2 hydroxyl groups form two water, giving:

$$NO_3^- + 2\mathrm{H_2O} + 4\mathrm{H_2O} = NH_4^+ + 3\mathrm{H_2O} + 2\mathrm{OH}^- + 2O_2 \tag{4-9}$$

Or

$$NO_3^- + 3\mathrm{H_2O} = NH_4^+ + 2\mathrm{OH}^- + 2O_2 \tag{4-10}$$

Proton Imbalance during Nitrite Reduction to Ammonium

Though rarely used as fertilizer, nitrite is an important intermediate molecule during the nitrate reduction to ammonia, and therefore also can be supplied as a nitrogen source. Due to this reason, the assimilation of nitrite is often studied in addition to nitrate and ammonium for comparison.

Similarly, for the case of nitrite media, when reduced to ammonia form, six electrons instead of eight electrons are required to bring nitrogen chemical valence down to negative three:

$$N(+3) + 6\mathrm{e}^- = N(-3) \tag{4-11}$$

Meanwhile, to balance the 2 oxygen atoms in nitrite ion, four instead of six protons are required. By adding the other four protons used in forming ammonia ion, this gives:

$$NO_2^- + 2\mathrm{e}^- + 8H^+ = NH_4^+ + 2\mathrm{H_2O} \tag{4-12}$$

Consequently, rewriting the above equation gives:

$$NO_2^- + 6\mathrm{e}^- + 6H^+ + 2H^+ = NH_4^+ + 2\mathrm{H_2O} \tag{4-13}$$

Likewise, adding 3/2 gas form oxygen molecules on both sides:

$$NO_2^- + 6\mathrm{e}^- + 6H^+ + 2H^+ + \frac{3}{2}O_2 = NH_4^+ + 2\mathrm{H_2O} + \frac{3}{2}O_2 \tag{4-14}$$

The water splitting still holds the same:

$$2\mathrm{H_2O} = 4H^+ + 4\mathrm{e}^- + O_2 \tag{4-15}$$

Multiply above equation by 3/2, then add with above nitrate conversion equation,

$$NO_2^- + 6\mathrm{e}^- + 6H^+ + \frac{3}{2}O_2 + 2H^+ + 3\mathrm{H_2O} = NH_4^+ + 2\mathrm{H_2O} + \frac{3}{2}O_2 + 6H^+ + 6\mathrm{e}^- + \frac{3}{2}O_2 \tag{4-16}$$

The part "$6\mathrm{e}^- + 6H^+ + \frac{3}{2}O_2$" appears on both sides will cancel with each other, above reduced to:

$$NO_2^- + 2H^+ + 3\mathrm{H_2O} = NH_4^+ + 2\mathrm{H_2O} + \frac{3}{2}O_2 \tag{4-17}$$

By adding 2 hydroxyl groups on both sides, it becomes:

$$NO_2^- + 2H^+ + 3H_2O + 2OH^- = NH_4^+ + 2H_2O + 2OH^- + \frac{3}{2}O_2 \quad (4\text{-}18)$$

Likewise, the 2 hydroxyl groups form two water when combined with the 2 protons on the left hand side, giving:

$$NO_2^- + 2H_2O + 3H_2O = NH_4^+ + 2H_2O + 2OH^- + \frac{3}{2}O_2 \quad (4\text{-}19)$$

Or

$$NO_2^- + 3H_2O = NH_4^+ + 2OH^- + \frac{3}{2}O_2 \quad (4\text{-}20)$$

From here, one could see the reduction of one nitrite will also generate two hydroxides in addition to the ammonium. Therefore, from the aspect of imbalanced proton, nitrite reduction has the same result as nitrate, even though they start from different chemical valence.

Biochemistry of Ammonia Assimilation

It has also been generally known that the assimilation of ammonia would also cause imbalanced proton, therefore eventually a change of substrate pH in the long term. Normally, the pH will drop as ammonia is being assimilated. However, detailed biochemical knowledge of this phenomenon is not well studied.

The following section will focus on the generation of imbalanced proton during ammonia assimilation to biomass. However, before digging into the detailed biochemistry of nitrogen metabolism, an ambiguous statement of related phenomenon in soil study needs to be clarified: the ammonia acidification in soil fertilization could be a mechanism that is totally different from the algae cultivation in liquid substrate (Borgognone et al. 2013, Riley and Barber 1971, Tischner 2000).

Related ammonia acidification in soil

Previous study in soil fertilization attributed this to the oxidation of ammonia under the existence of oxygen. Chemically, this is represented as (Lemke et al. 1998, Bloom, Sukrapanna, and Warner 1992):

$$NH_3 + 2O_2 = NO_3^- + \mathrm{H}^+ + \mathrm{H_2O} \quad (4\text{-}21)$$

Or in ion form:

$$NH_4^+ + 2O_2 = NO_3^- + 2\mathrm{H}^+ + \mathrm{H_2O} \quad (4\text{-}22)$$

Actually, this is a reversed reaction of the previously derived nitrate to ammonia conversion.

Another similar issue occurs with urea, which is also a very popular nitrogen source in agriculture,

$$(NH_2)_2CO + 4O_2 = 2NO_3^- + 2\mathrm{H}^+ + CO_2 + \mathrm{H_2O} \quad (4\text{-}23)$$

It is known that there are plenty of bacteria in nature that wound convert ammonium ion into nitrite form and also those that convert nitrite into nitrate.

However, when assimilated, the resulting nitrate still needs to be converted back into ammonia form before being transferred into biomass (Andreev 2001).

Assimilation of Ammonia into Biomass

The dominant nitrogen form is the nitrogen in amino acid or peptide form. In either case, nitrogen generally has to be converted into amino acid. In principle, there are two approaches by which ammonium ions are incorporated: reductive amination of alpha-ketoglutaric acids, and the formation of the amides of glutamic and aspartic acids (Britto and Kronzucker 2002).

Approach One: Direct Amination

The first approach starts with alpha-ketoglutarate, which is the corresponding anion of alpha-ketoglutaric acid. An ammonium ion is fixed onto alpha-ketoglutarate with the existence of NADH, to generate a glutamate and water. The reaction is as follows:

$$NH_4^+ + C_5H_4O_5^{2-}(\alpha - \text{ketoglutarate}) + NADH + \text{H}^+ = C_5H_8NO_4^-(glutamate) + NAD^+ + \text{H}_2\text{O} \quad (4\text{-}24)$$

Approach Two: Two Steps Amination

In algae and plants, a second approach is the adopted for the assimilation of most ammonium ions. This process has two steps. First, ammonium is fixed onto glutamate to generate a glutamine by the hydrolysis of an ATP.

$$NH_4^+ + C_5H_8NO_4^-(glutamate) + ATP = C_5H_{10}N_2O_3(glutamine) + \text{ADP} + \text{P}_\text{i} \quad (4\text{-}25)$$

One also needs to be aware that one water molecule consumed in ATP hydrolysis is just balanced by another water molecule generated during the preceding amination reaction, therefore there is no net generation or consumption of water.

Next, the glutamine will transfer its amino group onto another alpha keto-glutarate to generate two glutamates.

$$C_5H_4O_5^{2-}(\alpha - \text{ketoglutarate}) + C_5H_{10}N_2O_3(glutamine) + 2H^+ + 2e^- = 2\ C_5H_8NO_4^-(glutamate) \quad (4\text{-}26)$$

The net equation will be:

$$NH_4^+ + C_5H_4O_5^{2-}(\alpha - \text{ketoglutarate}) + ATP + 2H^+ + 2e^- = C_5H_8NO_4^-(glutamate) + \text{ADP} + \text{P}_\text{i} \quad (4\text{-}27)$$

Although the above two approaches deviate quite a lot from each other, their net equations could be reduced to similar form from the aspect of proton balance.

First, note that the reduction of NAD^+always generates a proton with NADH, i.e., the following equation:

$$NAD^+ + 2e^- + 2H^+ = NADH + H^+ \tag{4-28}$$

Which is actually:

$$NAD^+ + 2H = NADH + H^+ \tag{4-29}$$

Those two atomic hydrogens are the result of water photolysis. Given that light is sufficient this photolysis process could provide as much NADH/NADPH as necessary without generating imbalanced proton, charge, or electrons.

Back to the amination reaction, substitute this relation to the amination equation. The amination reaction of equation (4-24) becomes:

$$NH_4^+ + C_5H_4O_5^{2-}(\alpha - \text{ketoglutarate}) + NAD^+ + 2e^- + 2H^+ = C_5H_8NO_4^-(glutamate) + NAD^+ + \mathrm{H_2O} \tag{4-30}$$

Which equals to:

$$NH_4^+ + C_5H_4O_5^{2-}(\alpha - \text{ketoglutarate}) + 2e^- + 2H^+ = C_5H_8NO_4^-(glutamate) + \mathrm{H_2O} \tag{4-31}$$

Now add a water molecule on both sides of the amination reaction of equation (4-25), it becomes:

$$NH_4^+ + C_5H_4O_5^{2-}(\alpha - \text{ketoglutarate}) + ATP + \mathrm{H_2O} + 2H^+ + 2e^- = C_5H_8NO_4^-(glutamate) + \mathrm{ADP} + \mathrm{P_i} + \mathrm{H_2O} \tag{4-32}$$

This further simplified into:

$$NH_4^+ + C_5H_4O_5^{2-}(\alpha - \text{ketoglutarate}) + 2H^+ + 2e^- = C_5H_8NO_4^-(glutamate) + \mathrm{H_2O} \quad (4\text{-}33)$$

Therefore, both approaches are equivalent.

It appears that a proton is consumed during this reaction. However, additional discussion has to be addressed on this equation to clarify several important facts here.

First of all, in a photosynthetic growth, the ultimate energy source, as well as reducing power all comes from the photoreaction of water splitting. Therefore, the two protons and electrons ultimately come from the splitting of water. However, details are slightly different: the electron carrier here is NADH instead of the NADPH in water splitting. Those two chemicals can be converted between each other through:

$$NADPH + NAD^+ = NADP^+ + NADH \quad (4\text{-}34)$$

This is catalyzed by NAD $(P)^+$ transhydrogenase enzyme, therefore the net reaction is the splitting of one water molecule and consequentially half oxygen molecule is generated in the chloroplast. As long as imbalanced proton comes with same number of electrons on its side, the overall protons are balanced. Adding half oxygen molecule on both sides, then:

$$NH_4^+ + C_5H_4O_5^{2-}(\alpha - \text{ketoglutarate}) + 2H^+ + 2e^- + \frac{1}{2}\mathrm{O_2} =$$

$$C_5H_8NO_4^-(glutamate) + \mathrm{H_2O} + \frac{1}{2}\mathrm{O_2} \quad (4\text{-}35)$$

Substitute the photolysis of water:

$$\mathrm{H_2O} = 2H^+ + 2e^- + \frac{1}{2}\mathrm{O_2} \quad (4\text{-}36)$$

$$NH_4^+ + C_5H_4O_5^{2-}(\alpha - \text{ketoglutarate}) + \mathrm{H_2O} = C_5H_8NO_4^-(glutamate) + \mathrm{H_2O} + \frac{1}{2}\mathrm{O_2} \quad (4\text{-}37)$$

Cancel the water on both sides:

$$NH_4^+ + C_5H_4O_5^{2-}(\alpha - \text{ketoglutarate}) = C_5H_8NO_4^-(glutamate) + \frac{1}{2}\mathrm{O_2} \quad (4\text{-}38)$$

This means an alpha-ketoglutarate is converted into glutamate by fixing an ammonium ion and generate half oxygen molecule under light.

Now, the left hand side chemical, known as alpha − ketoglutarate$C_5H_4O_5^{2-}$, is actually an anion with two negative charges. These two negative charges are caused by the two negative charged carboxyl groups on both end of its carbon chain, and have the potential to form alpha-ketoglutaric acid when combined with two protons.

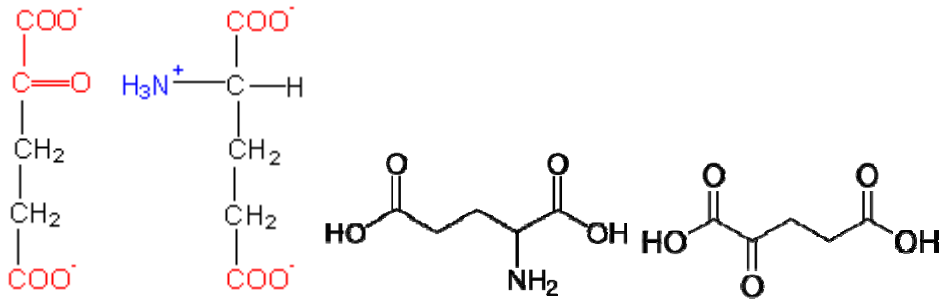


Figure 4-1. Alpha-ketoglutarate, glutamate, alpha-ketoglutaric acid, and glutamic acid

However, the situation is different at the right hand side chemical, glutamate $C_5H_8NO_4^-$, which also has two negatively charged carboxylic group while another positively charged amino-group is attached on the alpha carbon due to the amination reaction. Compared to the uncharged alpha carbon in alpha-ketoglutarate, an extra proton in addition of an amino group is transferred there. This proton is ionizable and could neutralize hydroxyl group in solution:

$$C_5H_8NO_4^-(glutamate) = C_5H_7NO_4^{2-}(glutamate) + \mathrm{H}^+ \quad (4\text{-}39)$$

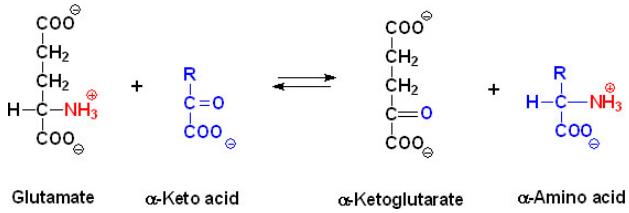
Rewrite the amination of alpha-ketoglutarate into:

$$NH_4^+ + C_5H_4O_5^{2-}(\alpha - \text{ketoglutarate}) = C_5H_7NO_4^{2-} + \mathrm{H}^+ + \frac{1}{2}\mathrm{O}_2 \quad (4\text{-}40)$$

Hence the accumulation of such proton would lead to the overall imbalanced proton.

Transamination between glutamate and other amino acids

Above discussion is only with the synthesis of glutamate. The transamination then spreads the stoichiometric relation of imbalanced proton to the synthesis of all amino acids. Starting from glutamate, the synthesis of other amino acid is achieved by transamination reaction. This moves the amino group in glutamate to other carbon skeleton. One needs to note that this process technically transfers $-NH_3^+$, instead of just a $-NH_2$. In the process, glutamate is converted back into alpha-ketoglutarate by losing its $-NH_3^+$ group. The glutamate/alpha-ketoglutarate system is recycled during the transamination and could be utilized for future ammonium assimilation:



As a result, glutamate does not appear in the net equation of the synthesis of other amino acid, and the net equation could be written as:

$$NH_4^+ + \alpha - \text{keto acid} \xrightarrow{algae} amino\ acid + H^+ \qquad (4\text{-}41)$$

The significance of this reaction is that the imbalanced proton relation spreads to all amino acid rather than only glutamate. With the fact that the dominate form of nitrogen is amino acid or peptide bond, this indicates the assimilation of one ammonium ion into biomass would introduce one imbalanced proton.

If one consider this in another way, suppose the nitrogen is supplied in the form of ammonia instead of ammonium ion, the three hydrogen atoms in ammonia would be barely enough to introduce an neutral amino group on the alpha-carbon without generating net proton, such that:

$$NH_3 + C_5H_4O_5^{2-}(\alpha - \text{ketoglutarate}) = C_5H_7NO_4^{2-} + \frac{1}{2}O_2 \quad (4\text{-}42)$$

Then a possible simpler expression of the whole process would be:

$$NH_4^+ \rightarrow NH_3\ (\text{assimilated}) + H^+ \quad (4\text{-}43)$$

If this proton is eventually secreted into substrate after ammonium ion is consumed, the environment will eventually becomes more acidic.

The whole process is more straightforward in the aspect of pure mass balance of atoms. The net result of alpha-ketoglutarate amination reaction is the reduction of the ketone structure, plus the addition of an amino group and a hydrogen. The first part is achieved by two NADH and two protons, which provides two electrons to reduced the ketone structure, taking out the oxygen and forming a water molecule. The second part of is achieved by using the electrons from NADH and the NH3 from ammonium ion. Combined with photolysis, the whole process eventual equals that alpha-ketoglutarate disproportionate (alpha carbon reduced while oxygen is oxidized) under photo energy by adding ammonium and yielding oxygen gas.

Proton Imbalance in Urea Assimilation

A mass balance study of the urea cycle is performed to support our explanation in the above sections. Net result of urea synthesis is the consumption of two ammonias and one carbon dioxide, plus the generation of one water molecule. With the experimental observation that urea based nitrogen source shows mostly balanced pH during growth, one could draw the conclusion that ammonia instead of ammonium is a balanced nitrogen source.

The mass balance of urea synthesis indicates NH_3 as balanced nitrogen source

Instead of using inorganic nitrogen sources, various cultivations have been design on feeding organic nitrogen source to enhance the growth (Liu et al. 2007). Urea is often known as a nitrogen source that does not cause significant imbalanced proton. By comparing the metabolism with other nitrogen sources, one should be able to estimate the imbalanced amount of proton of urea.

Urea is often generated as a product of catabolism. Its overall reaction equation can be given as:

$$NH_3 + CO_2 + aspartate + 3ATP + 2H_2O = CH_4N_2O(urea) + fumarate + 2ADP + 2Pi + AMP + PPi \quad (4\text{-}44)$$

Note the fumarate is produced from aspartate by removing NH3, therefore above reduces into:

$$2NH_3 + CO_2 + 3ATP + 2H_2O = CH_4N_2O(urea) + 2ADP + 2Pi + AMP + PPi \quad (4\text{-}45)$$

Since PPi (Pyrophosphate) can be decomposed into two phosphoric acids:

$$PPi + H_2O = 2Pi \quad (4\text{-}46)$$

Consequentially:

$$2NH_3 + CO_2 + 3ATP + H_2O = CH_4N_2O(urea) + 2ADP + 4Pi + AMP \quad (4\text{-}47)$$

With the relation that the hydrolysis of ADP creates AMP + Pi,

$$2NH_3 + CO_2 + 3ATP + 4H_2O = CH_4N_2O(urea) + 2ADP + 4Pi + AMP + 4H_2O \quad (4\text{-}48)$$

Which eventually gives,

$$2NH_3 + CO_2 = CH_4N_2O(urea) + H_2O \quad (4\text{-}49)$$

There is no net imbalanced proton during this process. Although this is the catabolism, the same mass balance should hold even if the reaction happens on the reversed direction. This indicates urea would virtually be assimilated as ammonia molecule. Therefore, if during ammonia assimilation the net protons are balanced, the urea assimilation should also be proton balanced.

Non Nitrogen Contributions

Although this experiment indicates that the nitrogen metabolism generally dominates the pH imbalance phenomenon, one needs to note that it is still not sufficient to ignore other possible reasons that also contribute to the change of system pH. It is known that various organism would also lead to pH imbalance while their corresponding nitrogen metabolism does not meet the same situation of this thesis. Similar observation is also obtained in our stand-alone cultivation on urea media. The net imbalanced pH, though small compared other nitrogen source, is not absolutely zero. This could be a result of very complicate combination of a variety of different metabolisms, such as the synthesis of lactic acid and so on. Fortunately, their contributions are small when compared with those of nitrogen metabolism. Therefore metabolic control based on nitrogen is capable to achieve the pH control goal.

Summary of Imbalanced Proton of Four Nitrogen Sources

Based on the above discussion, with the basic equation of phototrophic growth of algae:

$$CO_2 + \psi N_i + \delta H_2O \overset{\text{hv}}{\rightarrow} CH_xN_yO_z + \lambda O_2 + \phi \mathrm{H}^+ \quad (4\text{-}50)$$

One could write the equation with imbalanced proton for four different nitrogen sources.

For ammonium:

$$CO_2 + yNH_4^+ + \delta H_2O \xrightarrow{hv} CH_xN_yO_z + \lambda O_2 + yH^+ \quad (4\text{-}51)$$

Here ϕ is replaced with y, which is the nitrogen composition.

For nitrate:

$$CO_2 + yNO_3^- + \delta H_2O \xrightarrow{hv} CH_xN_yO_z + \lambda O_2 - yH^+ \quad (4\text{-}52)$$

With ϕ replaced with -y.

For nitrite:

$$CO_2 + yNO_2^- + \delta H_2O \xrightarrow{hv} CH_xN_yO_z + \lambda O_2 - yH^+ \quad (4\text{-}53)$$

ϕ is replaced with –y, same as nitrate.

For urea:

$$(1 - \frac{y}{2})CO_2 + \frac{y}{2}CH_4N_2O + \delta H_2O \xrightarrow{hv} CH_xN_yO_z + \lambda O_2 \quad (4\text{-}54)$$

Without net imbalanced proton, ϕ term disappears.

Chapter 5

Future Work: Model based Process Control for Algae Biofuel Production Systems

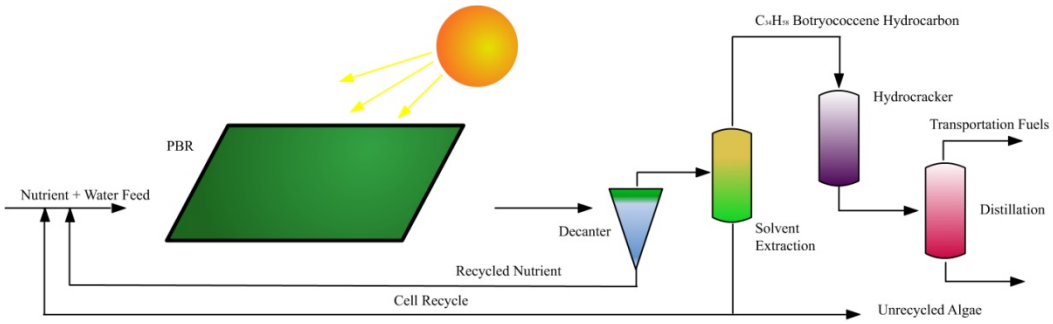


Figure 5-1. Schematic of Continuous Algae Biofuel Production System

Figure 5-1 shows the continuous algae biofuel production system designed for the future scale-up experiment, based on botryococcus, trickle bed reactor and our model based process control system. Compared with many existing bioengineering production systems, it uses continuous operation rather than batch production. This is feasible largely due to the constant high lipid content of botryococcus, which is a quite special organism in algae.

Architecture of the System

The core part is the photo bioreactor (PBR), where algae are growing and lipids are synthesized; Various PBR could be used, such as trickle-bed reactors which have the ability to maintain a high density. Part of the algae culture suspension will constantly flow out from the PBR and be delivered to a decanter where the algae cells are going to be separated from the supernatant. Those botryococcus algae has the ability to excrete lipids outside the cell, therefore

those cells will be then move to a solvent extraction unit. Meanwhile, the other part in the decanter: the supernatant will be recycled back to the PBR since it contains a large amount of unused inorganic nutrient. In the solvent extraction, the active cell part and lipid will be separated, after which the lipid content (botryococcene) will be sent to downstream process such as hydrocracker and distillation. Also, since algae could stay alive after the extraction, part of those living algae will be delivered back into the PBR as a seed to let the system continuously running. The recycling of algae is important since botryococcus (algae) grows slowly and the inoculation cost is very high. The recycled algae, recycled supernatant, plus the fresh feeding nutrient solution will be the input of PBR for continuous production.

Advantages of a continuous system are obvious: easy to scale up, much lower maintenance cost, and easier solution for the recycling of cells and nutrient. However, an essential problem will be how to keep such a system stable so that they could be maintained for long term. This presents a new challenge to process control.

Process Control Problems

Unlike most close system in traditional chemical engineering, the algae biofuel production system is an outdoor system, therefore it is always exposed to various controllability and observability issues.

Disturbance

Sunlight

Due to the nature of renewable biofuel, the energy source has to come from sunlight, which apparently undergoes a day and night cycle. Also, various weather conditions can greatly influence the amount of available sunlight as well. Unlike other conditions such as temperature, the production is much more sensitive to sunlight variation and much consideration has to be expended on it.

Temperature and Humidity

Temperature is also another issue due to the fact of being an outdoor system. It has to get equilibrated with the environment. Actually, temperature is highly related with the sunlight. From the observations in the lab shaker, the temperature in algae culture could be much higher than the environment with the direct radiation from sunlight. In general, algae have high tolerance of temperature, but extreme temperature would also cause damage.

Humidity affects the evaporation rate of photo bioreactor. In lab cultivation, humidity could be controlled by humidifying the gas that passes through the reactor. In the case of outdoor system, the higher evaporation due to lower humidity and sunlight radiation has to be considered to estimate the feeding of water.

Unobservable

Imbalanced proton/hydroxide

Although pH is easy to measure, the actual amount of proton or hydroxide generated or accumulated is almost impossible to accurately predict by comparing two different pH values since the culture suspension is too complicated.

Active biomass

Due to the fact that cell could store nutrient, but not necessarily assimilate them into the biomass, only part of a cell is the active part, therefore only this part should be used to predict the cell's future growth rate.

Nitrogen quota

This is the amount of stored nitrogen in the cell's nitrogen pool described in Droop model (Droop 1973, Droop et al. 1982) . The nitrogen assimilation is largely independent from the growth of whole cell.

Chlorophyll activity

With changes in sunlight caused by the day and night cycles, algae cells need time to adapt to the varying light intensity. Before suitable chlorophyll levels are reached, the cell is not able to grow at its maximal growth rate. There are techniques to measure such factors, but

automatic and continuous measurements might still be a problem due to the slow speed of the assay.

pH control issue

The pH value is a central problem of this thesis. For low density and small scale cell culture, there are several alternative methods to maintain a stable pH.

The first common solution is to use acid or base to control the pH. However, use of acid or base will bring a significant amount of counter ions into the system, which could accumulate and become toxic.

For example, in the later part of this thesis, one would expect the amount of imbalanced proton to be close to the mole number of total nitrogen assimilated into the cell. To maintain ultra high culture, a nitrogen mole concentration close to 0.2 mol/L media is used. If ammonium chloride is fed as nitrogen source and sodium hydroxyl is used to control pH, this could end up with a NaCl concentration close to 0.2 mol/L media. Similarly, if potassium nitrate is used as media and hydrochloride acid is used as the control chemical, the media will have a final KCl concentration of around 0.2 mol/L. Neither of above case is desired to maintain a long term continuous production system.

Another solution is using pH buffer to hold the pH at a certain level. This is effective but not feasible in large scale due to the cost of buffer. Urea media is also used to culture algae and it could automatically keep a much stable culture pH. However, urea itself is an organic chemical, therefore not a suitable choice for the source of renewable energy. Besides, the cultivation on urea would still cause a relatively small change of pH, which may not be due to the proton imbalance during nitrogen metabolism. To build a complete pH control system, one needs

chemicals that could bring pH to any direction on demand while the extent could also be quantitatively estimated.

Considering the above drawbacks, we purposed an approach to use incremental feeding of different nitrogen source to control the pH. The quantitative study of this technique will be the center work of this thesis.

Control Strategy & Controller Structure

Due to the above reasons, a model based process control system has to be implemented in order to maintain continuous algae based biofuel production. This includes three major parts:

1. Process model which describe the status of algae as well as the relation with substrate condition
2. Sensor technique and the ability to continuously acquire up-to-date weather data.
3. Adaptive method to estimate unobservable, measurement noise, prediction error, as well as adjusting kinetics parameters.
4. Constrained optimization method to find the best control solution under uncontrollable outdoor condition.

Control Problem Definition & Controlled Outputs

State variables: cell density (active biomass, lipid content, nitrogen pool); cumulated imbalanced proton (pH); Light adaption index; Nutrient concentration in photo bioreactor.

Input variables: cell density; pH; lipid contents; temperature; humidity; light intensity; nitrogen concentration; weather prediction.

Manipulated variable: nutrient/water feeding rate; recycling ratio of algae.

Disturbance: sunlight; temperature; humidity.

Controlled outputs: include system pH, cell density in photo bioreactor. A control of pH is the central issue to maintain the system. Cell density is controlled to achieve the maximal production in high sunlight days and minimal maintenance cost in low sunlight days.

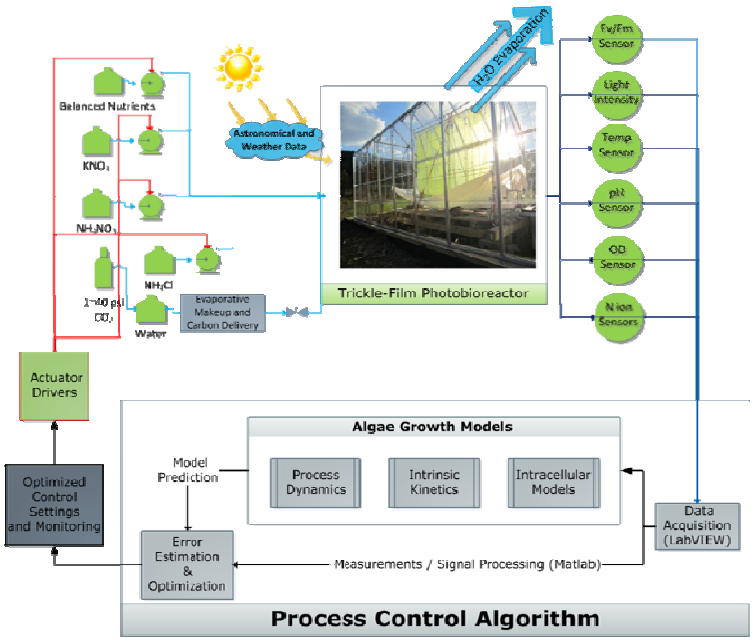


Figure 5-2. Schematic of Model based Process Control for Continuous Algae Biofuel Production System

Process model: photo bioreactor

A modified version of the Droop model is designed to estimate the imbalance pH.

Starting from a recent version of Droop model [ref] is given as a multivariable initial condition problem (Droop et al. 1982). In a heterotrophic growth, the status of algae is described by three state variables: active biomass (x), intracellular nitrogen storage (Q), and lipid storage (P). Their relations are described as:

Active Biomass:

$$\frac{dx}{dt} = \mathrm{ux} - \mathrm{x}\frac{\mathrm{f_0}}{\mathrm{V}} - \mathrm{x}\frac{\left(\mathrm{f_1^i} + \mathrm{f_2^i} - \mathrm{f_0}\right)}{\mathrm{V}}$$

Nitrogen Substrate:

$$\frac{dS_1}{dt} = -\rho\mathrm{x} + \mathrm{S_1^i}\frac{\mathrm{f_1^i}}{\mathrm{V}} - S_1\frac{\mathrm{f_0}}{\mathrm{V}} - S_1\frac{\left(\mathrm{f_1^i} + \mathrm{f_2^i} - \mathrm{f_0}\right)}{\mathrm{V}}$$

Carbon Substrate:

$$\frac{dS_2}{dt} = -\frac{\mathrm{ux}}{\mathrm{Yxs}} - \frac{\pi\mathrm{x}}{\mathrm{Yps}} + \mathrm{S_2^i}\frac{\mathrm{f_1^i}}{\mathrm{V}} - S_2\frac{\mathrm{f_0}}{\mathrm{V}} - \mathrm{k_m x} - S_2\frac{\left(\mathrm{f_1^i} + \mathrm{f_2^i} - \mathrm{f_0}\right)}{\mathrm{V}}$$

Nitrogen storage in cell:

$$\frac{dQ}{dt} = \rho\mathrm{x} - \frac{\mathrm{ux}}{\mathrm{Yxq}} - \mathrm{Q}\frac{\mathrm{f_0}}{\mathrm{V}} - \mathrm{Q}\frac{\left(\mathrm{f_1^i} + \mathrm{f_2^i} - \mathrm{f_0}\right)}{\mathrm{V}}$$

Lipid storage:

$$\frac{dP}{dt} = \pi\mathrm{x} - \mathrm{P}\frac{\mathrm{f_0}}{\mathrm{V}} - \mathrm{P}\frac{\left(\mathrm{f_1^i} + \mathrm{f_2^i} - \mathrm{f_0}\right)}{\mathrm{V}}$$

Liquid volume:

$$\frac{dV}{dt} = \mathrm{f_1^i} + \mathrm{f_2^i} - \mathrm{f_0}$$

Here, x is active biomass (g/L); S_1 is nitrogen substrate concentration (g/L); S_2 is carbon substrate concentration (g/L); Q is nitrogen storage in cell (g/L); P is lipid storage (g/L); V is liquid volume (L). u, π, ρ, are the kinetic coefficients of cell growth, lipid storage and nitrogen storage rates (1/h). $\mathrm{f_1^i}$, $\mathrm{f_2^i}$, $\mathrm{f_0}$ are the flow rate (L/h) of two nutrient sources and outlet, and the $\left(\mathrm{f_1^i} + \mathrm{f_2^i} - \mathrm{f_0}\right)$ will cancel with each other for a continuous system. Yxs, Yxq, Yps being the yield ratio of biomass from carbon, nitrogen and the yield ratio of lipid from carbon. $\mathrm{k_m}$ is the maintenance constant (1/h) of algae (Siegler et al. 2011, Siegler et al. 2012, Surisetty et al. 2010).

In a phototrophic growth, most of the above could hold while certain major modification has to be made. First of all, the carbon source comes from dissolved CO_2 instead of feeding

nutrient. Since the concentration of dissolved CO_2 is in equilibrium with CO_2 in air (discussed in appendix) . The substrate CO_2 will mainly be a balanced result between mass transfer and consumption rate. Second, the coefficient for cell growth rate will be modified to include the effect of light, temperature and pH value, since those are not constant condition as in a close reactor. A modified version of growth rate should looks like:

$$\mu = \mu_{max}(pH, T)\left(\frac{N}{K_N + N}\right)\left(\frac{I}{I + K_{sI} + \frac{I^2}{K_{iI}}}\right)\left(\frac{CO_2}{K_{CO_2} + CO_2}\right)$$

$$\mu_{max}(pH, T) = \mu_{max}\left[\left(\frac{1}{1 + e^{-\alpha(pH - pH_{min})}}\right) + \left(\frac{1}{1 + e^{\alpha(pH - pH_{max})}}\right)\right]\left[\left(\frac{1}{1 + e^{-\alpha(T - T_{min})}}\right) + \left(\frac{1}{1 + e^{\alpha(T - T_{max})}}\right)\right]$$

Here μ_{max} is the maximal inherent growth rate (1/h); N is substrate nitrogen concentration (g/L); I is light intensity ($\mu E/m^2/s$); Term CO_2 denotes the substrate CO_2 concentration (g/L); T and pH are system temperature and pH, respectively; K_N, K_{sI}, K_{iI}, K_{CO2} are all model parameters for kinetics.

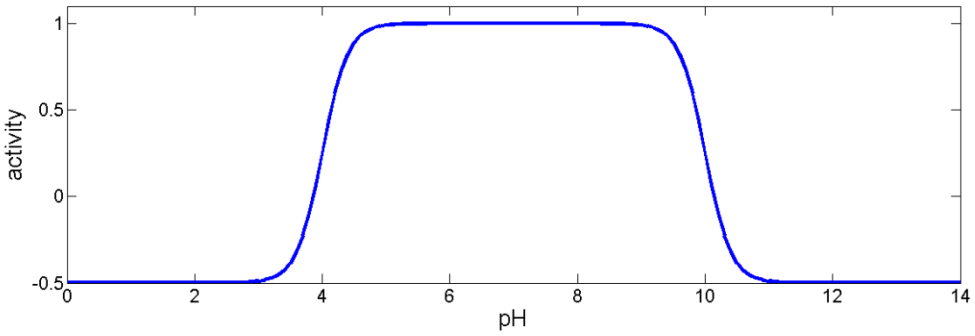


Figure 5-3. Plot of growth activity vs pH.

The four factors on the first row equation are respectively the adjustment according to pH and temperature, nitrogen concentration, light intensity, and CO_2 concentration. The effect of pH and temperature are modeled using combination of Sigmoid function to simulate the plateau shape relation, i.e., growth will not be affected much unless temperature and pH are in

unacceptable range. Figure 5-3 shows an example plot of the effect of pH. The effect of temperature on growth rate is similar as that of pH.

Next, the light adaption index, or more specifically, the chlorophyll activity, is a state variable that is highly dependent on light intensity while a historical or lag effect is included. Detailed equations are still under study, but one simple and rough form of this kinetics could be simulated using a capacitor charging and discharging model that accommodates both historical effect and saturation kinetics:

$$\frac{dA}{dt} = \mathrm{k_{A1}A_{sat}} - \mathrm{k_A IA}$$

Where the adaption of light could be considered as an analogy of capacitor charging, A_{sat} is the saturated chlorophyll activity and k_{A1}, k_A are related kinetic parameters with the relation of $\mathrm{k_{A1}} = \mathrm{k_A I}$. A_{sat} is also a function of the light intensity. When no light is radiated at night, the chlorophyll will gradually lose its activity until totally shut off (discharging). In the day time, activity is recovered (charging). The stronger the light intensity is, the higher chlorophyll activity will be.

Finally, the most important change is the pH control part.

To include the control of pH, one has to introduce another variable, imbalanced proton. We use I_p to express it. Also, due to the fact that we are going to use multiple nitrogen sources, we use different symbols to denote nitrate and ammonium formed nitrogen source.

The cumulated imbalanced proton I_p can be expressed as:

$$\frac{dI_p}{dt} = \phi_{\mathrm{NO3}}\mathrm{ux} + \phi_{\mathrm{NH4}}\mathrm{ux} - I_p\frac{\mathrm{f_0}}{\mathrm{V}} - I_p\frac{\left(\mathrm{f_1^i} + \mathrm{f_2^i} - \mathrm{f_0}\right)}{\mathrm{V}}$$

Here the symbol ϕ_{NO3} and ϕ_{NH4} denote the ϕ value of nitrate and ammonium based media. Due to the preferential assimilation of nitrogen, only one term will be non-zero at a moment.

The amount of imbalanced proton is similar to the analytical proton numbers. However, the imbalanced proton is more flexible so that one could choose the "zero value" to monitor and modify its change over time. The pH value is not used as the major observable of this imbalanced proton since the relation between imbalanced protons and changing pH is too complicated to reach an accurate prediction. However, a titration curve based on imbalanced proton vs pH model will still be adapted as the observation model with a high observation noise. In other word, tracking current accumulation of imbalanced proton is mainly based on the observation of cell growth, and the prediction is indirectly calculated using the ϕ value relation. Process control program will mainly rely on the estimated imbalanced proton as the key indicator for predictive control for future feeding strategy. However, the pH value is still considered to be an observation, but high tolerance range (measurement noise) will be assigned so that the sensor measurement of pH is not heavily weighted unless pH is too far away from desired value.

The advantage of imbalanced proton and optical density based pH control is that the system could avoid the prediction from pH to analytical concentration of imbalanced proton, which is fairly hard. However, the reliability of this approach is highly dependent on the accuracy of the ϕ value, although this parameter could be also used as an adaptable model parameter.

Sensor and weather data

Optical Density Sensor

Besides ordinary sensor such as temperature, pH, light intensity, the use of our special developed optical density sensor, provides a non-intrusive, continuous method to automatically acquire the cell growth density in the reactor and pipeline. This sensor has been developed for

one year as an essential component in our process control. It is now ready for the monitoring of low and medium cell densities, but further testing is required for ultra high density measurement.

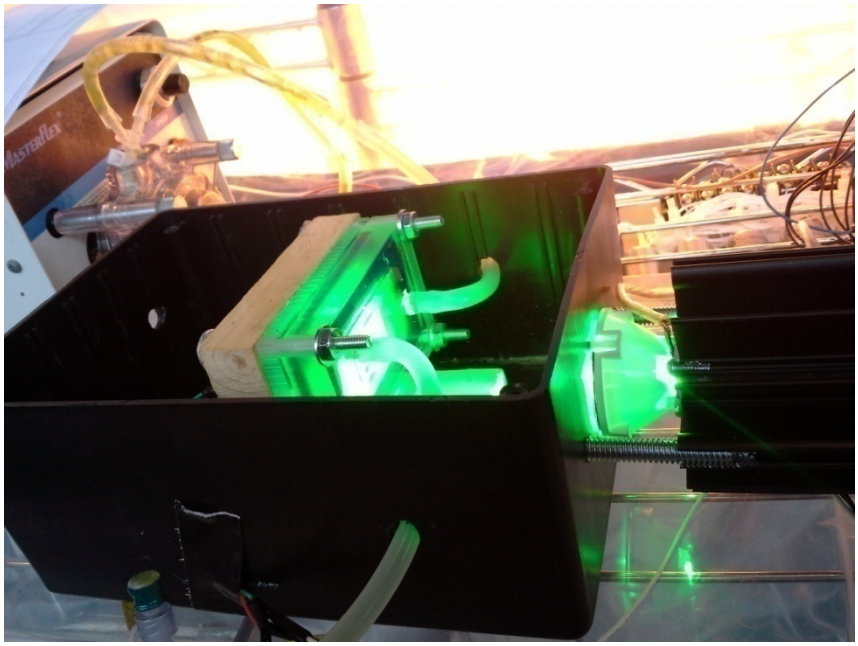


Figure 5-4. Optical density sensor in test period

Internet based Weather Prediction Data

Another important aspect in the predictive control is the up-to-date weather prediction information. A geo-based sunlight model could be used as part of the process program. However, since sunlight is heavily influenced by the actual weather and the weather prediction has a limited time range for high accuracy, external weather predictions based on local current weather measurement are better solutions. There are various Internet based open weather application program interface (API) that can be used to provide the latest weather prediction given the geographic location of the reactor. This data could be accessed simultaneously, formatted by local program, and fed into the process control program to obtain future sunlight and temperature information for making the optimization decision (Robles, Kim, and Kim 2010).

Actuator

Major actuators are the feeding pumps and valves. An outdoor system has many uncontrollable variables, but one aspect that can be controlled is the feeding rate (different nitrogen sources and water), harvesting rate and recycling ratio. This includes:

Feeding pumps for nutrient

The feeding of nutrient mainly affects two aspects: the growth rate of algae and metabolic pH control. In a continuous algae biofuel production system, most algae is recycled and the recycling ratio could be set according to the desired cell density. This has to be in accordance with the nutrient feeding strategy so that the growth of algae could make up the loss of algae. In other words, the algae biomass is at steady state.

In the aspect of metabolic pH control, different nitrogen sources are switched by valves so that various nitrogen sources could be moved into bioreactor on demand.

Feeding pumps for water

Feeding of water affects the biomass concentration of the photo bioreactor. Water is both essential chemical and the hydrogen sources used to synthesize lipid. Also because of evaporation, water has to be sufficiently supplied to prevent the reactor from drying out.

Stream divider for recycled algae

The stream divider is used to control the ratio of recycling of algae. Combined with the feeding of nutrient, this system could control the cell density in photo bioreactor. In the case that

low sunlight would happen in the future days (weather prediction), the cell density in the reactor could be set to low in order to reduce the maintenance cost.

Model Predictive Controller

Formulation

In general, the controller looks for the optimized solution to maximize the lipid production (in a period of future time) as a function of feeding strategy and recycling ratio.

Max $P(\mathrm{N}_1, \mathrm{N}_2, \mathrm{W}, \mathrm{R}, \mathrm{X}, \mathrm{Y})$

Here N_1 and N_2 denote feeding rate of different nitrogen sources; W is the feeding rate of water; R is the recycling rate of algae; X is a vector collection of current system variables (observed and estimated); Y is the vector that includes the input variables from sensors and weather prediction.

Typical time period of the optimization could be a couple of weeks, depending on the actual algae growth rate and local weather condition.

The metabolic pH control is represented as the system constrains:

$$pH(\mathrm{N}_1, \mathrm{N}_2, \mathrm{W}, \mathrm{R}, \mathrm{X}, \mathrm{Y}) < pH_{max}$$

$$pH(\mathrm{N}_1, \mathrm{N}_2, \mathrm{W}, \mathrm{R}, \mathrm{X}, \mathrm{Y}) > pH_{min}$$

The whole system is then a constraint optimization process control. The maximal and minimal allowed pH is set to be a stable and safe pH boundary.

Unscented Kalman Filter as Data Assimilation Method

Unscented Kalman filter is a recently developed non-linear version of the Kalman filter method (LaViola, Aac, and Aac 2003, Anderson 2001). The mathematic detail of unscented Kalman filter is complicated and could be found in professional literature in such fields (Houtekamer and Mitchell 1998, Sinopoli et al. 2004), therefore only brief introduction is given here.

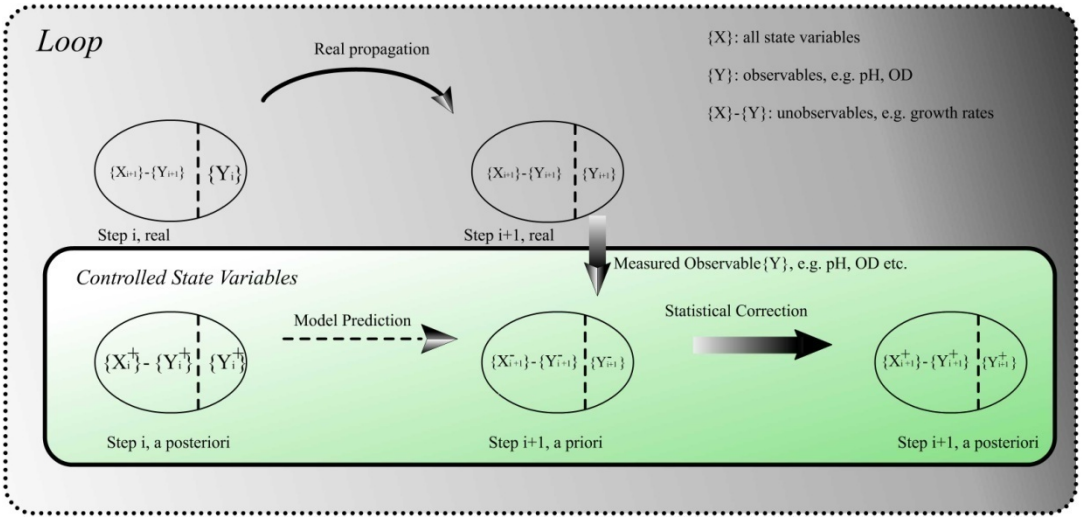


Figure 5-5. Illustration of Principle of Generic Kalman Filter

Figure 5-5 shows the two steps update of the state variables of current process. The basic idea of Kalman filter is to get the best prediction for the future state by combining the result from both model prediction and experimental observation. There are several reasons to use such combination:

1. Process model is not accurate.
2. Measurement uncertainty.
3. Many state variables in the process model are not observable, but derivation from process model would lead to high deviation.

4. Parameters in process model need to be adapted in each run.

Kalman filter method provides a way to update the state variables based on the measurement value. It stores the state variables, which is used to make model prediction, and covariance matrix, which is used to estimate the reliability of current model. Each update of the process state is consisted of two steps. First, the algorithm will make prediction based on the current process model. Next, measured value such as sensor readings will be used to compare with the model prediction. Adjustment of the model prediction will be made using the current covariance matrix, the difference between prediction and measurement, and measurement noise. Another important feature is that model parameters could also be treated as process state variables so that parameters can also be adjusted while the system is running (Daum 2005, Sinopoli et al. 2004, Wan, van der Merwe, and Nelson 2000).

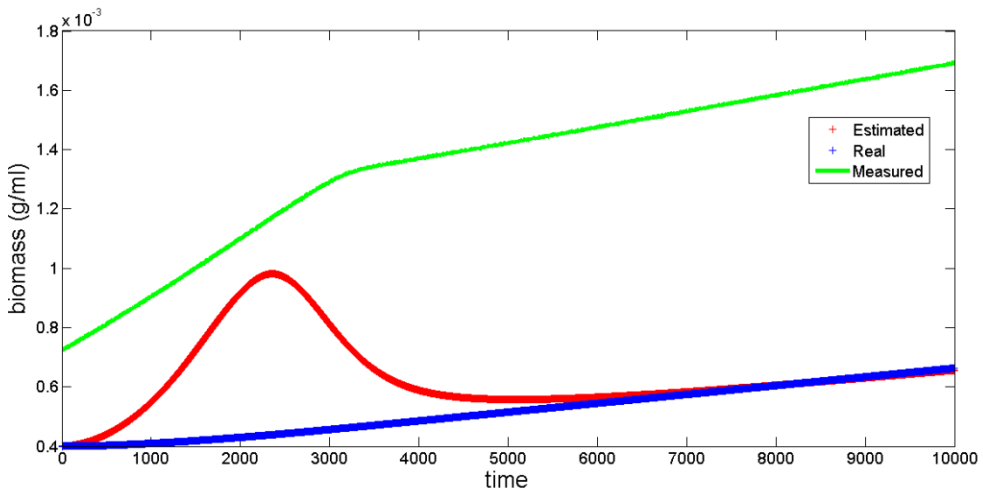


Figure 5-6. Simulation of parameter adaption using unscented Kalman filter based on Droop model. Red is model prediction, blue is the simulated real process, and green is the simulated system observation.

Figure 5-6 shows a simulated example of parameter adaption using Kalman filter. The red curve in the plot is generated by intentionally setting the growth rate coefficient to be much higher than the simulated “real process”. The green curve is the measurement of total dry weight (with red curve being only part of it). Initially, the wrong process model gave a very high

prediction than the actual process. After comparing its results with measurement, the wrong growth rate was then brought to match the results from the measurement. Eventually, the growth rate coefficient was brought to a suitable value and the two different curve overlap.

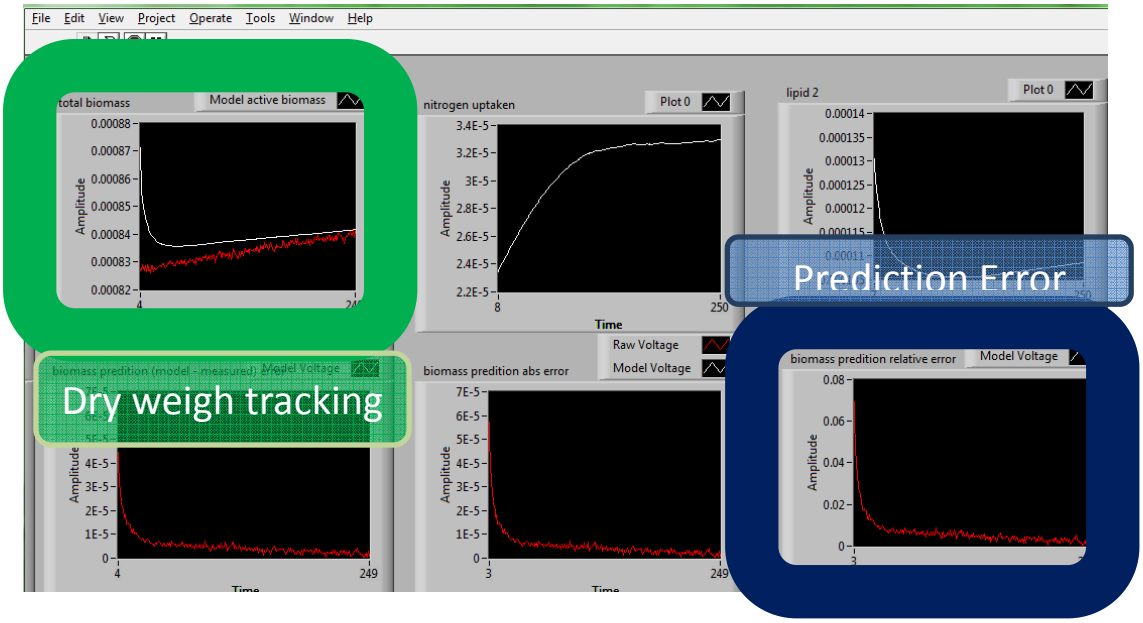


Figure 5-7. Screenshot of LabVIEW in a real running of parameter adaption using unscented Kalman filter based on Droop model.

Figure 5-7 is a real run of parameter adaption using unscented Kalman filter during a algae cultivation in our lab. The red curve are measurement values while the white curve is the model prediction. One could see that the prediction error is drastically reduced after comparing with the real measure. Both state variable and model parameter are modified to converge to a certain value.

Mesh Adaptive Direct Search (MADS) as Optimization Strategy

There are numerous non-linear global optimization algorithms. Mesh adaptive direct search (MADS) is the one algorithm we tried in the current version of lab study (Audet, Savard, and Zghal 2010). The general idea of MADS is to search over on all possible direction of in the search space at a current state point. The distance of each search on each different direction will be adjusted by the searching result. The optimized result is reached when the program cannot find a better point based on current convergence conditions. This algorithm is also implemented in other related algae biofuel optimization research. Future work might be required to find the best available optimization algorithm for our system.

Chapter 6

Conclusion

This work aims at developing an understanding of the requirements for a model-based process control strategy of continuous algae biofuel production system, with a focus on quantitative evaluation of the amount of imbalanced protons in the phototrophic growth of algae. This work relates the macroscopic phenomena of pH change during algae growth to the biomolecular physiology of nitrogen uptake and metabolism, to provide a foundation for a model-based algae pH control system that will be effective for high-density algae cultivation.

We emphasize the following conclusions:

1. The magnitude of proton imbalance during algae growth could be quantitatively defined and experimentally characterized. The net proton uptake or secretion is proportional to the biomass accumulated with a fixed number, and is largely independent of the growth history.
2. One mole of ammonium assimilation will lead to the secretion of a nearly identical molar quantity of protons. We attribute this to the amination reaction that creates ionizable protons while feeding ammonium into protein biosynthesis.
3. One mole of nitrate assimilation will lead to the uptake of roughly one mole of protons. This is due to the generation of two hydroxides during the reduction of nitrate into ammonium and the subsequent generation of one proton during the ammonium assimilation.
4. These results suggest that once a nitrogen source is metabolized to ammonia, the remainder of metabolism is stoichiometrically balanced with respect to proton

uptake and secretion. A different way of viewing this is that the stoichiometry of the metabolism is proton-balanced from the conversion of ammonia to the rather constant composition of biomass. Given the important role of proton gradients in biological energetics, this observation requires additional confirmation with other nitrogen sources (and discussion about its biological relevance).

5. Given the dynamic nature of algae growth conditions such as light, temperature, CO_2 and O_2 levels, some fluctuation of proton imbalance is not unexpected for short-term growth. Some fluctuations are observed in this work for the 'artificial' growth of a 16-hr photoperiod and removal of CO_2 at night. However, when viewed over an extended batch growth period, these values fluctuate around the long term value. We expect that the majority of process control can be achieved through modeling of the overall stoichiometry, with minor fluctuations being managed through adaptive process control algorithms.
6. The predictability of nitrate versus ammonium uptake is key to implementing such a control strategy, with the greatest concern being the loss of predictability of uptake if CO_2 becomes limiting (which imposes more complex regulation) and even secretion of nitrogen. Therefore the work presented here for stoichiometry will have to be integrated into an overall process design and photobioreactor operation model that includes CO_2 and O_2 gas transport.
7. The knowledge of nitrogen use stoichiometry in this thesis provides a more rational basis for examining ultra-high density algae culture systems where nitrogen is the dominant contributor to counter-ions. The accumulation of counter-ion salts can be managed with NH_4NO_3 and other forms of nitrogen, while including an understanding of the overall system time constants of mixing and nutrient uptake.

8. This work has therefore set the stage for a model-based process control system for continuous algae biofuel production that ranges from metabolic use of nitrogen to overall bioprocess design and implementation.

These observations are based on experiments using *Chlorella vulgaris*, but are expected to be generally applicable to eukaryotic microalgae in general as a result of what appears to be conserved genetic basis of nitrogen use. Similarly, the biochemical explanation for the proton imbalance is based on pathways toward nitrogen assimilation to proteins that are common to most photosynthetic organisms. While the stoichiometry would be expected to be similar for cyanobacteria, it is not clear that the nitrogen use regulation is similar due to the organization of nitrogen assimilation genes on operons. Ongoing work is being done in examining nitrogen regulation in cyanobacteria. In addition, these relations would not be applicable to photoheterotrophic growth (using non-CO_2 carbon sources), or conditions of nitrogen fixation from N_2 (which is possible in cyanobacteria that have specialized physiology to avoid the oxygen inhibition of this reaction). None-the-less, since micro-algae are the major candidates for biofuels production, this work is a significant step towards the goal of algae biofuel production. In addition to setting the stage for a bioprocess control strategy, this work illustrates the magnitude of the issue of 'ignoring' the proton balance where the molar exchange of protons is nearly 1:1 with the nitrogen which is the major media component of for algae growth.

Appendix A

Quantitative Speciation Calculation

To determine such a multi variable equilibrium system, speciation calculation is used to obtain numerical result.

Mass balance of ions in aqueous system

First of all, the conservation law of each element and charge has to be met. The conservation of element (or mass balance) means the total analytical concentration of each element should equate to the sum of all forms of chemicals with such element. In general, it can be written as:

$$[A]_{analytical} = k_1[A_1] + k_2[A_2] + \cdots + k_i[A_i] \qquad \text{(A-1)}$$

Where 1 to i represents different chemical forms that contains element A, k is coefficient for the chemicals that have different number of element A from the form where its analytical concentration is given.

For example, the mass balance of the carbon from carbonic acid will be as follows:

$$[H_2CO_3]_{analytical} = [H_2CO_3] + [HCO_3^-] + [CO_3^{2-}] \qquad \text{(A-2)}$$

In this case, all chemicals on the right hand side come from the left side. Carbon is actually chosen as the element to write the conservation equation.

One thing should be noted is that: If carbonate is chosen as the conserved unit, the above equation will not change, since there are equivalent and indicate certain redundancy. Therefore, one could write the mass balance in multiple ways, but only one non-redundancy form will be used to solve the problem.

Typically, the relation between concentrations of different forms of the same chemicals is given by the equilibrium constant. For the case of carbonic acid, one has:

$$[H_2CO_3] = \frac{[H^+][HCO_3^-]}{K_{C1}} \qquad \text{(A-3)}$$

$$[HCO_3^-] = \frac{[H^+][CO_3^{2-}]}{K_{C2}} \qquad \text{(A-4)}$$

Combine them,

$$[H_2CO_3] = \frac{[H^+]^2[CO_3^{2-}]}{K_{C1}K_{C2}} \qquad \text{(A-5)}$$

Substitute this back into above mass balance equation (A-2),

$$[H_2CO_3]_{analytical} = \frac{[H^+]^2[CO_3^{2-}]}{K_{C1}K_{C2}} + \frac{[H^+][CO_3^{2-}]}{K_{C2}} + [CO_3^{2-}] \qquad \text{(A-6)}$$

Now the right hand side becomes a function which only depends on concentration of carbonate and pH. Since the analytical concentration is known, the above equation (A-6) becomes a simpler algebra non-linear equation:

$$f([CO_3^{2-}], [\mathrm{H}^+]) = 0 \qquad \text{(A-7)}$$

Similarly, if phosphoric acid presents, there will be:

$$[H_3PO_4]_{analytical} = \frac{[H^+]^3[PO_4^{3-}]}{K_{p1}K_{p2}K_{p3}} + \frac{[H^+]^2[PO_4^{3-}]}{K_{p2}K_{p3}} + \frac{[H^+][PO_4^{3-}]}{K_{p3}} + [PO_4^{3-}] \qquad \text{(A-8)}$$

Or

$$g([PO_4^{3-}], [\mathrm{H}^+]) = 0 \qquad \text{(A-9)}$$

Note that this equation is cubic with respect to proton concentration, due to the fact that phosphoric acid could be dissociated in three steps.

For each ligand, there should be one independent mass balance equation.

Charge balance of ions in aqueous system

Finally, charge balance has to be meet, which usually gives another equation:

$$k_{p1}[p_1] + k_{p2}[p_2] + \cdots + k_{pi}[p_i] = k_{n1}[n_1] + k_{n2}[n_2] + \cdots + k_{nj}[n_j] \quad \text{(A-10)}$$

In other words, sum of the charge from all cations should equate to the sum of the charge from all anions.

For example, an aqueous solution with carbonic acid and phosphoric acid has:

$$[H^+] = [HCO_3^-] + [CO_3^{2-}] + [H_2PO_4^-] + 2[HPO_4^{2-}] + 3[PO_4^{3-}] + [\mathrm{OH}^-] \quad \text{(A-11)}$$

By using the equilibrium constant, the above equation becomes:

$$[H^+] = \frac{[H^+][CO_3^{2-}]}{K_{C2}} + 2[CO_3^{2-}] + \frac{[H^+]^2[PO_4^{3-}]}{K_{p2}K_{p3}} + 2\frac{[H^+][PO_4^{3-}]}{K_{p3}} + 3[PO_4^{3-}] + \frac{\mathrm{Kw}}{[\mathrm{H}^+]} \quad \text{(A-12)}$$

Or,

$$h([CO_3^{2-}], [PO_4^{3-}], [\mathrm{H}^+]) = 0 \quad \text{(A-13)}$$

By combining mass balance and charge balance, the equations are closed, therefore they could be solved numerically as a non-linear (more specifically, high order polynomial equations) algebra equations problem, and the Newton-Raphson method is typically used. To make iteration more stable, ion concentrations are often converted into their minus logarithm form. When solved, the concentration of each ion, as well as the pH will be obtained.

For simplicity, the above equations are referred as speciation equations in this paper.

Appendix B

Buffering Effect in Algae Culture

An important consideration is the theoretical accuracy of our method in a buffered growth media. The most difficult part for qualitative measurement of the ϕ value is minimizing the interference from complicated chemical properties of algae culture. From both theoretical calculation and experimental observation, carbonate is the dominant buffer system in algae culture. Other buffering systems, such as phosphoric salts, as well as organic ligand generated by algae also exist and contribute to the complexity of the culture media. Theoretical consideration and a speciation calculation are performed in this appendix. The result shows that disturbance from other organic acids or bases secreted by algae does not cause large measurement error as long as nitrogen based proton imbalance is the dominant factor.

CO_2 buffering

Start from the simplest case, a pure chemical carbonate buffering system mainly involves the following chemicals:

$$CO_2(\mathrm{g}) \leftrightarrow CO_2(\mathrm{l}) + \mathrm{H_2O} \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow H^+ + CO_3^{2-} \qquad \text{(B-1)}$$

Gas phase CO_2 concentration usually fixed at the determined value of the ambient equilibrium CO_2 concentration for the specific growth condition. The liquid phase is generally a diluted solution, therefore the dissolved concentration is associated with gas phase CO_2 through Henry's law:

$$CO_2(\mathrm{l}) \rightleftharpoons k_{Henry} p_{CO2} \qquad \text{(B-2)}$$

The above equilibrium is valid only when the equilibrium is rapid relative to the transport and consumption of CO_2, which can be modeled as:

$$\frac{dCO_2(l)}{dt} = k_L a(CO_2^*(l) - CO_2(l)) \qquad \text{(B-3)}$$

With the following equilibrium reactions:

$$CO_2(\text{l}) \rightleftharpoons H_2CO_3 \qquad \text{(B-4)}$$

$$H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \qquad \text{(B-5)}$$

$$HCO_3^- \rightleftharpoons H^+ + CO_3^{2-} \qquad \text{(B-6)}$$

With equlibirium equations:

$$K_{C1} = \frac{[H^+][HCO_3^-]}{H_2CO_3} \qquad \text{(B-7)}$$

$$K_{C2} = \frac{[H^+][CO_3^{2-}]}{HCO_3^-} \qquad \text{(B-8)}$$

Other buffering

When other buffering is present, there will be many other dissociation equilibriums; take phosphoric acid as example:

$$H_3PO_4 \rightleftharpoons H^+ + H_2PO_4^- \qquad \text{(B-9)}$$

$$H_2PO_4^- \rightleftharpoons H^+ + HPO_4^{2-} \qquad \text{(B-10)}$$

$$HPO_4^{2-} \rightleftharpoons H^+ + PO_4^{3-} \qquad \text{(B-11)}$$

With equilibrium equations:

$$K_{P1} = \frac{[H^+][H_2PO_4^-]}{H_3PO_4} \qquad \text{(B-12)}$$

$$K_{P2} = \frac{[H^+][HPO_4^{2-}]}{H_2PO_4^-} \qquad \text{(B-13)}$$

$$K_{P3} = \frac{[H^+][PO_4^{3-}]}{HPO_4^{2-}} \qquad \text{(B-14)}$$

Other buffer system could be modeled similarly to phosphoric acid.

Imbalanced proton during nitrogen assimilation

For cultivation on nitrate media, assuming imbalanced proton value is proportional to the assimilated nitrate:

$$NO_3^- \xrightarrow{\text{algae}} \alpha OH^- \qquad \text{(B-15)}$$

The full growth equation should also include the secretion of other chemicals:

$$CO_2 + H_2O + NO_3^- \xrightarrow{\text{algae}} \alpha OH^- + \beta A^- + \gamma B^- + biomass \qquad \text{(B-16)}$$

Where, A^- and B^- are unknown anion or a group of molecules that have net negative charge. For simplicity, we assume that they carry a single net charge. A^- represents the ions that completely dissociated, while B^- represents ions that only partially dissociated and therefore need a pKa value to be fully described. In a biochemistry viewpoint, ion A^- typically could be simple inorganic ions used to exchange with $NO3^-$ in the substrate, and B could be net organic ions that are generated during metabolism.

Finally, the sum of the all three ions should equate to 1.

Similarly, for ammonium media:

$$CO_2 + H_2O + NH_4^+ \xrightarrow{\text{algae}} \alpha H^+ + \beta C^+ + \gamma D^+ + biomass \qquad \text{(B-17)}$$

Where C^+ and D^+ stand for the ions that completely and partially dissociated, respectively.

Note that one important difference between ammonium and nitrate is that ammonium itself is partially dissociated, and is therefore acidic. Considering the dissociation equilibrium of ammonium, or possibly ion C and D generated from algae's metabolism, one has additional equilibrium:

$$NH_4^+ + OH^- \leftrightarrow NH_3 \cdot H_2O \qquad \text{(B-18)}$$

With an equilibrium of:

$$K_{N1} = \frac{NH_3 \cdot H_2O}{[NH_4^+][OH^-]} \qquad \text{(B-19)}$$

Note that hydrolysis is just another form of same ion equilibriums, and its equilibrium does not need to be included in the analysis, otherwise system will be over-defined.

Feasibility of ϕ measurement in algae culture based on speciation calculation

To evaluate the pH behavior of an algae culture's supernatant, we make the following assumptions:

i. System is consisted of two phases: algae cell as solid phase and supernatant as liquid phase
ii. Liquid phase is purely aqueous, i.e., no oil phase.
iii. No fast redox reaction occurs in the supernatant.
iv. Supernatant is dilute and a homogeneous solution.
v. No generation of inorganic sediment .

A complete list of components of our chlorella culturing media can be found in appendix C. The system can be described as a combination of a set of completely and partially dissociated chemicals.

Analysis of reliability of measured ϕ value

From the above equations, with the above assumptions, the presence of buffer could add huge complexity in the mass balance of ions. However, this will not affect the neutralization taking place in the system, as long as the buffer's counter ion completely dissociate. This is because completely dissociated chemical components will not appear in the mass balance, and the

same amount of proton and hydroxyl will neutralize each other. Therefore no change is necessary to any of above mass balance equations. For charge balance, both sides of equation will be added with their counter ions while nothing else needs to be modified. As a result, neutralization between strong acid and base in buffer solution acts as if there is no buffer.

Therefore, for the simplest case, nitrate assimilation can be modeled as:

$$NO_3^- \xrightarrow{\text{algae}} \alpha OH^- + \beta A^- \qquad \text{(B-21)}$$

where A- is completely dissociated, then one can obtain the exact value of hydroxyl generated throughout the process by adding hydrochloric acid to bring the pH back to the original pH (the pH before algae growth), regardless of the buffering effect.

However, if nitrate assimilation also exchange nitrate with partially dissociated components:

$$NO_3^- \xrightarrow{\text{algae}} \alpha OH^- + \beta A^- + \gamma B^- \qquad \text{(B-22)}$$

The amount of hydrochloric acid needed to bring pH back to the original pH is higher than the amount of hydroxyl generated, since B^- will hydrolysis and brings up the pH. Due to the existence of buffer, the amount of acid needed to counter the effect caused by the hydrolysis of B^- varies with the concentration of buffer as well as the rest of the chemicals. This part cannot be exactly evaluated since little is known about the concentration or the pKa value of this B^- component at the time when ϕ is measured. Therefore, the more the partially dissociated components is, the more inaccurate the measurement would be. The situation for ammonium based media is similar except that it must also consider ammonium hydrolysis.

Even so, the scale of such inaccuracy can still be estimated by doing calculation using the concentration of the prepared media. For example, a speciation calculation is performed using WFAMC media, which is the media used in algae culture. B^- is chosen to have a pKa value of

4.75, which is the same value as acetic acid, since B^- is likely to be such an organic component. A summary of the results are given in the following table.

Table 6-1. Percentage of Measurement Error Caused by Weak Dissociation Component.

NO_3^- molar concentration	H^+ used on OH^- (mol)	HCl used on OH^-(%)	HCl used on B^- (%)	pH=6		pH=7		pH=8	
				H^+By B^-	Error	H^+By B^-	Error	H^+By B^-	Error
0.022	0.0176	80%	20%	2.25E-04	1.3%	2.35E-05	0.1%	1.37E-06	<0.1%
0.022	0.011	50%	50%	5.62E-04	5.1%	5.88E-05	0.5%	4.91E-06	<0.1%
0.022	0.0044	20%	80%	8.98E-04	20.4%	9.40E-05	2.1%	8.45E-06	0.2%

This calculation assumes different percentage of the generation of B^- : 20%, 50%, 80%. The amount of acid used to counter the hydrolysis of B^- is calculated in the right side of the table. This amount is also compared with the acid used to counter their corresponding hydroxide as a percentage. Since the dissociation is influenced by pH, three typical cultivation pH are used to include different situation.

For the above calculation, one could draw such conclusions:

1. The error caused by B^- is much less than the percentage of B^-. This is easy to understand since usually only a small portion of B^- could be hydrolyzed. This indicates that the inaccuracy is negligible as long as hydroxyl is the dominant.

2. The accuracy does not change with the amount of nitrate being assimilated. As a result, during one experiment, given the same growth condition, measured ϕ values should not change as the cultivation is going on.

3. Those partially dissociated components could be a source of additional buffer, based on its partial dissociated behavior. This could be a reason of increased algae buffering capability as algae grow (Scherholz and Curtis 2013).

In other words, the measurement error caused by other possible ions generated during the metabolism is negligible in the measurement of nitrate's ϕ value. Similar conclusion could be

derived from the case of titration using base. The amount of base used to counter the generated proton should also be unaffected by the additionally generated ions.

Appendix C

Cultivation Media

B.3: Wayne's Freshwater Algal Medium for Chlorella (1X WFAMC)

	MW	[final]	[stock]	prep / L	/ 250 mL
KNO_3	101.11	--	-na-	2.2	0.55
MR26 Phosphates (50x, 1M) (pH 6.8) 1M				1.3 mL	0.325 mL
K_2HPO_4 (dibasic)	174.18	0.150 g/L	115 g/L		
KH_2PO_4 (monobasic)	136.09	0.059 g/L	44.9 g/L		
pH to 6.8 with KOH or H_3PO_4					
WFAM MICROnutrients (1000x)			g/L stock	1 mL	0.25 mL
H_3BO_3 (boric acid)	61.83	1/1000th or mg/L	1.86		
$MnCl_2 \cdot 4H_20$	197.41		0.54		
$ZnSO_4 \cdot 7H_20$	287.56		0.066		
$ZnSO_4 \cdot H_20$	179		0.0411		
$ZnSO_4$ (anhydrous)	161.47		0.0371		
Na_2MoO_4-$2H_2O$	241.95		0.031		
$(NH_4)_6Mo_7O_{24} \cdot 4H_20$	1235.86		0.0229		
$CoCl_2 \cdot 6H_20$	237.93		0.030		
$CuSO_4 \cdot 5H_20$	249.7		0.0075		
Fe-EDTA$\cdot 2H_20$ (F)	403.1	0.024 g/L	4.0 g/L (4 mg/mL)	6 mL	1.5 mL
After autoclaving add Mg & Ca solutions aseptically					
Magnesium Solution (1M, filter sterilized)			g / 50mL stock	1 mL	0.25 mL
$Mg(NO_3)_2 \cdot 6H_20$	256.41	0.132 g/L	6.6		
$MgSO_4 \cdot 7H_20$	246.5	0.121 g/L	6.03		
$MgSO_4$ (anhydrous)	120.0	0.0588	2.94		
Calcium Solution (1M, filter sterilized)			g / 50mL stock	0.088 mL	0.022 mL
$CaCl_2 \cdot 2H_20$	147	0.0132 g/L	7.5		
$CaCl_2$ (anhydrous)	111	0.0100	5.66		

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