1 Integrated Co-limitation Kinetic Model for Microalgae Growth in 2 Anaerobically Digested Municipal Sludge Centrate

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10 **Abstract**

11 Microalgae have a substantial potential as a renewable energy resource, but sustainable
12 microalgae-based bioenergy production has to find an inexpensive water and nutrient
13 resource. Wastewater has been considered as such a resource to reduce environmental
14 impacts and economic burdens of microalgae cultivation. Understanding of microalgae
15 growth kinetics in wastewater is necessary to improve microalgae productivity for
16 successful implementation of the integrated microalgae-wastewater system. This study
17 presents an integrated co-limitation kinetic model of microalgae growth in anaerobically
18 digested municipal sludge centrate. Nitrogen (N), dissolved carbon dioxide (CO₂)
19 concentrations, light intensity (I), and temperature (T) were selected as major growth
20 factors in the model. The model framework was constructed by combining threshold and
21 multiplicative structures to explain the relationship among these factors. Depending on
22 culture conditions, the integrated model consists of alternative rate expressions for factors
23 selected. These expressions include the Andrews and Monod models for both CO₂ and N,
24 and the Chalker and Muller-Fuega models combined with the Arrhenius equation for

light intensity and temperature. The model was calibrated and validated using batch studies with anaerobically digested municipal sludge centrate as wastewater source. Criteria to select the rate expressions considering limitation conditions include $\text{NH}_4\text{-N} \leq 150 \text{ mg L}^{-1}$, aqueous $\text{CO}_2 \leq 50 \text{ mg L}^{-1}$, and light intensity $\leq 90 \text{ } \mu\text{mol photon m}^{-2} \text{ s}^{-1}$. Above these ranges, the rate expressions considering both limitation and inhibition were applied to the model. The model was shown to have a reasonable growth rate prediction of *Chlorella sp.* under different nutrient levels of the centrate.

Keywords

Microalgae growth, Kinetic model, wastewater, biomass production

Nomenclatures

I	Incident light intensity, $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$
I_{av}	Average irradiance in the culture, $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$
I_e	Average irradiance at the energy compensation point, $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$
I_K	Light Saturation point where is I at $\mu = \mu_{\text{max}}$, $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$
K_{i, CO_2}	Inhibition constant of CO_2 , mg L^{-1}
$K_{i, \text{NH}_4\text{-N}}$	Inhibition constant of $\text{NH}_4\text{-N}$, mg L^{-1}
K_{S, CO_2}	Half-saturation constant of CO_2 , mg L^{-1}
$K_{S, \text{NH}_4\text{-N}}$	Half-saturation constant of $\text{NH}_4\text{-N}$, mg L^{-1}
S_{CO_2}	Aqueous carbon dioxide concentration in the solution, mg L^{-1}
$S_{\text{NH}_4\text{-N}}$	Ammonium nitrogen concentration in the solution, mg L^{-1}
T	Temperature, $^{\circ}\text{C}$
T_{ref}	Reference Temperature (20°C)

49	Y_N	Microalgae yield coefficient; ratio of biomass amount produced per amount
50		of nitrogen consumed, $M M^{-1}$
51	Y_P	Microalgae yield coefficient; ratio of biomass amount produced per
52		amount of phosphorus consumed, $M M^{-1}$
53	d	Diameter of the reactor, m
54	$f(CO_2)$	A function of carbon dioxide concentration
55	$f(I)$	A function of light intensity
56	$f(N)$	A function of nitrogen concentration
57	$f(P)$	A function of phosphorus concentration
58	$f(T)$	A function of temperature
59	k	Attenuation rate, ($0.2 m^2 g^{-1}$)
60	n	The number of the specific growth rates calculated based on the
61		experimental data
62	t_1	time of initial microalgae concentration during exponential growth, day
63	t_2	time of maximum microalgae concentration during exponential growth,
64		day
65	x	Microalgae cell concentration in the reactor, $g m^{-3}$
66	x_1	Microalgae concentrations at initial microalgae concentration during
67		exponential growth, $mg L^{-1}$
68	x_2	Microalgae concentrations at maximum microalgae concentration during
69		exponential growth, $mg L^{-1}$
70	θ	Arrhenius temperature coefficients for growth
71	μ	Specific growth rate, day^{-1}

72	μ_{max}	Maximum specific growth rate, day ⁻¹
73	$\mu_{measured}$	Specific growth rate calculated from experimental results, day ⁻¹
74	μ_{model}	Specific growth rate estimated from the models, day ⁻¹

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76 **1. Introduction**

77 Microalgae-based bioenergy has received considerable interest because of
78 distinctive advantages over other energy crops, including high solar energy yield, high
79 biomass productivity, and low land use [1,2]. However, traditional microalgae cultivation
80 systems face significant challenges due to the high dependency on fertilizer and
81 freshwater as well as difficulties in full-scale bioreactor design [3,4]. To minimize
82 resource consumptions for water and nutrients, microalgae cultivation integrated with
83 wastewater has been proposed [5,6]. Although this integrated system can significantly
84 reduce the operational costs and environmental impacts for both microalgae cultivation
85 and wastewater treatment, the productivity of microalgae is low due to inconsistent
86 nutrient composition in wastewater [7,8]. In addition, the performance of such systems
87 varied with the types of wastewater and microalgae species [6,8,9]. Thus, understanding
88 of microalgae growth in wastewater is the key to optimize the integrated systems for
89 successful implementation.

90 To date, growth kinetic models have been developed by considering the effect of
91 a single limiting factor or multiple limiting factors [10,11]. Due to the recognition of co-
92 limitation, the models considering multiple factors were preferred as they provided a
93 better explanation of the growth which is based on either threshold or multiplicative
94 theories [12-15]. The multiplicative theory assumes that all resources simultaneously
95 affect the overall growth rate, while the threshold theory considers that the overall growth

rate is affected only by the most limited resource among all resources required by cell growth [16]. Kinetic modeling studies have mostly adopted the threshold theory to explain N-P co-limitation on microalgae growth [16-18]. On the other hand, the multiplicative theory was often applied to describe the effect of N, light, and CO₂ on microalgae growth [19-22]. Currently, there is no growth kinetic model that considers major multiple factors including N, P, light, CO₂, and temperature, and their different co-limitation effects in the modeling framework [11]. In addition, the existing modeling studies were conducted under artificial nutrient medium so that most of the studies considered only nutrient limitation conditions for microalgae growth without investigating growth inhibition caused by high nutrient concentrations. In that sense, the application of these existing kinetic models is limited for microalgae growth in wastewater because inhibition of nutrients on the growth may occur for microalgae growth in wastewater.

There have been a few attempts to develop kinetic models for microalgae growth in wastewater conditions [23-27]. Ruiz et al. [23] proposed a kinetic model for wastewater photobiotreatment with microalgae. They applied two different kinetic models for algae growth and nutrient uptake. The growth kinetic model was based on the Verhulst model, also called as the logistic model, while the nutrient uptake model for N and P was based on the Quiroga second-order equation [28]. Both models were validated using experimental data for the growth of *Chlorella vulgaris* in secondary effluent of a conventional wastewater treatment plant. This growth model considered microalgae biomass concentration as the only variable in the model expression without including other growth factors particularly relevant to wastewater. Besides, to apply the growth

model, the parameters ($\mu_{\max}$, $\mu_{N,\max}$, $\mu_{P,\max}$, X_0 , $X_{\max}$, S_{na} , Y_N , Y_P) in both growth and nutrient uptake models have to be determined. Wu et al. [24] developed an integrated kinetic model for growth of *Scenedesmus sp.* LX1 in domestic secondary effluent in open pond systems. They applied the multiplicative theory to describe combined effects of N, P, and light intensity on the microalgae growth. However, previous studies have pointed out that N-P co-limitation on the growth follows the threshold instead of multiplicative relationship [29]. Furthermore, this model does not take into consideration of the effect of temperature which is one of the important factors for the growth [30] and cannot be easily controlled for the open pond systems. Coppens et al. [25] developed a kinetic model based on the multiplicative theory considering inorganic carbon, N, P and light to determine the nutrient recovery potential of nitrate-storing diatoms from marine wastewater. The proposed kinetic model was able to describe the *Phaeodactylum tricornutum* growth in synthetic marine wastewater. However, similar to the limitation of Wu et al. [24], they did not consider the different co-limitation effects of the selected factors in model framework and the impact of temperature for outdoor application. Kasiri et al. [26] developed a non-linear dynamic model that describes the growth rate and uptake rate of *Chlorella kessleri* cultivated in oil-sands process water. The growth model in that study was based on multiplicative theory, including CO₂, light intensity, and phosphate factors, while the nutrient uptake model considered CO₂, phosphate, nitrate and ammonium. Temperature and pH were kept constant and not considered in the growth model. It was concluded that the model adequately described the algal growth rate as well as the uptake rate of selected factors in oil sands process water. However, the criteria for selecting relevant growth factors were not clear and nitrogen as one of the

important growth factors for microalgae was not considered in the growth model [5]. Halfhide et al. [27] investigated the performance of photobioreactor for growth of *Chlorella* sp. in anaerobically digested municipal sludge centrate. In their study, both nutrient and light were considered, and light was selected as the most limiting factor in their model based on the threshold theory. However, previous studies suggested that nutrient and light follows the multiplicative instead of threshold relationship. Also, since temperature was not included, this model is unsuitable for outdoor cultivation system.

These existing kinetic models were developed for the same domain, microalgae growth in wastewater; however, their applications are limited due to specific study conditions. Thus, a new kinetic model, which can be applied to broader conditions, is needed. Another limitation for current kinetic models for microalgae growth in wastewater is that these models fail to consider all important growth factors, including N, P, CO₂, light intensity, and temperature in the model development under wastewater conditions [5]. Furthermore, the previous studies did not consider different co-limitation effects of growth factors in the model framework. Lastly, most of the models for microalgae growth in wastewater were focused on the secondary effluent. According to Prescott [31], green microalgae require higher N and P concentrations than other microalgal species. Besides, the use of high strength wastewaters is economically beneficial because such wastewaters are able to support higher algae biomass concentrations [32] and consequently reduce reactor volume requirements and harvesting costs. Therefore, centrate from dewatering of anaerobic digested sludge as a side stream of municipal wastewater is a better source for microalgae cultivation because it contains very rich nutrients, especially N and P, compared to other wastewaters. Yuan et al. [33]

and Halfhide et al. [27] pointed out that using centrate for algae cultivation will be advantageous for wastewater treatment systems because the centrate, containing high N and P loading (up to 30% for N, 26-90% for P), flows back into the mainstream and negatively impacts the performance of biological treatment in typical advanced wastewater treatment plants [34,35].

In order to fill the gaps mentioned above, this study aims at developing an integrated co-limitation kinetic model for microalgae growth in wastewater, especially the centrate. This kinetic model considered all growth factors relevant to the centrate and different co-limitation effects (threshold and multiplicative) of selected growth factors in the model framework.

2. Methodology

2.1 Model development

Model factor selection: Microalgae growth is a complex process that is affected by environmental factors and nutrition factors in aquatic systems. The environmental factors include illumination, CO₂ level, temperature, and pH, while nutrition factors consist of macronutrients (i.e., N, P, sulfur, potassium, and magnesium) and micronutrients (i.e., iron (Fe), manganese (Mn), cobalt (Co), zinc (Zn), boron (B), copper (Cu), nickel (Ni) and molybdenum) [77].

Considering wastewater as a culture medium for microalgae growth, not only the growth factors mentioned above but also other factors (such as predator) need to be considered. Wastewater typically contains a sufficient amount of macronutrients such as N and P, and micronutrients [5], but the composition of nutrients and their concentrations vary with types of wastewater [36]. Thus, depending on wastewater types, the influence

of the factors on microalgae growth will be different. In this study, the centrate was used as the microalgae growth medium. Due to the characteristics of the centrate, several factors, including heterotrophic growth, heavy metals, predators, and pH, were excluded in the model. Since organic carbon in the centrate is poorly bioavailable [33], heterotrophic growth was not considered. In terms of heavy metals, microalgae are typically tolerant to high level of heavy metals, such as Ti, Pb, Mg, Zn, Cd, Sr, Co, Hg, Ni, and Cu because polyphosphate in the algae enables storage of these metals [37]. As a result, microalgae are used for removal of heavy metals from aqueous solutions [38]. Thus, heavy metals will not significantly affect microalgae growth. Predators may play a role as a potential inhibiting factor for microalgae growth [39,40]. According to Arauzo [41], over 2.5 mg L⁻¹ un-ionized ammonia causes a significant decrease of the zooplankton (Predators) population. Since the centrate usually contains a high ammonia concentration (>300 mg L⁻¹) [44-46], the inhibiting effect of predators is negligible. pH is one of the most relevant environmental factors that affect the growth of microalgae, and most microalgae prefer a neutral pH [5]. Since anaerobic digestion typically maintains neutral pH for methanogen, pH of the centrate is usually neutral which contains sufficient HCO₃⁻ alkalinity [42]. According to Goldman et al. [78], pH in the culture medium can be altered by a biological transformation of nitrogen species (e.g. NO₃⁻, NH₄⁺, and Urea). For anaerobically digested centrate, however, the pH change due to NH₄⁺ uptake should be insignificant because sufficient HCO₃⁻ alkalinity is present in the medium [78]. Thus, pH is not considered as one of the growth factors in this study. In terms of the environmental factors, CO₂, temperature, and light intensity are important for photosynthesis which is directly related to autotrophic microalgae growth. In terms of the

nutrition factors, N and P are the most important macronutrients for microalgae growth because they are cell elements. Therefore, by considering microalgae growth factors and constituents in centrate, CO₂, N, P, temperature, and light intensity were selected as major growth factors in the model development.

Model framework construction: In order to explain the effects of the selected factors on microalgae growth, this study proposed a new integrated co-limitation model framework (Eq. 1), which considers both multiplicative and threshold co-limitations. In terms of N and P, De Groot [29] concluded that they follow the threshold relationship. Thus, in the model, it is assumed that the co-limitation of N and P on microalgae growth was based on the threshold theory, while the effect of other factors follows the multiplicative theory.

$$\mu = \mu_{max} \left((f(N), f(P)) \cdot f(CO_2) \cdot f(I) \cdot f(T) \right) \quad (1)$$

where μ is the specific growth rate; μ_{max} is the overall maximum specific growth rate; $f(N)$ is a function of nitrogen concentration; $f(P)$ is a function of phosphorus concentration; $f(CO_2)$ is a function of carbon dioxide concentration; $f(I)$ is a function of light intensity; and $f(T)$ is a function of temperature.

Since N is more limited than P based on the centrate characteristics, $f(P)$ is eliminated from the overall rate expression, and the integrated model is reduced to Equation 2.

$$\mu = \mu_{max} (f(N) \cdot f(CO_2) \cdot f(I) \cdot f(T)) \quad (2)$$

Rate expression selection for individual factors: Expressions of $f(N)$, $f(CO_2)$, $f(I)$, and $f(T)$ were adopted from existing microalgae growth kinetic models considering only a single factor. The expressions of $f(N)$ and $f(CO_2)$ were selected based on the following assumptions; i) microalgae growth depends on N and aqueous CO₂ concentrations in

wastewaters because intercellular N and C storages are insignificant for the growth [43];
ii) most of the bioavailable N sources in the centrate are in the form of ammonium ($\text{NH}_4\text{-N}$); iii) inhibition of N and CO_2 on microalgae growth may occur because of high concentrations of NH_3 in centrate ($>300 \text{ mg L}^{-1}$) and CO_2 ($>15\%$) [44-46]. Based on the assumptions above, the Andrews model was selected as the mathematical expression for $f(N)$ and $f(\text{CO}_2)$ to explain both limitation and inhibition effect. Although the Monod model is not able to describe the inhibition effect at high concentrations, it is widely used to describe microalgae growth under low and moderate substrate concentrations ($\text{N} \leq 100 \text{ mg L}^{-1}$, CO_2 in mixture gas $\leq 5\%$) with fewer kinetic parameters [47-50]. Thus, the Monod model and the Andrews model were adopted for $f(N)$ and $f(\text{CO}_2)$ expressions under low to medium and high substrate concentrations, respectively.

In terms of selecting expressions for $f(I)$, the following assumptions were made: i) the cultivation systems contain high concentrations of microalgae because that is desirable for bioenergy feedstock production, and ii) microalgae cultivation systems are under outdoor conditions in order to reduce the energy cost from artificial illumination. Considering the first assumption, light limitation on microalgae growth may occur due to light attenuation caused by high density of the microalgae cells as well as high chromaticity of wastewaters. Among existing models considering light intensity, it was reported that the Chalker [51] model was the best model to describe light limitations under low and medium light intensity conditions [52]. Considering the second assumption, photoinhibition for microalgae growth occurs during the central hours of the daylight period [53]. According to Martínez et al. [54], the Muller-Feuga [55] model

provided a good description of photoinhibition at high light intensity. Thus, depending on the level of light intensity, the Chalker or Muller-Feuga model was adopted for $f(I)$.

In terms of expressions for $f(T)$, the Arrhenius equation is most commonly used to describe the effect of temperature on microalgae growth [10,56-58]. Thus, in this study, the Arrhenius equation was used for $f(T)$ and combined with expressions for light intensity since the temperature in cultivation systems will be affected by radiant heat [59].

The selected expressions were summarized in Table 1. In the integrated model, each factor has two possible expressions. Thus, depending on the initial cultivation condition, one of expressions for each factor was selected for the integrated model.

<Table 1>

2.2 Experimental methods

Microalgae and culture medium: The Anaerobically Digested municipal sludge Centrate (ADC) was used as the culture medium in the experiments. The ADC was collected from the Northeast Water Reclamation Facility (located in Clearwater, FL). The characteristics of ADC are shown in Table 2. In order to prepare the ADC culture medium, glass fiber filters with pore size of 0.45 μm (Fisher Scientific; Pittsburgh, PA, G4) were used to remove particulates in the ADC. The filtered ADC was stored in a refrigerator at 4°C before the cultivation experiment.

Chlorella sp. was harvested from a secondary clarifier at the Howard F. Curren Advanced Wastewater Treatment Facility in Tampa, FL. The *Chlorella* sp. was initially cultivated in the Bold 1 NV medium [60] and then cultivated in the medium containing 50% filtered ADC and 50% deionized water for adaptation before switching to 100% filtered ADC medium. The *Chlorella* sp. was inoculated at 22±1°C in a temperature-

controlled room in 1L Erlenmeyer flasks with a working volume of 500mL. The cultures were kept suspended by aeration (0.03% CO₂). A 24-hour continuous light (about 2000 lux) was provided by 13W fluorescent lamps.

<Table 2>

Experimental set-up: The experiments were conducted in 1.2 L batch-type photobioreactors containing 900 mL of the medium for 7-14 days. During the experiment, a sterile CO₂-air mixture (with a flow rate of 300ml/min) was supplied to the culture through a fine bubble diffuser. The reactors were illuminated by 13 W fluorescent lamps (24:0 h light-dark cycles) located outside of the reactor to provide the desired light intensity, and the reactors were located in a temperature-controlled room at 22±1°C. For each series of experiments, the initial microalgae concentration (expressed as Chl a) was kept constant around 0.7 ± 0.2 mg L⁻¹. The standard cultivation conditions were: NH₄-N concentration of about 340 mg L⁻¹, light intensity of 5000 lux, and aeration with a CO₂ and air mixture gas (5% CO₂). In order to evaluate the effect of each selected factor, the conditions of the factor of interest was modified, while others were kept the same as the standard condition. The NH₄-N concentration in ADC was varied between 9-586 mg L⁻¹ by dilution (The PO₄-P concentration was maintained by spiking the ADC with Monopotassium phosphate (KH₂PO₄)). The CO₂ and air mixture was varied between 0.003%-15% CO₂. Lastly, illumination was varied between 500-15000 lux. Due to radiation, culture temperature varied from 21°C to 39°C with varying illumination. All kinetic experiments were duplicated, and the samples were collected daily for 7-14 days.

Analytical methods: Since the growth media is the real centrate, there is a possibility to contain bacteria in the centrate. According to Mara [61], chlorophyll a concentration is a

good measure of the amount of microalgae biomass and was used to represent microalgae biomass concentration in the study. Chlorophyll a was analyzed using the ethanol extraction method according to the Dutch Standard [62]. For measurements of NH₄-N, NO₃-N, and alkalinity, samples were filtered through 0.45 µm membranes (No. 6876-2504, Whatman). NH₄-N was measured by a modified Willis et al. method [63], while NO₃-N and alkalinity were measured by Standard methods [64]. The pH was measured by a calibrated Orion GS9156 pH electrode meter (Thermo Fisher Scientific Inc., Waltham, MA). The temperature inside the reactor was measured using Liquid-in-Glass Partial Immersion Thermometers (Thermo Fisher Scientific Inc., Waltham, MA). Incident light intensity (I_0) from the exterior of each reactor was measured using a “30 light meter Onset® HOBO U12 data logger” (Pocasset, MA). Average irradiance (I_{av}) within each reactor was calculated using Equation 3 [54,65].

$$I_{av} = \frac{I_0}{k \cdot d \cdot x} (1 - \exp(-k \cdot d \cdot x)) \quad (3)$$

Where k is the attenuation rate ($0.2 \text{ m}^2 \text{ g}^{-1}$) [77], d is the diameter of the reactor (m), and x is the microalgae cell concentration in the reactor (g m^{-3}).

2.3 Calibration and Validation

Based on the growth curve of *Chlorella sp.* (biomass concentrations vs. time), the specific growth rates were calculated using Equation 4.

$$\mu_{measured} = \frac{\ln(x_2/x_1)}{t_2 - t_1} \quad (4)$$

Where $\mu_{measured}$ is the specific growth rate calculated from experimental results (day^{-1}), x_2 and x_1 are the microalgae concentrations (mg L^{-1}) at maximum and initial microalgae

concentration during exponential growth respectively, and t_2 and t_1 are the time (days) of maximum and initial microalgae concentration during exponential growth.

Parameters of the integrated model from Table 1 were calibrated sequentially to minimize overfitting issue. Figure 1 shows a flow chart of the process to determine the criteria for selecting rate expressions and kinetic parameters for the integrated model. This process can be divided by two steps. Step 1 mainly focused on determination of kinetic parameters for selected rate expressions using experimental data. Step 2 is to determine limitation criteria and an overall maximum growth rate (μ_{max}). In step 1, the kinetic parameters were determined by fitting the selected models to experimental data. These parameters were estimated by minimizing an Objective Function (OF, Eq. 5) using the Solver add-in in Microsoft Excel (Generalized Reduced Gradient Nonlinear solver tool):

$$OF = \sqrt{\frac{1}{n-1} \sum_{i=1}^n (\mu_{measured} - \mu_{model})^2} \quad (5)$$

where μ_{model} is the specific growth rate estimated from the models (day^{-1}) and n is the number of the specific growth rates ($\mu_{measured}$) calculated based on the experimental data.

In step 2, the criteria to select the appropriate expression between two options for each factor was selected, and then the μ_{max} in the integrated model was determined using other 20 data points. In this step, the kinetic parameters determined in step 1 were applied. The range of the criteria for different growth factors was adopted from the literature, including 150~300 mg L^{-1} for N, 40~220 mg L^{-1} for aqueous CO_2 , and 70~200 $\mu\text{mol photon m}^{-2} \text{ s}$ for light intensity.

<Figure 1>

Once the model was calibrated, predicted growth rates using the integrated kinetic model were compared to calculated growth rates from another set of experimental data to evaluate whether the model can represent the real system. The goodness of fit (R^2) of more than 0.8 is generally considered as a good degree of agreement between simulation and experimental data in the case of microalgae growth simulations [66].

3. Results and Discussions

3.1 Microalgae growth in the centrate

Figure 2 shows microalgae growth under different culture conditions with varying initial $\text{NH}_4\text{-N}$ concentrations, aqueous CO_2 concentrations and light intensities. In this study, *Chlorella* sp. showed effective growth in the real centrate. Li et al. [67] reported that microalgae (*Chlorella vulgaris*) growth rates in centrate are comparable with those using different types of municipal wastewater (e.g., activated sludge extract, primary settled sewage, and primary clarifier effluent). In addition, they observed the highest final biomass of the microalgae grown in the centrate among different wastewaters.

Figure 2 (a) shows the changes in chlorophyll a concentration at various initial $\text{NH}_4\text{-N}$ concentrations ranging from 9.9-586 mg L^{-1} . It was observed that the final chlorophyll a concentration increased from 1.8 mg L^{-1} to 15.8 mg L^{-1} with the increase in initial $\text{NH}_4\text{-N}$ concentration. Previous study of Aslan & Kapdan [48] observed similar results of the increase of final chlorophyll a concentrations as a result of the increase of initial $\text{NH}_4\text{-N}$ concentrations using *Chlorella vulgaris*. Figure 2 (b) shows the variation of chlorophyll a concentration at different aqueous CO_2 concentrations. The culture with 12.9 mg L^{-1} aqueous CO_2 resulted in the highest final chlorophyll a concentration. In the

study of Mortensen & Gislerød [68], 140 mg L⁻¹ dissolved CO₂ was an optimal condition for growth of *Chlorella sorokiniana* in artificial growth medium at 28°C. Our optimum aqueous CO₂ concentration for microalgae growth is much lower than that of Mortensen & Gislerød [68] because cultivation conditions such as temperature and growth medium are different. According to Beardall & Raven [69], increases in cultivation temperature result in increased metabolic activity and growth of microalgae. Our cultivation temperature was 22±1°C, which is 6°C lower than the study of Mortensen & Gislerød [68] so that lower temperature leads to lower metabolic activity which requires less CO₂. In addition, Mortensen & Gislerød [68] cultivated the microalgae in artificial growth medium containing the essential nutrients under the balanced condition for optimum growth. However, the centrate contains imbalanced nutrients for microalgae growth which may cause nutrient limitation or inhibition.

Figure 2(c) shows the result for variation of chlorophyll a with time at different initial light intensities. Due to radiation from light, the temperature of culture increased from 21°C to 40°C with the increase in initial incident light intensity. In Figure 2 (c), L5 (under light intensity of 77 μmol photon m⁻² s⁻¹) showed the longer lag phase than other conditions, which is mainly due to unhealthy inoculum. The final chlorophyll a significantly increased from 6.8 mg L⁻¹ to 28 mg L⁻¹ with the increase in the light intensity as well as temperature, and the culture with 459 μmol photon m⁻² s⁻¹ (40°C) resulted in the highest final chlorophyll a concentration. As shown in Figure 2, the initial NH₄-N and aqueous CO₂ concentrations as well as initial light intensity significantly affect total chlorophyll a concentration of microalgae, which is in agreement with the results of previous studies [45,70,71].

Based on the experimental data during the exponential growth stage in the growth curves of *Chlorella sp.* (chlorophyll a concentrations vs. time), the specific growth rates were calculated using Equation 4. Under the different initial $\text{NH}_4\text{-N}$ concentrations, the specific growth rates were varied from 0.56 to 1.1 day^{-1} . When initial CO_2 concentrations varied, the specific growth rates changed from 0.76 to 1.18 day^{-1} . Under light intensity conditions of 6.8-459 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$, the specific growth rates varied from 0.22 to 1.73 day^{-1} . Compared to studies of Lam & Lee [8] and Li et al. [67], the growth rates in this study are within the ranges of the previous studies.

<Figure 2>

3.2 Parameter determination

Figure 3 shows the observed μ_{measured} and fitted μ as a function of initial $\text{NH}_4\text{-N}$ concentrations, initial aqueous CO_2 concentrations, and initial I_{av} . It was observed that the Monod and Chalker expressions produced a good agreement with experimental data at low and moderate substrate and light conditions, respectively ($\text{NH}_4\text{-N} \leq 150 \text{ mg L}^{-1}$, $\text{CO}_2 \leq 50 \text{ mg L}^{-1}$, and light intensity $\leq 90 \mu\text{mol photon m}^{-2} \text{s}^{-1}$). The Andrews and Muller-Feuga expressions fit better to the growth rate data at all ranges of $\text{NH}_4\text{-N}$ and CO_2 concentrations and light intensity; however, both require fitting one more parameter. The Monod and Chalker expressions are kept in the integrated model because of their simple mathematical formula and fewer parameters to be determined. The difference should be made, however, in applying these expressions because they were developed based on different assumptions. The Monod and Chalker expressions were developed considering non-inhibitory conditions, while the Andrews and Muller-Feuga expressions were developed considering both limitation and inhibitory conditions.

Estimated kinetic parameter values (highlighted in grey) are shown in Table 3 and compared to values obtained from literature. The half saturation constant in the Monod expression for N was comparable with the values from Kim et al. [72]. They studied N and P removal from municipal secondary wastewater effluent by *Chlorella vulgaris* and determined the maximum specific growth rate (μ_{max} , h⁻¹) and the half saturation coefficient ($K_{S,N}$, mg L⁻¹) in the Monod equation to be 0.01245 h⁻¹ (0.299 d⁻¹) and 0.0696 mg L⁻¹, respectively.

The I_K of the Chalker model in our study was lower than the value of Kurano & Miyachi [52]. They examined the effect of light intensity on microalgae growth in the artificial growth medium, and reported that the growth rate of *Chlorococcum littorale* is in accordance with Chalker model (μ_{max} =0.115 h⁻¹ and I_k =150 μ mol photon m⁻² s⁻¹). Lower I_K value in our study is attributed to the lower light intensity range used in the experiment (0-90 μ mol photon m⁻² s⁻¹) than that of the Kurano & Miyachi's study (2.3-1060 μ mol photon m⁻² s⁻¹). In addition, the differences for cultivation temperature, microalgal species, and nutrient conditions may also cause the difference.

As shown in Table 3, the value of K_{S,CO_2} for the Monod model was in agreement with Novak and Brune [73], who reported that K_{S,CO_2} for *Chlorella* sp. varies in a range of 0.03–0.36 mmol L⁻¹ (1.32–15.8 mg L⁻¹). In terms of the Andrews model for CO₂, the value of K_{S,CO_2} is higher while the value of K_I is lower than those in the previous study of Ketheesan & Nirmalakhandan [22]. They adopted the Andrews model to explain the effect of CO₂ on growth of *Scenedesmus* sp. and *Nannochloropsis salina* in the airlift-raceway reactor using the artificial growth medium. The difference is probably caused by different microalgal species (*Scenedesmus* sp. and *N. salina* in Ketheesan &

Nirmalakhandan [22] vs *Chlorella sp.* in our study), different growth medium (artificial growth medium in Ketheesan & Nirmalakhandan [22] vs real centrate in our study). Growth condition as well as microalgal species affect the growth kinetics [74].

The estimated kinetic parameters for the selected expressions were applied, and then the overall maximum growth rate for the integrated model as shown in Equation 2 was determined using other experimental data. The overall maximum growth rate was 0.7 day⁻¹ (R²=0.82) which is comparable to that (0.677 day⁻¹) from Li et al. [67] (growth of *Chlorella sp.* in centrate). The R²s between the experimental and the predicted growth rates at different culture conditions are all above 0.8 (Figure 3).

<Figure 3>

<Table 3>

3.2 Model performance

Figure 4 presents the comparison of the growth rates predicted by the integrated model (μ_{model}) versus the calculated growth rates ($\mu_{measured}$) based on another 20 sets of experimental data that were not used for model calibration. The result in Figure 3 apparently shows that most of data points are closer to the line of y=x and the R² of the linear trend line of y=x was 0.91. This result indicates that the predicted growth rates are close to measured growth rate and the integrated model developed is able to predict the microalgae growth rate in centrate.

<Figure 4>

The integrated model was also tested using published data from the studies that applied wastewaters as a growth medium. The N: P ratios of these wastewaters were below 16, which indicate N is more limited than P for microalgae growth and meets the

requirement for Equation 2. Figure 5 shows the microalgae biomass concentration predicted using our model and the microalgae biomass concentration obtained from literature. In the study of Yuan et al. [33], *Chlorella sp.* was cultivated in the centrate obtained from a lab-scale anaerobic digestion reactor with the initial cultivation conditions of 208.2 mg N L⁻¹, 0.02 pCO₂ (29.6 mg CO₂ L⁻¹), 9000 lux (121.5 μmol m⁻² s⁻¹), and 20 °C. The model used the Andrews expression for N, Monod expression for CO₂, and Muller-Feuga expression for light intensity combined with Arrhenius equation for temperature to predict the microalgae growth under given conditions. In Cabanelas et al. [75]’s study, *Chlorella vulgaris* was grown in anaerobically digested municipal sludge centrate obtained from their local wastewater treatment plant with the initial cultivation conditions of 130 mg N L⁻¹, 0.01 pCO₂ (14.8 mg CO₂ L⁻¹), 150 μmol m⁻² s⁻¹, and 20 °C. In this case, the integrated model combining the Monod expression for N, Monod expression for CO₂, Muller-Feuga model for light intensity, and Arrhenius equation for temperature was applied. As shown in Figure 5 (a) and (b), the model was able to describe the microalgae growth trend during the experiment period (R²=0.85 in figure 5 (a), R²=0.92 in figure 5 (b)). Figure 5 (c) shows the simulation result compared with the experimental data obtained from Cheng et al. [76]. Cheng et al. [76] used *Chlorella pyrenoidosa* to remove the nutrients from undiluted anaerobically digested effluent of swine manure. Their initial conditions were 1093 mg N L⁻¹, 0.15 pCO₂ (222 mg CO₂ L⁻¹), 6000 lux (81 μmol m⁻² s⁻¹), and 27 °C. Thus, the integrated model was constructed by integrating the Andrews expressions for N and CO₂, Chalker expression for light intensity, and Arrhenius equation for temperature. The model was not able to capture the growth trend between day 2 and day 6; however, the result shows that the overall model

simulation agrees with the experimental data to a good degree ($R^2=0.88$). Although the wastewater used in Cheng et al. [76] is not the same as anaerobically digested municipal sludge centrate, the model can still describe the overall microalgae growth. Thus, the integrated model framework developed in this study is applicable to *Chlorella* species grown in wastewaters that have N:P ratios below 16.

<Figure 5>

3.3 Model limitations

Although the integrated model developed in this study is useful to predict microalgae growth in wastewaters, the model has several limitations for application. First, the parameters provided in this study are obtained from the experiments using the centrate and indigenous *Chlorella* sp. According to Liu and Zachara [79], kinetic parameters estimated from batch experimental data result in major uncertainties of the model predictions. Careful manipulation of experimental conditions can improve accuracy of kinetic parameters and therefore reduce uncertainties of model predictions. Since the experimental design of 24:0 h light-dark cycle was used in this study, the kinetic parameters determined for light intensity may not be applicable for cultivation conditions with different light-dark cycles. In addition, the estimated kinetic parameters for microalgae growth may be different, depending on the sources of the wastewater or microalgae species. Thus, in order to increase the usability of the model as well as to improve the model prediction, the model should be tested for other types of wastewaters and the calibration may be required for different types of wastewaters. Second, appropriate rate expressions for P in the integrated model need to be investigated. In this study, microalgae growth in the centrate was not limited by P so that P was not

considered as a major factor in the final growth kinetic model. Thus, developing the robust integrated model would require suitable rate expressions of P for future applications. In addition, organic carbon needs to be considered as growth factors in order to explain microalgae growth in wastewater containing organic carbon (such as effluent of primary wastewater treatment), since microalgae (e.g., *Chlorella sp.*) have an ability to use organic carbon as a carbon source for their growth. Future research may focus on these limitations to improve the applicability of the model to various types of wastewaters.

4. Conclusions

To describe the microalgae growth in anaerobically digested municipal sludge centrate, an integrated co-limitation kinetic model was constructed by incorporating N, CO₂, light, and temperature factors. The model framework combining threshold and multiplicative theories was able to explain the relationship of the selected factors on microalgae growth. The model was calibrated using experimental data from lab scale batch reactors cultivating *Chlorella sp.* in the centrate and validated through the experimental data in this study as well as data obtained from literature. The model developed was able to predict the microalgae growth rate well for all the growth conditions investigated. This model can be used in bioreactor design as well as process control and optimization of microalgae cultivation systems integrated with wastewater.

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Figure 1 The process for determining the criteria and kinetic parameters for the integrated model

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a)

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b)

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c)

Figure 2 Total algae chlorophyll a concentrations under different growth conditions: a) varying initial $\text{NH}_4\text{-N}$ concentration ($\text{N1}=9.9 \text{ mg L}^{-1}$, $\text{N2}=27.1 \text{ mg L}^{-1}$, $\text{N3}=47.2 \text{ mg L}^{-1}$, $\text{N4}=88.8 \text{ mg L}^{-1}$, $\text{N5}=139.2 \text{ mg L}^{-1}$, $\text{N6}=226.1 \text{ mg L}^{-1}$, $\text{N7}=586.1 \text{ mg L}^{-1}$); b) varying initial aqueous CO_2 concentration ($\text{C1}=8.5 \text{ mg L}^{-1}$, $\text{C2}=12.9 \text{ mg L}^{-1}$, $\text{C3}=19 \text{ mg L}^{-1}$, $\text{C4}=26 \text{ mg L}^{-1}$, $\text{C5}=92 \text{ mg L}^{-1}$, $\text{C6}=122 \text{ mg L}^{-1}$, $\text{C7}=200 \text{ mg L}^{-1}$); c) varying initial light intensity with temperature ($\text{L1}=6.8 \text{ } \mu\text{mol photon m}^{-2} \text{ s}^{-1}$, $\text{L2}=12.2 \text{ } \mu\text{mol photon m}^{-2} \text{ s}^{-1}$, $\text{L3}=17.6 \text{ } \mu\text{mol photon m}^{-2} \text{ s}^{-1}$, $\text{L4}=27 \text{ } \mu\text{mol photon m}^{-2} \text{ s}^{-1}$, $\text{L5}=77 \text{ } \mu\text{mol photon m}^{-2} \text{ s}^{-1}$, $\text{L6}=86.4 \text{ } \mu\text{mol photon m}^{-2} \text{ s}^{-1}$, $\text{L7}=459 \text{ } \mu\text{mol photon m}^{-2} \text{ s}^{-1}$).

c)

Figure 3 Specific growth rate as a function of growth factors: a) specific growth rate vs. initial $\text{NH}_4\text{-N}$ concentration; b) specific growth rate vs. aqueous CO_2 concentration; c) specific growth rate vs. light intensity and temperature.

Figure 4 Plot of predicted specific growth rate versus calculated growth rate based on experimental data.

(c)

Figure 5 Published experimental data and modeling results for validation: (a) data obtained from Yuan et al. [33] ($R^2=0.87$); (b) data obtained from Cabanelas et al. [75] ($R^2=0.93$); and (c) data obtained from Cheng et al. [76] ($R^2=0.86$).

818 Table 1 Overall expression for the integrated model

Factors	Model	Consideration	Rate expressions
N	Monod model	Limitation	$\mu = \mu_{max,NH4-N} \frac{S_{NH4-N}}{K_{S,NH4-N} + S_{NH4-N}}$
	Andrews model	Limitation and inhibition	$\mu = \mu_{max,NH4-N} \frac{S_{NH4-N}}{K_{S,NH4-N} + S_{NH4-N} + S_{NH4-N}^2 / K_{i,NH4-N}}$
CO ₂	Monod model	Limitation	$\mu = \mu_{max,CO2} \frac{S_{CO2}}{K_{S,CO2} + S_{CO2}}$
	Andrews model	Limitation and inhibition	$\mu = \mu_{max,CO2} \frac{S_{CO2}}{K_{S,CO2} + S_{CO2} + S_{CO2}^2 / K_{i,CO2}}$
Light and Temperature	Chalker model combined with Arrhenius equation	Limitation	$\mu = \mu_{max,I} \cdot \theta^{T-20} \cdot \tanh\left(\frac{I_{av}}{I_K}\right)$
	Muller-Fuega model combined with Arrhenius equation	Limitation and inhibition	$\mu = \mu_{max,I} \cdot \theta^{T-20} \cdot \frac{2 * (1 - I_e/I_K) * (I_{av}/I_K - I_e/I_K)}{(1 - I_e/I_K)^2 + (I_{av}/I_K - I_e/I_K)^2}$

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821 Table 2 Characteristics of the centrate

Source	Average Concentration (mg L ⁻¹)
pH	7.81±0.15
Alkalinity	751±50
COD	811±74
TN	445±153
NH ₄ -N	397±145
NO ₃ -N	0.5±0.3
NO ₂ -N	n.d.
TP	238±59

822 *n.d.: Not detected

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824 Table 3 Calibrated parameters for the integrated model

Factors	Applicable ranges	Models	Parameters	Comparison
N	$\text{NH}_4\text{-N} \leq 150 \text{ mg L}^{-1}$	Monod model	$K_{s,N}=0.1 \text{ mg L}^{-1}$	$\mu_{\max}=0.01245 \text{ h}^{-1}$ $K_{s,N}=0.0696 \text{ mg L}^{-1}$ [72]
	$\text{NH}_4\text{-N} > 150 \text{ mg L}^{-1}$	Andrews model	$K_{s,N}=1.78 \text{ mg L}^{-1}$ $K_{i,N}=364 \text{ mg L}^{-1}$	Best fit value
CO_2	Aqueous $\text{CO}_2 \leq 50 \text{ mg L}^{-1}$	Monod model	$K_{s,\text{CO}_2}=3.60 \text{ mg L}^{-1}$	$\mu_{\max}=0.014\text{-}0.07 \text{ h}^{-1}$ ($0.336\text{-}1.68 \text{ d}^{-1}$) $K_{s,\text{CO}_2}=0.03\text{-}0.36 \text{ mM}$ ($1.32\text{-}15.8 \text{ mg L}^{-1}$) [73]
	Aqueous $\text{CO}_2 > 50 \text{ mg L}^{-1}$	Andrews model	$K_{s,\text{CO}_2}=4.26 \text{ mg L}^{-1}$ $K_i=250 \text{ mg L}^{-1}$	$\mu_{\max}=2.0 \text{ d}^{-1}$ $K_{s,\text{CO}_2}=0.0009 \text{ mol/m}^3$ (0.04 mg L^{-1}) $K_i=180 \text{ mol/m}^3$ (7922 mg L^{-1}) [22]
Light and Temperature	Light intensity $\leq 90 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$	Chalker model combined with Arrhenius equation	$I_K=16.98 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ $\Theta=1.35$	$\mu_{\max}=0.115 \text{ h}^{-1}$ ($\mu_{\max}=2.76 \text{ d}^{-1}$) $I_K=150 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ [52]
	Light intensity $> 90 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$	Muller-Feuga model combined with Arrhenius equation	$I_e=1 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ $I_K=54.7 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ $\Theta=1.16$	Best fit value
Maximum growth rate (d^{-1})			$\mu_{\max}=0.7 \text{ d}^{-1}$	$\mu_{\max}=0.677 \text{ d}^{-1}$ [67]

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