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# Minimal Medium for Optimal Growth and Lipid Production of the Microalgae *Scenedesmus dimorphus*

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*The culture of Scenedesmus dimorphus at the laboratory scale has often been in standard microalgae media, such as 3N-Basal Bold medium (3N-BB), which contains 15 different chemicals. Given the extremely tight profit margins of large-scale production of biofuel from microalgae, it is important to identify the minimally sufficient quantities of nutrients necessary to maximize lipid productivity. The individual and interactive effects of six groupings of the components of 3N-BB medium on growth rate, lipid content, and total biomass yield of S. dimorphus were determined. Trace metal and vitamin concentrations were reduced to 1/6 the level of 3N-BB medium without adversely affecting growth rates and biomass concentration, while concentrations of CaCl<sub>2</sub> and K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub> were reduced to 1/10 that of 3N-BB without adversely affecting biomass and lipid concentrations. Both lipid productivity and lipid content were maximized at the lowest NaNO<sub>3</sub> concentration (1/10 that in 3N-BB) and independent of MgSO<sub>4</sub> concentration, while the interaction of these two chemicals enhanced biomass concentration.*

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**Keywords:** algae, medium, lipids, Scenedesmus dimorphus, nutrients

## INTRODUCTION

The freshwater microalgae *Scenedesmus dimorphus* has garnered interest as a biofuel source because of its relatively high growth rate and lipid content. Specific growth rates of *S. dimorphus* have been reported between 0.8 day<sup>-1</sup> [1] and 1.6 day<sup>-1</sup> (the latter for *S. obliquus*, a taxonomic synonym for *S. dimorphus* [2]). Lipid content in *S. dimorphus* has been reported to be between 18% and 32% (w/w) [3–7].

While nitrogen content in the media is necessary for algae biomass production, it has been often reported that the depletion of nitrogen from the media generally causes an increase in lipid content [8] at the expense of total biomass concentration (e.g., *Chlorella vulgaris* [9,10]; *Nannochloropsis* sp. *F&M-M24* [4]). Similar results have been obtained with some species of *Scenedesmus* [11], with an increase in lipid

content of *S. TR-84* from 20% (in nitrogen-sufficient condition, at day 5) to 45% (in nitrogen-deficient condition, at day 14) [12], and for *S. obliquus*, 21% in nitrogen-sufficient to 46% in nitrogen-deficient conditions [13]. However, conflicting results have been obtained by others, who showed that lipid content was unaffected by nitrogen concentration, for *S. dimorphus* [3] and *S. DM* [4]. The type of nitrogen source also affects biomass productivity, with greater growth rates of *Scenedesmus* sp. *LX1* obtained in ammonia and urea, relative to nitrate, but with ammonia inhibiting maximum concentration because of pH effects [14,15].

The culture of *S. dimorphus* at the laboratory scale has often been in standard microalgae media, such as 3N-Basal Bold medium (3N-BB). This media contains a total of 15 chemicals, including a variety of salts, trace elements, and vitamins that have been found useful for algae growth, but not necessarily optimized for *S. dimorphus*. Given the extremely tight profit margins to be expected from large-scale production of biofuel from microalgae, it is important to identify the minimally sufficient quantities of nutrients necessary to maximize lipid productivity. As has been reported with nitrogen, some nutrients have opposing effects on lipid content and biomass production for the same species. There has been much research on the effects of individual nutrients, and in some cases, ratios of nitrogen/phosphorous, on growth of *Scenedesmus* [1,11,12,14–18]. However, the complexity of metabolic networks in the cell almost certainly results in interaction among the various nutrients in terms of their effects on the cell. The use of statistical experimental design techniques is an efficient method for medium design when a large number of components are involved, with potential interactions among the components. This method allows a rapid screening for the determination of optimal levels of each nutrient, as well as identification of the few components that warrant additional investigation. This approach was used by Kathiresan *et al.* [19] to optimize media composition for biomass, phycobiliprotein, and phycoerythrin production by the red microalgae *Porphyridium purpureum*. Mandal and Mallick [7] used a central composite design to determine the effect of cultivation time, nitrate, phosphate, and thiosulfate on the two outcomes of biomass concentration and lipid content of *S. obliquus*. A similar

experimental design methodology is used here to determine the individual and interactive effects of six groupings of the components of 3N-BB medium on the four outcomes of biomass concentration, lipid content, growth rate, and lipid concentration for *S. dimorphus*. The maximization of growth rate is important to minimize reactor volume (in continuous processes) or time of culture (for batch). The lipid concentration reflects the sometimes conflicting properties of lipid content and biomass concentration and is ultimately the most important outcome to be maximized. Three components were considered individually: sodium nitrate, magnesium sulfate, and calcium chloride. Monobasic and dibasic potassium phosphate were varied together as one component because of their role as a pH buffer. The final two components are the trace metal solution, containing five metal salts dissolved in a Na<sub>2</sub>EDTA solution, and a vitamin solution, containing biotin, B1, and B12.

## MATERIALS AND METHODS

### Algae Culture

*S. dimorphus* was originally obtained from the UTEX culture collection (ID# 746 Austin, TX). Algae stock was thawed from liquid nitrogen (in 5% DMSO) and maintained in 1.5% agar prepared with 3N-BB medium, containing the following components, per 1 L of DI water: 0.75 g NaNO<sub>3</sub>, 0.025 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.075 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.075 g K<sub>2</sub>HPO<sub>4</sub>, 0.175 g KH<sub>2</sub>PO<sub>4</sub>, 0.025 g NaCl, 6 mL trace metal stock solution (containing 0.75 g/L Na<sub>2</sub>EDTA, 0.097 g/L FeCl<sub>3</sub>·6H<sub>2</sub>O, 0.041 g/L MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.005 g/L ZnCl<sub>2</sub>, 0.002 g/L CoCl<sub>2</sub>, and 0.004 g/L NaMoO<sub>4</sub>), and 2 mL of each vitamin stock solution (H (biotin) at 0.04 g/L, B<sub>1</sub> (thiamine) at 1.105 g/L, and B<sub>12</sub> (cyanocobalamin) at 0.1575 g/L, all in 50 mM HEPES solution). All chemicals were from Sigma. Seed cultures for growth experiments were prepared by inoculating cells from agar into a 250 mL Erlenmeyer flask with 3N-BB medium, sparged with 5% CO<sub>2</sub> in air at a flow rate of 0.1 LPM, and agitated on a stir plate at room temperature. Illumination was provided by 14 W, 48" Accupro fluorescent bulbs on a 12 h on/12 h off cycle, with a measured intensity of 150–200 ft candles at the liquid surface maintained in a sterile hood.

### Lipid Analysis

Seventy-five to hundred milliliters of the algae suspension was divided into 50 mL centrifuge tubes and centrifuged for 1.5 h at 2000 rpm. The supernatant was discarded and the pellets were rinsed with DI water, vortexed, and combined in a 1.5 mL microcentrifuge tube, where they were centrifuged for 20 min at 14,000 rpm. The supernatant was discarded and the resulting pellets were dried in an oven at 45–50°C for 1–2 days. The dried pellet was ground to a fine powder in mortar and pestle and transferred to a glass test tube. Five milliliters of hexane–isopropanol solution (3:2 v/v; Acros, NJ) were added to the powdered biomass, the samples were shaken at 200 rpm for 3 h on a shaker platform, and the supernatant removed. The solvent extraction procedure was repeated with 5 mL of fresh solvent, and after the second shaking period, the solvent was removed by filtration through 0.65- $\mu$ m-pore filter paper (Miconsep, Honeoye, NY). The combined solvents were dried in the fume hood at room temperature and weighed to obtain the mass of the lipid fraction. These values were used to calculate lipid content ( $\gamma_L$ , g lipid/gdw biomass).

### Growth Experiments

Experiments were conducted in 250-mL Erlenmeyer flasks with 150 mL working volume. Inoculum volume was determined so as to provide a starting  $A_{600}$  of about 0.01 in the experimental flasks. Illumination was provided by seven 14-

W, 24" fluorescent tubes (four Coralite Aquapro T-5/10,000K and three Accupro AFL/F14T5/14W/830) on a 12 h on/12 h off cycle, with a measured intensity of 515–550 ft candles at the liquid surface. Temperature and agitation control were provided by a Labline Orbital shaker bath at 30°C  $\pm$  1°C and 150 rpm of agitation. Gas (5% CO<sub>2</sub> in air v/v) was sparged into each flask through a 200  $\mu$ L pipette tip at 0.1  $\pm$  0.02 liters per minute. Flasks were vented through 1- $\mu$ m syringe filters. Each flask was sampled once daily and the absorbance at 600 nm measured ( $A_{600}$ ). Absorbance was found previously to be linear with cell concentration for  $A_{600} < 1.0$ , with a slope of 0.62 gdw/L per  $A_{600}$  (unpublished data). Samples with higher cell concentrations ( $A_{600} > 1.0$ ) were diluted with 3N-BB, and the absorbance reported as "adjusted absorbance."

Specific growth rates ( $\mu$ , in day<sup>-1</sup>) were calculated from the slope of the natural log of the adjusted  $A_{600}$  versus time. Biomass concentrations ( $X_{\text{final}}$ , in gdw/L) were calculated from adjusted absorbance from the final day of the experiment.

### Experimental Design

This study was performed in two experiments. The first experiment employed a two-level, six-factor fractional factorial design intended to probe the significance of media constituents on *growth rate and biomass concentration*. This first experiment consisted of five blocks of eight flasks each, containing a different 1/8 (calculated from  $1/(2^{6-3})$ ) fractional factorial design. Blocking was employed because the experimental set-up allowed the running of only eight flasks at a time. The six factors each consisted of one of the following components: sodium nitrate, magnesium sulfate, monobasic and dibasic potassium phosphate (varied together as one component, hereafter called potassium phosphate), calcium chloride, trace metal solution, and vitamin solution (biotin, B1, and B12). Monobasic and dibasic potassium phosphates were varied as a single unit in order to maintain their buffering capacity. Table 1 contains the media composition for each treatment (flask). "1" signifies that the component is at the concentration used in the 3N-BB medium, while a "–1" denotes a concentration at 1/6th the level in 3N-BB.

In the second experiment the objective was expanded to include outcomes of lipid content, in addition to growth rate and biomass concentration. The scope was narrowed by eliminating the media components found to have insignificant effects in Experiment 1. The range over which the media constituents were varied was increased to elicit a more significant cell response. The effects of four components were investigated, i.e., sodium nitrate, magnesium sulfate, potassium phosphate (monobasic and dibasic potassium phosphate varied together as one component), and calcium chloride. The experimental design was a 2<sup>4</sup> full factorial design run in two blocks of eight flasks each. Table 3 shows the experimental compositions for each treatment. "1" signifies that the component is at twice the concentration used in the 3N-BBM, while a "–1" denotes a concentration at 1/10th the level in 3N-BBM. Cell suspensions at the end of the experiment, on day 13, were analyzed for lipid content as described above.

Fitted model coefficients, lower and upper confidence limits on predicted values, and *P*-values were calculated using Minitab® software.

## RESULTS

### Experiment 1

The specific growth rates were calculated for each flask from the adjusted absorbance measurements during the exponential growth phase. The exact period of the exponential growth phase was variable within each experiment, and the identification of this period affects the calculated growth rates. Therefore, the specific growth rates were calculated for

**Table 1.** Experimental design and results from Experiment 1.

Treatment	Component level						Specific growth rate $\mu$ (day <sup>-1</sup> )			Biomass (gdw/L)
	NaNO <sub>3</sub>	CaCl <sub>2</sub>	MgSO <sub>4</sub>	Potassium phosphates	Trace metals	Vitamins	Growth day 4–8	Growth day 4–7	Growth day 5–8	
1	-1	-1	-1	1	1	1	0.606	0.691	0.529	0.40
2	1	-1	1	-1	1	1	0.684	0.770	0.615	0.39
3	-1	1	1	1	-1	1	0.581	0.672	0.482	0.52
4	1	1	-1	-1	-1	1	0.589	0.653	0.523	0.44
5	1	1	-1	1	1	-1	0.629	0.711	0.556	0.45
6	-1	1	1	-1	1	-1	0.654	0.735	0.605	0.47
7	1	-1	1	1	-1	-1	0.589	0.657	0.505	0.41
8	-1	-1	-1	-1	-1	-1	0.656	0.729	0.610	0.47
9	-1	1	1	1	1	1	0.411	0.465	0.364	0.22
10	1	1	-1	-1	1	1	0.445	0.487	0.405	0.26
11	-1	-1	-1	1	-1	1	0.339	0.373	0.315	0.16
12	1	-1	1	-1	-1	1	0.468	0.508	0.431	0.32
13	1	-1	1	1	1	-1	0.387	0.432	0.346	0.20
14	-1	-1	-1	-1	1	-1	0.432	0.475	0.398	0.29
15	1	1	-1	1	-1	-1	0.435	0.479	0.391	0.28
16	-1	1	1	-1	-1	-1	0.492	0.551	0.449	0.38
17	1	-1	1	1	1	1	0.369	0.404	0.364	0.31
18	-1	-1	-1	-1	1	1	0.466	0.506	0.474	0.41
19	1	1	-1	1	-1	1	0.388	0.422	0.381	0.27
20	-1	1	1	-1	-1	1	0.422	0.456	0.427	0.38
21	-1	1	1	1	1	-1	0.373	0.409	0.362	0.33
22	1	1	-1	-1	1	-1	0.43	0.468	0.421	0.40
23	-1	-1	-1	1	-1	-1	0.375	0.416	0.366	0.36
24	1	-1	1	-1	-1	-1	0.431	0.457	0.430	0.48
25	1	1	1	1	1	1	0.257	0.274	0.232	0.27
26	-1	1	-1	-1	1	1	0.378	0.400	0.370	0.44
27	1	-1	-1	1	-1	1	0.325	0.357	0.283	0.40
28	-1	-1	1	-1	-1	1	0.379	0.415	0.350	0.49
29	-1	-1	1	1	1	-1	0.293	0.315	0.252	0.34
30	1	-1	-1	-1	1	-1	0.383	0.405	0.361	0.54
31	-1	1	-1	1	-1	-1	0.259	0.272	0.244	0.31
32	1	1	1	-1	-1	-1	0.334	0.380	0.303	0.32
33	1	-1	-1	1	1	1	0.316	0.357	0.285	0.28
34	-1	-1	1	-1	1	1	0.326	0.349	0.303	0.35
35	1	1	1	1	-1	1	0.289	0.330	0.260	0.24
36	-1	1	-1	-1	-1	1	0.339	0.389	0.290	0.43
37	-1	1	-1	1	1	-1	0.307	0.345	0.267	0.39
38	1	1	1	-1	1	-1	0.361	0.417	0.311	0.41
39	-1	-1	1	1	-1	-1	0.278	0.317	0.240	0.27
40	1	-1	-1	-1	-1	-1	0.359	0.398	0.326	0.44

A component level of “1” indicates that the concentrations used are those in the 3N-BB medium; “-1” indicates the concentration is at 1/6th the level in the 3N-BB medium.

three periods of time for each experiment (days 4–7, days 4–8, and days 5–8). The results of these calculations for Experiment 1 are shown in Table 1.

A general linear model was developed for the relationship of the growth responses to the six factors and all of their two factor interactions. Table 2 contains the model coefficients and *P*-values used to determine which of the factor or factor interactions were statistically significant. None of the interactions was found to be significant (all interaction *t*-tests using a null hypothesis of coefficient values = 0.00 yielded *P*-values  $\gg$  0.05). The model was then recalculated with only the significant first-order factor terms included, also shown in Table 2. For this reduced model, only the constant term and the coefficient for potassium phosphate ( $K_2HPO_4/KH_2PO_4$ ) were found to be significantly different from zero (*P* < 0.001). The final model

equations for  $\mu$  (specific growth rate) with all nonsignificant terms removed are:

$$\begin{aligned}
 \mu_{\text{days 4-7}} &= 0.4661 - 0.0313 f_{K_2HPO_4/KH_2PO_4} \\
 \mu_{\text{days 4-8}} &= 0.4209 - 0.0355 f_{K_2HPO_4/KH_2PO_4} \\
 \mu_{\text{day 5-8}} &= 0.3857 - 0.0345 f_{K_2HPO_4/KH_2PO_4}
 \end{aligned}
 \tag{1}$$

where  $\mu$  is in day<sup>-1</sup>, and  $f_i$  is the coded factor for species *i*, with values between -1 and 1, where -1 and 1 are as defined in the Experimental Design section, relative to concentrations in the 3N-BB medium. From Eq. 1 (plotted in Figure 1), it can be concluded that the higher level of potassium phosphate negatively affected the observed growth rates (i.e., a negative coefficient) and the other factors have no statistically significant effect on growth rate.

**Table 2.** Experimental design and results from Experiment 2.

Flask	Component level				Specific growth rate $\mu$ (day <sup>-1</sup> )			Biomass day 13 (gdw/L)	Lipid content day 13 (wt %)	Lipid concentration day 13 (g/L)
	NaNO <sub>3</sub>	CaCl <sub>2</sub>	MgSO <sub>4</sub>	Potassium phosphates	Days 4–7	Days 4–8	Days 5–8			
1	-1	-1	1	-1	0.43	0.54	0.35	0.89	16.9	0.15
2	1	-1	1	1	0.49	0.58	0.43	1.80	2.60	0.05
3	-1	-1	-1	1	0.40	0.49	0.34	0.87	15.5	0.14
4	-1	1	1	1	0.41	0.52	0.32	0.98	11.5	0.12
5	1	-1	-1	-1	0.40	0.49	0.32	1.04	10.3	0.09
6	-1	1	-1	-1	0.41	0.51	0.32	0.97	20.5	0.20
7	1	1	-1	1	0.40	0.51	0.34	0.84	10.0	0.08
8	1	1	1	-1	0.46	0.55	0.39	1.61	2.20	0.04
9	-1	1	-1	1	0.46	0.49	0.42	0.91	28.0	0.27
10	1	-1	1	-1	0.45	0.50	0.38	1.71	8.80	0.01
11	-1	-1	1	1	0.38	0.44	0.31	0.85	26.8	0.25
12	-1	-1	-1	-1	0.43	0.44	0.41	0.63	21.3	0.13
13	1	1	-1	-1	0.46	0.53	0.38	1.08	8.00	0.10
14	1	1	1	1	0.46	0.51	0.41	1.34	7.10	0.09
15	1	-1	-1	1	0.42	0.41	0.45	0.71	7.70	0.06
16	-1	1	1	-1	0.52	0.55	0.49	0.80	27.6	0.23

“1” denotes that the component is at a concentration that is 2× that of 3N-BB; “-1” denotes that the component is at a concentration that is 1/10 that of 3N-BB.

The regression model was also employed to fit an equation using the final biomass concentration from this experiment (at day 9) as the response. The first calculation utilized all six factors as well as all possible two-factor interactions. From this calculation the only significant factor coefficients found were those for potassium phosphate ( $P = 0.001$ ) and the NaNO<sub>3</sub>-CaCl<sub>2</sub> interaction ( $P = 0.043$ ), as shown in Table 2. All others were found to be nonsignificant ( $P > 0.18$ ). A second model was then fitted using the significant terms as well as terms for the first-order NaNO<sub>3</sub> and CaCl<sub>2</sub> factors included to satisfy model hierarchy requirements. This second calculation produced the following reduced model:

$$X_{\text{final}} = 0.3621 - 0.0424 f_{\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4} - 0.0087 f_{\text{NaNO}_3} - 0.0026 f_{\text{CaCl}_2} - 0.0187 f_{\text{NaNO}_3} f_{\text{CaCl}_2} \quad (2)$$

Analysis of this model predicts that the lowest biomass concentration will occur with all three of these factors at their highest (i.e., 3N-BB) levels (Figure 2a). Because of the interaction term in Eq. 2, the highest predicted biomass yields occur when potassium phosphate and sodium nitrate are at their lowest levels and CaCl<sub>2</sub> at its highest (Figure 2a). The predicted value for  $X_{\text{final}}$  for this case, as shown in Table 5 ( $X_{\text{final}} = 0.43$  gdw/L), is significantly greater than that for the 3N-BB levels ( $X_{\text{final}} = 0.29$  gdw/L), as evidenced by the absence of overlap of the 95% confidence intervals. The predicted biomass concentration for the case of all three factors at their lowest value ( $X_{\text{final}} = 0.40$  gdw/L) is close to the highest predicted yield and not significantly different from it, but is significantly greater than that for the 3N-BB case (from comparison of confidence intervals).

In summary, these results suggest that all six components could be reduced to 1/6th the value in the 3N-BB with a significant increase in biomass concentration and without a significant negative effect on growth rate.

## Experiment 2

The growth curves of both blocks in Experiment 2 are shown in Figure 3. Specific growth rates were calculated

over three windows of time from these curves for days 4–7, days 4–8, and days 5–8 (Table 3). The final biomass concentrations, lipid contents, and lipid concentrations (all at day 13) are also shown in Table 3. Regression models were fit using this data for the six responses against the four factors and all of their possible two factor interactions (Table 4).

From inspection of the  $P$ -values in Table 4, none of the medium components show significant effect on the growth rates calculated from the various time intervals. On the other hand, the complete model for the biomass response contained both significant and insignificant terms. A reduced model was calculated containing significant terms (as well as first-order terms to satisfy hierarchy requirements for significant interactions):

$$X_{\text{final}} = 1.0644 - 0.0269 f_{\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4} + 0.2019 f_{\text{NaNO}_3} + 0.0019 f_{\text{CaCl}_2} + 0.1831 f_{\text{MgSO}_4} - 0.0506 f_{\text{NaNO}_3} f_{\text{CaCl}_2} + 0.1656 f_{\text{NaNO}_3} f_{\text{MgSO}_4} - 0.0669 f_{\text{NaNO}_3} f_{\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4} - 0.0669 f_{\text{CaCl}_2} f_{\text{MgSO}_4} \quad (3)$$

The large number of two-way interactions can be visualized using the interaction plots shown in Figure 4. These plots show that at low concentration, sodium nitrate dominates, inhibiting the effects of the other components and producing relatively low biomass yields (Figures 4a–4c). With sodium nitrate at its high concentration level, magnesium sulfate has a relatively large effect producing higher biomass yields at its high level (Figure 4b). With either sodium nitrate or magnesium sulfate at their high levels, calcium chloride produces higher biomass yields at its low level (Figures 4a and 4d). These observations likely result from the needs for nitrogen for protein synthesis, the major component of growing cells. Ca<sup>++</sup> and Mg<sup>++</sup> ions are important cofactors in many enzymes of the intermediary metabolism, which may account for the interactions of these two components.

Regression was also employed to fit both full and reduced models to the lipid data (Table 4). The only significant factor

affecting the values of both lipid content ( $y_L$ , in g lipid/gdw biomass) and lipid concentration ( $L_{final}$ , in g/L) was sodium nitrate. The reduced model equations are:

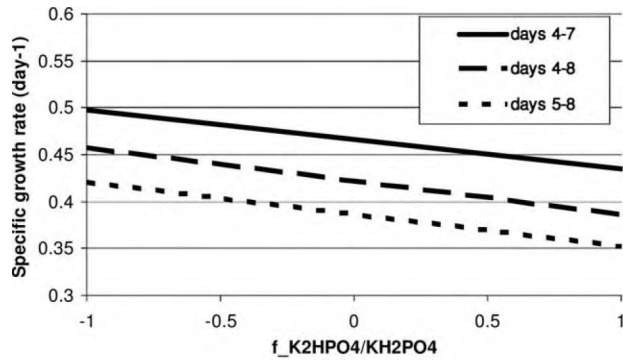
$$y_L = 0.1405 - 0.0696 f_{NaNO_3} \quad (4)$$

$$L_{final} = 0.1309 - 0.0484 f_{NaNO_3} \quad (5)$$

High levels of nitrate, which promote increases in biomass concentration, also inhibit lipid accumulation (Figure 5), as shown by the  $P$ -value of 0.000 for the model coefficient of  $-0.0696$  (Table 4). In fact,  $NaNO_3$  is the only component that significantly affects lipid content in these results. The final lipid concentration in the flask, which is calculated from the product of the lipid content and final biomass concentration, is, not surprisingly, significantly affected only by  $NaNO_3$ , with an overall negative effect.

## DISCUSSION

Much of the lab-scale research on algae growth uses general algae medium formulations, such as 3N-BB, which have been developed over time and found to support a variety of algae genus and species. While the medium is relatively inexpensive to prepare for laboratory research, costs for medium constituents can make or break large-scale algae

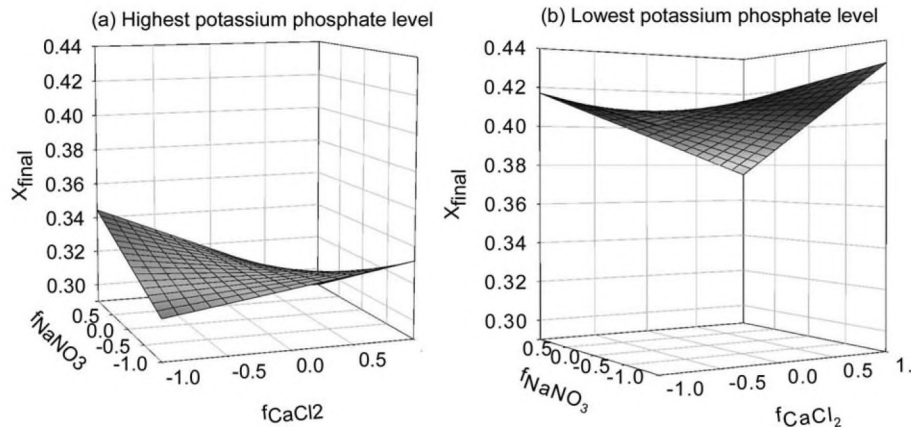


**Figure 1.** Effect of  $K_2HPO_4 \cdot KH_2PO_4$  on specific growth rate, from Eq. 1 using Experiment 1 data. The abscissa represents the range of  $f_{K_2HPO_4/KH_2PO_4}$ , where “-1” and “1” represent 1/6th and 1 $\times$ , respectively, the concentration of  $K_2HPO_4 \cdot KH_2PO_4$  in 3N-BB medium.

culture for commodity products such as fuel. The objective of this research is to determine the minimal nutrient concentrations necessary to achieve maximal algae growth and lipid productivity. The motivation is not only to make algae oil production economically feasible, but environmentally sound as well. The six major groupings of medium constituents were screened in the first phase of this research to identify those which most influenced biomass productivity. The investigation of medium components was then broadened to include effects on lipid content and biomass concentration in early stationary phase, in addition to growth rate. The focus was narrowed to the four major salts, while the range of concentrations was expanded, to a 20-fold variation.

## Growth Rate

A high growth rate is crucial for biomass production in industrial scale processes in order to reduce the capital equipment (for continuous processes) and land costs. The finding that all the tested media components could be reduced to 1/6 the level in the 3N-BB medium without influencing growth rate or final biomass concentration is a positive one, and can significantly reduce nutrient costs at the large scale. The only exception was potassium phosphate, in which lower concentrations actually yielded higher growth rates (Eq. 1). This is significant given that phosphate fertilizers have more than doubled in cost between 2005 and 2011 [20] making it the single most expensive macronutrient. Statistical analysis of the data shows that using 1/6 the level of potassium phosphates used in 3N-BB in fact boosts the growth rate of *S. dimorphus* anywhere from 8% to 66% (at 95% confidence), as determined from the model in Eq. 1. These experiments could not distinguish whether this effect is due to the  $K^+$  or  $PO_4^{3-}$  ions. However, since it has been shown that excess phosphorus competes with iron and manganese uptake by plants [21], both of which are essential nutrients in plants [22,23], it is more likely that the phosphate ions are the inhibitory substances. Further investigation using other phosphate salts and potassium salts may resolve this question. The negative effect of the potassium phosphates on growth rate was not corroborated in the second experiment. This may result from nonlinearity in the response curve, i.e., the growth rate may increase at 1/6 the 3N-BB concentration, but then decrease to basal growth rate at 1/10 the concentration. At this lowest potassium phosphate concentration, the phosphorous may no longer compete with the iron or manganese. The lack of a phosphorous effect on



**Figure 2.** Effects of interactions of  $NaNO_3$  and  $CaCl_2$  on biomass concentration, using Eq. 2 developed from Experiment 1 data. The abscissas represent the range of the factors, where “-1” and “1” represent 1/6th and 1 $\times$ , respectively, the concentration of that factor in 3N-BB medium. The ordinate represents the biomass concentration, in gdw/L. (a) Calculated using  $f_{K_2HPO_4/KH_2PO_4} = 1$  for minimum biomass; (b) Calculated using  $f_{K_2HPO_4/KH_2PO_4} = -1$  for maximum biomass.

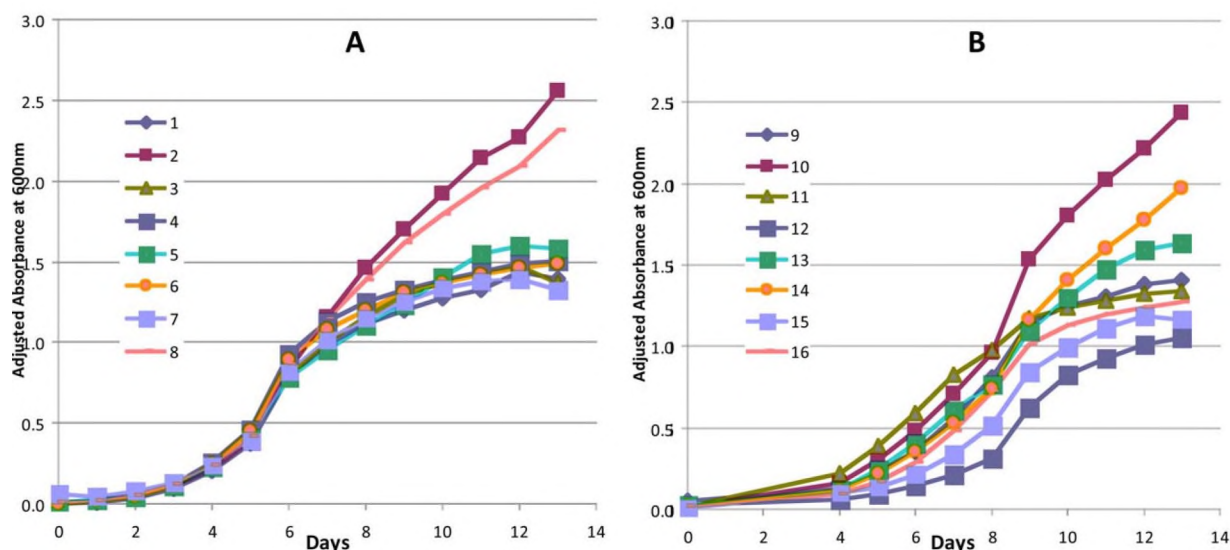
**Table 3.** Model results from Experiment 1 data.

Factor	$\mu$ Days 4–7		$\mu$ Days 4–8		$\mu$ Days 5–8		Biomass concentration	
	Regression coefficient	<i>P</i> -value*	Regression coefficient	<i>P</i> -value	Regression coefficient	<i>P</i> -value	Regression coefficient	<i>P</i> -value
<b>Regression coefficients, including all factors and their second-order interactions</b>								
Constant	<b>0.4661</b>	<b>0.000</b>	<b>0.4209</b>	<b>0.000</b>	<b>0.3857</b>	<b>0.000</b>	<b>0.3621</b>	<b>0.000</b>
NaNO <sub>3</sub>	0.0038	0.588	0.0038	<b>0.489</b>	0.0020	0.674	-0.0094	0.373
CaCl <sub>2</sub>	-0.001	0.879	-0.0027	<b>0.585</b>	-0.0034	0.449	-0.0070	0.469
MgSO <sub>4</sub>	-0.0017	0.791	-0.0031	<b>0.540</b>	-0.0051	0.265	-0.0129	0.194
Potassium phosphates	<b>-0.0294</b>	<b>0.000</b>	<b>-0.0291</b>	<b>0.000</b>	<b>-0.0336</b>	<b>0.000</b>	<b>-0.0376</b>	<b>0.001</b>
Trace metals	0.0046	0.512	0.0049	0.365	0.0062	0.213	-0.0060	0.569
Vitamins	-0.0032	0.621	-0.0029	0.563	-0.0028	0.537	-0.0133	0.181
NaNO <sub>3</sub> *CaCl <sub>2</sub>	-0.0062	0.349	-0.0062	0.222	-0.0059	0.201	<b>-0.0211</b>	<b>0.043</b>
NaNO <sub>3</sub> *MgSO <sub>4</sub>	-0.0056	0.390	-0.0057	0.262	-0.0043	0.344	-0.0127	0.200
NaNO <sub>3</sub> *Phosphates	0.0062	0.349	0.0065	0.202	0.0095	0.048	0.0025	0.795
NaNO <sub>3</sub> *trace metals	0.0011	0.879	-0.0003	0.954	-0.0006	0.902	0.0034	0.741
NaNO <sub>3</sub> *Vitamins	-0.0026	0.688	-0.0014	0.776	0.0007	0.884	-0.0134	0.178
CaCl <sub>2</sub> *MgSO <sub>4</sub>	0.0059	0.496	0.0032	0.633	0.0035	0.558	-0.0008	0.949
CaCl <sub>2</sub> *phosphates	0.005	0.562	0.0058	0.389	0.0068	0.258	0.0081	0.530
CaCl <sub>2</sub> *trace metals	0.0013	0.836	0.0021	0.677	0.0031	0.484	0.0061	0.530
CaCl <sub>2</sub> *vitamins	-0.0048	0.576	-0.0028	0.673	-0.0023	0.703	-0.0015	0.906
MgSO <sub>4</sub> *phosphates	-0.0049	0.566	-0.0050	0.455	-0.0063	0.296	0.0013	0.920
MgSO <sub>4</sub> *trace metals	-0.0069	0.299	-0.0098	0.280	-0.0098	0.280	-0.0102	0.297
MgSO <sub>4</sub> *vitamins	-0.0023	0.787	-0.0024	0.836	-0.0024	0.836	0.0031	0.806
Phosphates*trace metals	-0.0001	0.989	-0.0020	0.821	-0.0020	0.821	0.0011	0.909
Phosphates*vitamins	-0.0024	0.776	-0.0054	0.649	-0.0054	0.649	0.0026	0.836
Trace metals*vitamins	0.0027	0.674	0.0096	0.293	0.0096	0.293	-0.0097	0.323
<b>Regression coefficients for model reduced to significant terms</b>								
Constant	<b>0.4661</b>	<b>0.000</b>	<b>0.4209</b>	<b>0.000</b>	<b>0.3857</b>	<b>0.000</b>	<b>0.3621</b>	<b>0.000</b>
NaNO <sub>3</sub> **	N/A	N/A	N/A	N/A	N/A	N/A	-0.0087	0.320
CaCl <sub>2</sub> **	N/A	N/A	N/A	N/A	N/A	N/A	-0.0026	0.769
Phosphates	<b>-0.0313</b>	<b>0.000</b>	<b>-0.0355</b>	<b>0.000</b>	<b>-0.0345</b>	<b>0.000</b>	<b>-0.0424</b>	<b>0.000</b>
NaNO <sub>3</sub> *CaCl <sub>2</sub>	N/A	N/A	N/A	N/A	N/A	N/A	<b>-0.0187</b>	<b>0.038</b>

Statistically significant results are in bold italics.

\* *P* values for *t*-test H<sub>0</sub>: Factor coefficient = 0.0; H<sub>A</sub>: Factor coefficient < > 0.0.

\*\* Because the NaNO<sub>3</sub>\*CaCl<sub>2</sub> interaction is significant for biomass, the first-order terms have been included to satisfy model hierarchical requirements.



**Figure 3.** Growth curves from Experiment 2. (a) Treatments 1–8 in block 1; (b) Treatments 9–16 in block 2. Media compositions for each flask are given in Table 3. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

**Table 4.** Predicted biomass concentration and 95% confidence limits, as calculated from Eq. 2, derived from Experiment 1 data.

$f_{\text{NaNO}_3}$	$f_{\text{CaCl}_2}$	$f_{\text{potassium phosphate}}$	Predicted biomass concentration (gdw/L), $X_{\text{final}}$	95% lower confidence limit	95% upper confidence limit
-1	1	-1	0.429	0.390	0.469
-1	-1	-1	0.397	0.358	0.436
1	1	1	0.290	0.250	0.329

“-1” refers to concentrations at 1/6 that of 3N-BB; “1” refers to concentrations equal to that of 3N-BB.

**Table 5.** Model results from Experiment 2 data.

Model term	$\mu$ Days 4-7	$\mu$ Days 4-8	$\mu$ Days 5-8	Biomass		Lipid content		Lipid concentration	
	<i>P</i> -value*	<i>P</i> -value	<i>P</i> -value	Regression coefficient	<i>P</i> -value	Regression coefficient	<i>P</i> -value	Regression coefficient	<i>P</i> -value
<b>Regression coefficients, including all factors and their second-order interactions</b>									
Constant	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	1.0644	<b>0.000</b>	0.1405	<b>0.001</b>	0.1309	<b>0.001</b>
Block	0.352	0.148	0.083	0.0606	0.034	-0.0286	0.119	-0.0221	0.159
NaNO <sub>3</sub>	0.590	0.605	0.504	0.2019	<b>0.000</b>	-0.0696	<b>0.009</b>	-0.0484	<b>0.019</b>
CaCl <sub>2</sub>	0.352	0.192	0.697	0.0019	0.927	0.0031	0.839	0.0051	0.710
MgSO <sub>4</sub>	0.267	0.148	0.628	0.1831	<b>0.001</b>	-0.0111	0.484	-0.0010	0.944
Potassium phosphates	0.459	0.421	0.922	-0.0269	0.234	-0.0040	0.796	-0.0046	0.740
NaNO <sub>3</sub> *CaCl <sub>2</sub>	0.459	0.834	0.354	-0.0506	0.058	-0.0058	0.711	-0.0124	0.388
NaNO <sub>3</sub> *MgSO <sub>4</sub>	0.459	0.677	0.504	0.1656	<b>0.001</b>	-0.0080	0.609	0.0004	0.976
NaNO <sub>3</sub> *phosphates	0.459	0.834	0.150	-0.0669	<b>0.025</b>	0.0016	0.916	-0.0078	0.575
CaCl <sub>2</sub> *MgSO <sub>4</sub>	0.912	0.477	0.354	-0.0669	<b>0.025</b>	-0.0115	0.470	-0.0190	0.211
CaCl <sub>2</sub> *phosphates	0.590	0.754	0.449	-0.0219	0.318	0.0019	0.903	0.0052	0.705
MgSO <sub>4</sub> *phosphates	0.590	0.916	0.245	0.0219	0.318	-0.0054	0.729	-0.0048	0.728

Model term	Biomass		Lipid content		Lipid concentration	
	Regression coefficient	<i>P</i> -value	Regression coefficient	<i>P</i> -value	Regression coefficient	<i>P</i> -value
<b>Model data from regression — Model reduced to significant terms</b>						
Constant	1.0644	<b>0.000</b>	0.1405	<b>0.000</b>	0.1309	<b>0.000</b>
NaNO <sub>3</sub>	0.2019	<b>0.000</b>	-0.0696	<b>0.000</b>	-0.0484	<b>0.000</b>
CaCl <sub>2</sub> **	0.0019	0.929				
MgSO <sub>4</sub>	0.1831	<b>0.000</b>				
Potassium phosphates**	-0.0269	0.230				
NaNO <sub>3</sub> *CaCl <sub>2</sub>	-0.0506	<b>0.046</b>				
NaNO <sub>3</sub> *MgSO <sub>4</sub>	0.1656	<b>0.000</b>				
NaNO <sub>3</sub> *phosphates	-0.0669	<b>0.016</b>				
CaCl <sub>2</sub> *MgSO <sub>4</sub>	-0.0669	<b>0.016</b>				

Statistically significant results are in bold italics. Blank entries for regression coefficients in reduced model indicate insignificant terms.

\* *P* values for *t*-test HO: Factor coefficient = 0.0; HA: Factor coefficient < > 0.0.

\*\* Nonsignificant first-order terms have been included in the reduced model when they also appear in interactions to satisfy model hierarchy requirements.

growth rate at 1/10 3N-BB concentration, corresponding to 5.3 mg P/L, is corroborated by the very low reported value for *K<sub>s</sub>* for phosphorous: 0.27 mg P/L [24]. On the other hand, the concentration of nitrogen at 1/10 3N-BB levels is 12 mg/L, which is identical to the reported *K<sub>s</sub>* for nitrogen [17]. Although we did not observe any effect of vitamin levels (B<sub>1</sub> and B<sub>12</sub>) on growth rate, it has been shown that vitamins B<sub>6</sub> and B<sub>2</sub> assist algae in response to stress by heavy metals [25].

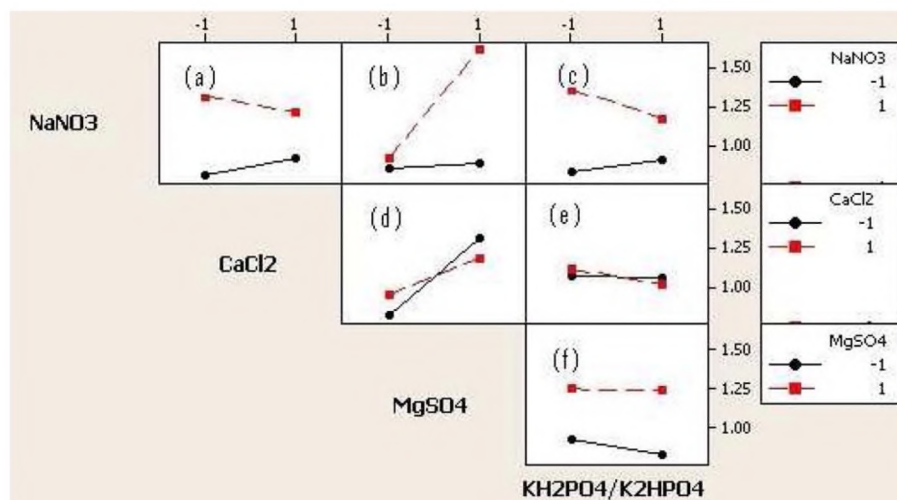
The calculated growth rates are functions of the specific window of time considered to be “exponential phase.” To

ensure that the potential subjectivity in indentifying the exponential phase, and the subsequent variation in specific growth rate calculations, did not influence the conclusions, the statistical analyses were conducted using specific growth rates calculated for three different windows of time. In all cases, the same conclusions with regard to media component effects on growth rate were obtained.

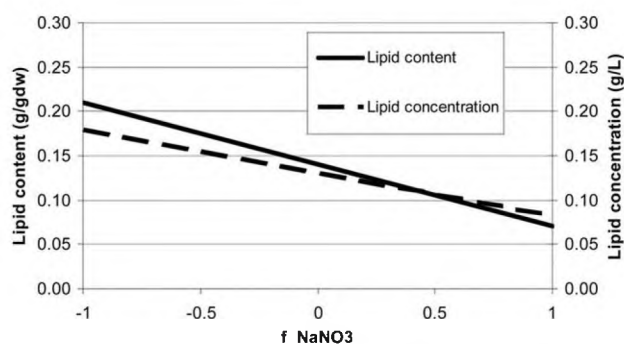
#### Biomass Concentration

The second experiment showed that higher biomass yields resulted from those cultures supplemented with





**Figure 4.** Effects of interactions of the four factors from Experiment 2 on biomass concentration. The abscissas represent the range of the factors, where “-1” and “+1” represent 1/10 $\times$  and 2 $\times$  the concentration of that factor relative to the 3N-BBM. The ordinate represents the biomass concentration, in gdw/L. (a–c) The interactions of NaNO<sub>3</sub> with CaCl<sub>2</sub>, MgSO<sub>4</sub>, and potassium phosphates, respectively. (d and e) The interactions of CaCl<sub>2</sub> with MgSO<sub>4</sub> and potassium phosphates, respectively. (f) The interaction of MgSO<sub>4</sub> and potassium phosphates. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]



**Figure 5.** Effect of NaNO<sub>3</sub> on lipid content and lipid concentration, from Eqs. 4 and 5, respectively, using Experiment 2 data. The abscissa represents the range of  $f_{\text{NaNO}_3}$ , where “-1” and “1” represent 1/10th and 2 $\times$ , respectively, the concentration of NaNO<sub>3</sub> in 3N-BB medium.

excess NaNO<sub>3</sub> and MgSO<sub>4</sub>, separately and in combination. It is not surprising that these two chemicals are limiting for biomass concentration, given that nitrogen is essential for protein synthesis and magnesium is an essential component in the reaction center of chloroplasts. The concurrent excess of these two components exhibits a synergistic effect on biomass by allowing cultures to maintain active cell division throughout the 13-day study period. Low levels of these components do not seem to have an effect on growth rate *per se*, but rather force an early stationary phase due to nutrient depletion, which reduces the final biomass concentration (Figure 4b). This effect was not observed in the first set of experiments (Table 3), in which the experiments were only carried out to day 9, and thus many of the cultures did not reach stationary phase, and nutrients may not yet have been depleted. Biomass concentration was unaffected by either reduced (1/6) or enhanced (2 $\times$ ) levels of trace metals, consistent with a previous environmental study using CoCl<sub>2</sub> [25].

### Lipid Yield

There is ample evidence in the literature that higher levels of lipid accumulation occur during periods of stress, when active replication is halted or slowed by nitrogen limitation, both in a variety of algae genus [8] and the *Scenedesmus* genus [12,13]. Our data supports this conjecture as well, showing that low levels of NaNO<sub>3</sub> led to higher cellular lipid content, up to 28% by dry weight, while excess NaNO<sub>3</sub>, in combination with excess of other nutrients, results in lipid content as low as 2.2% (Table 3). Equation 4 suggests maximum lipid content occurs with the minimum NaNO<sub>3</sub> concentration tested here, which is equivalent to 0.055 g NO<sub>3</sub><sup>-</sup>/L. This confirms previous results showing that 0.04 g NO<sub>3</sub><sup>-</sup>/L was optimal for lipid content for *S. obliquus* [7]. Limitations in light and/or CO<sub>2</sub> may have caused the absence of this observed effect of nitrogen on lipid accumulation for *S. dimorphus* from several other reports in the literature [3,4]. We did not observe a significant effect of the potassium phosphates on lipid content, over the range of concentrations evaluated here (0.02, 0.16, and 0.32 g PO<sub>4</sub><sup>3-</sup>/L), whereas an optimum has been reported at the low end of this range, at 0.03 g PO<sub>4</sub><sup>3-</sup>/L [7].

The actual lipid productivity of the cultures is indicated by the final lipid concentration, and optimal media composition must take into account the trade-offs associated between the constituents of this property, i.e., lipid content and biomass concentration. Of the four components tested explicitly (NaNO<sub>3</sub>, CaCl<sub>2</sub>, MgSO<sub>4</sub>, and K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>), only NaNO<sub>3</sub> affected the lipid concentration over the 20-fold variation in concentrations tested here. The small negative coefficient for NaNO<sub>3</sub> (-0.048, Eq. 4) indicates that the effect on lipid content slightly outweighs the effect on biomass concentration. The final lipid yield is not the only important productivity metric; the time to reach this (i.e., the growth rate) also strongly influences the process economics. Given that the lowest NaNO<sub>3</sub> concentration did not negatively affect growth rate, it appears that the lower NaNO<sub>3</sub> concentration (1/10 that in 3N-BB) may be optimal for large-scale algae oil production. However, this recommendation is tempered by two caveats. First, these experiments were limited to 13 days of culture. Over a longer period of time, even the *S. dimorphus*

cultures with initially high NaNO<sub>3</sub> concentrations will eventually be depleted of nitrogen, and will increase in lipid content (unpublished data). In this circumstance, the overall lipid concentration may then be further increased due to the higher biomass concentration, provided that light limitations do not occur, with the trade-off that a longer period of time is needed. Secondly, there are ways to optimize the process, such as by means of two reactors (or ponds) in series, the first to maximize biomass yield, and the second to accumulate lipids.

These experimental results were conducted in 250 mL flasks in which every effort was made to ensure that all other operating parameters (light, temperature, shaking, air/CO<sub>2</sub> supply) was consistent among the flasks. In a different, or larger, culture system, one of the above operating parameters may be more limiting for growth, which may suggest that medium concentrations could be reduced even further. Conversely, a greater availability of energy or carbon for growth in an alternative system will require a reexamination of these results and possible upward adjustment of recommended concentrations.

#### ACKNOWLEDGMENTS

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