Application of Chemostat culture to Nutrient uptake rate measurements by the macroalgae *Saccharina japonica* var. *religiosa* (Phaeophyceae) and *Ulva australis* (Ulvophyceae)

Ryosuke Okazaki[1], Narumi Teramoto[1], Andrew K Carlson[1], Kiyoko Nakanishi[1]

Isao Kudo[1,2]

1. Graduate School of Environmental Science, Hokkaido University, Sapporo 060-0810, Hokkaido, Japan

2. Faculty of Fisheries Sciences, Hokkaido University, Hakodate 042-8611, Hokkaido, Japan

Author for correspondence: ikudo@fish.hokudai.ac.jp

SUMMARY

In this study, we applied a chemostat culture method, for the first time, to measure the nutrient uptake rate of macroalgae. We examined two methods of measuring the nutrient uptake rate of two macroalgae, *Saccharina japonica* var. *religiosa* and *Ulva australis,* by comparing nutrient uptake kinetics between the chemostat culture and batch culture. In the chemostat culture, the nutrient concentration was kept constant by monitoring the change in nutrient concentration using an Auto Analyzer in real time and adding nutrients

to compensate for the macroalgae's nutrient consumption. The nutrient uptake in the chemostat culture could be best fitted to the Michaelis-Menten saturation kinetics. In the batch culture, the nutrient concentration decreased with time, either constantly or exponentially due to a rapid uptake of nutrients by the macroalgae. The nutrient uptake rate in the batch culture generally showed a scattered relationship with nutrient concentration, with a weak fitting to the Michaelis-Menten saturation kinetics. This discrepancy seemed to be partly because the change in nutrient concentration was large between the sampling intervals in the batch culture. Determining an appropriate sampling interval for detectable concentration change is difficult unless the nutrient concentration is measured in real time. Therefore, the application of the chemostat culture method to the measurement of the uptake rate by macroalgae could greatly improve our understanding of nutrient uptake kinetics.

Key index words: batch culture; chemostat culture; macroalgae; Michaelis-Menten curve; nutrient uptake kinetics

INTRODUCTION

The batch culture method has generally been used to measure the nutrient uptake rate of macroalgae (D'Elia & DeBoer 1978; Haines & Wheeler 1978; Harlin & Craigie 1978;

Wallentinus 1984; Harrison *et al*. 1986; Pedersen 1994; Pedersen & Borum 1997; Martinez *et al*. 2012). In this method, the uptake rate by macroalgae is measured by incubating a thallus sample in a tank and observing the decrease in nutrient concentration in the surrounding seawater. The uptake rate is calculated by dividing the amount of decrease in concentration by the sampling interval. This rate is further normalized by algal biomass (dry weight) or surface area. This method is convenient, but when a sample has a high nutrient uptake rate, the large drawdown of nutrients within the tank becomes an issue. Generally, the nutrient uptake rate is expressed as a function of the nutrient concentration according to the Michaelis-Menten equation, $V = V_{max}\ S/(K_s + S)$, where V is the uptake rate (μmol g dry wt^{-1} h^{-1}), V_{max} is the maximal uptake rate, S is the concentration of limiting nutrient (μM), and K_s is the half-saturation constant representing the value of S, where $V = V_{max}/2$. The species-specific nutrient uptake kinetic parameters, V_{max} and K_s, have been used to explain species competition for a limiting nutrient (Dugdale 1967; Tilman 1977; Button 1985).

In a batch culture, nutrient concentration within the tank decreases with time due to nutrient uptake, resulting in incorrect evaluation of the relationship between nutrient concentration and uptake rate. A larger tank or shorter sampling interval could be used to reduce the effects of the decreasing nutrient concentration. However, too small of a

change in nutrient concentration could result in larger errors in calculating the uptake rates. Therefore, to ensure precise uptake rate measurement, it is necessary to monitor the nutrient concentration in the incubation tank in real time and keep the concentration at a designated value by compensating for the consumption by algae.

The use of a chemostat culture can allow for the measurement of the nutrient uptake rate while keeping the nutrient concentration constant in the incubation tank. Chemostat is one of the continuous culture methods for keeping the concentration of chemicals at a constant level by monitoring the concentration in real time and supplying chemicals to compensate for their decrease (consumption). The continuous culture generally supplies fresh medium to the culture tank and removes the effluent from the tank at a constant flow rate (Tilman & Kilham 1976). This method can control the growth rate of microalgae (μ) by changing the dilution rate (D, d^{-1}). D is defined as f/v, where f is the volume (L) replaced daily by the fresh medium and v is the volume (L) of the culture. In this method, the limiting nutrient concentration in the culture tank is depleted at a steady state. Thus, the continuous culture cannot control the nutrient concentration at a designated level in the culture tank. In the chemostat culture, a target nutrient concentration in the incubation tank is monitored in real time, and the nutrient decrease rate is calculated to recover the nutrient losses with an appropriate nutrient addition. Generally, an Auto Analyzer is used

62 to measure nutrient concentrations in seawater (Grasshoff *et al*. 1999). This instrument
63 has the high analytical sensitivity and accuracy required to measure the nutrient
64 concentration in seawater. However, the instrument takes 5-10 minutes to display the
65 results after introducing the sample. This time lag in measurement has to be considered
66 to keep the nutrient concentration at a certain level in the chemostat culture. The
67 objectives of this study were to develop a method of measuring the nutrient uptake rate
68 of macroalgae in a chemostat culture by using an Auto Analyzer in real time and to
69 compare methods to verify the effectiveness of the chemostat culture.
70
71 MATERIALS AND METHODS
72 Sample collection
73 Samples of *Ulva australis* were collected in August 2015 and those of *Saccharina*
74 *japonica* var. *religiosa* in April 2019 from the shore of Oshoro Bay, Hokkaido (43°12’28”
75 N; 140°51’53” E). The weight of *Ulva* ranged from 8.5 to 11.2 g wet wt. The length and
76 weight of *Saccharina* (only 1st age) without sorus ranged from 170 to 250 mm and 13.3
77 to 31.9 g wet wt, respectively. Macroalgae thalli were sampled from the rocky substrate
78 without damaging the holdfasts and were transported to the laboratory under dark and
79 cool conditions. Epiphytes were removed by gentle brushing under running seawater. The

macroalgae were maintained for at least 24 h in the incubator at the in situ temperature in Oshoro Bay (August 2015: 24.0 ± 0.5°C, April 2019: 7.0 ± 0.5°C) and at 120 μmol photons m^{-2} s^{-1} using fluorescent lamps with daylight spectrum.

Chemostat culture experiment

Three liters of prescreened (100 μm mesh) seawater collected from Oshoro Bay were poured into an incubation tank and stirred constantly with a magnetic stirrer in a temperature-controlled incubator. The stirrer equipped with guard frame was used and a stainless mesh was placed in the incubation tank to prevent possible damage to thallus. Rotation rate was set at 600 rpm, generating water motion at approximately 3.4 cm s^{-1}. In the experiment using *Saccharina*, the uptake rates of either NH_4-N or NO_3-N were measured. The PO_4-P concentration was adjusted to a sufficient level (>4.0 μM). At the beginning of each uptake experiment, NH_4-N, NO_3-N, and PO_4-P concentrations in the tank were the sum of those contained in the original seawater and the spiked amounts of nutrient stock solution. In contrast, in the experiment using *Ulva*, the uptake rates of NO_3-N and PO_4-P were measured. In the NO_3-N uptake experiment, the NO_3-N concentration was adjusted to 0.5-20 μM, while the PO_4-P concentration was adjusted to a sufficient level (>4.0 μM). In the PO_4-P uptake experiment, the PO_4-P concentration was adjusted to 0.1-4.0 μM, while the NO_3-N concentration was adjusted to a sufficient level (>20 μM).

98 The NH_4-N, NO_3-N, and PO_4-P concentrations in the original seawater were < 0.1 μM in
99 August 2015 and April 2019 (except NO_3-N at 5 μM in April 2019). The nutrient stock
100 solutions were prepared from special reagent grade chemicals such as KNO_3, $(NH_4)_2SO_4$,
101 and KH_2PO_4 at 10,000, 5,000, and 5,000 μM, respectively.
102 A macroalgal thallus was placed in the tank. The density of macroalgae varied from 1.5
103 to 5.3 g wet wt L^{-1} depending on species. Every 10 minutes, an aliquot (10 mL) of
104 seawater was sampled from the tank and filtered using a GF/F filter (pore size 0.7 μm).
105 The filtered sample was placed to the sample tray manually. Nutrient concentrations
106 (NH_4-N, NO_3-N, and PO_4-P) were then quickly measured with an Auto Analyzer
107 (QuAAtro, BRAN+LUEBBE, Tokyo, Japan) (Grasshoff *et al*. 1999). The Auto Analyzer
108 took 5-8 minutes to obtain the analytical results. The schematic time course change of
109 concentration and procedures in the chemostat culture are shown in Fig. 1.
110 The nutrient decrease rate (μmol L^{-1} min^{-1}), $R_{0\text{-}10}$, was calculated from the change in
111 nutrient concentration between t = 0 and t = 10 minutes divided by 10 (min). The amount
112 of the first nutrient addition at t = 20 was calculated to recover the nutrient loss, assuming
113 that $R_{0\text{-}10}$ would continue until t = 20 because the result sampled at t = 10 was obtained at
114 t = 15-18. The amount of nutrient addition at t = 20, A_{20} (μL), was calculated as follows:

115 $$A_{20} = \frac{\{(C_T - C_{10}) + R_{0-10}\} \times W}{N \times 10^{-6}} \quad (1)$$

where C_T is the target nutrient concentration (μM); C_{10} is the concentration at t = 10; N is the nutrient concentration in the nutrient stock solution (μM); and W is the seawater volume (L) in the tank.

The nutrient decrease rate after t = 30 was calculated from the change in concentration and the added amount of nutrient in each tank, as follows:

$$R_{(t-10)-t} = \frac{(C_t - C_{t-10}) + C_{A(t-10)})}{10} \quad (2)$$

where C_t is the nutrient concentration (μM) at time t and $C_{A(t-10)}$ is the increase in nutrient concentration by adding nutrient at time = t-10, calculated as

$$C_{A(t-10)} = \frac{A_{t-10} \times N \times 10^{-6}}{W} \quad (3)$$

where A_{t-10} is the amount of added nutrient (μL) at time = t-10.

Because there were biological fluctuations in the nutrient uptake by macroalgae, the average value of the two most recently calculated nutrient decrease rates, $\overline{R_t}$, was used for the calculation of A_t after t = 30, as follows:

$$\overline{R_t} = \frac{R_{(t-20)-(t-10)} + R_{(t-10)-t}}{2} \quad (4)$$

A_t was calculated as

$$A_t = \frac{\{(C_T - C_{t-10}) + \overline{R_{t-10}}\} \times W}{N \times 10^{-6}} \quad (5)$$

This operation was continued for 60-90 minutes until nutrient concentrations and nutrient

uptake rates were stabilized. Four settings could be simultaneously run at one time, with a 5-minute delay for two of four settings. The calculation for the chemostat operation was conducted with an Excel (Microsoft, Redmond, USA) worksheet. This worksheet will be provided upon request by the corresponding author.

Nutrient uptake rates (R) were converted to those based on dry weight as follows:

$$V_{t-(t+10)} = \frac{\{(C_t - C_{t+10)} + C_{A(t)}\} \times W \times 60}{\Delta t \times B} \quad (6)$$

where $V_{t-(t+10)}$ is the nutrient uptake rate between time t to t+10 (μmol g dry wt^{-1} h^{-1}); B is the dry weight of the sample (g dry wt) and Δt is the sampling interval (min).

The thallus was dried at 55 °C on aluminum foil until a constant dry weight was achieved, typically for 4 to 5 days.

The average and standard deviation (SD) of the uptake rate for each concentration setting were calculated from the five to eight uptake rate data from a single experiment. The average and SD of the nutrient concentration were also calculated. The results between t = 0 and t = 20 were not included in the calculation for the average nutrient uptake rate and concentration. Four to five settings of different concentrations were applied to fit the Michaelis-Menten curve.

The precision of analysis, expressed as the coefficient of variation (CV) on the Auto Analyzer, was about 1%, and the detection limit (c.a., 0.01 μM) was defined as three times

the CV of the lowest standard solution.

The experimental reproducibility (CV%) using different strands varied from 3% to 20%. The control experiment was conducted in the same way without macroalgae. The effect of microalgae (phytoplankton) in the 100 μm mesh screened seawater on the measured uptake rates was negligible because no change in the nutrient concentrations was observed in the control experiment. Thus, the obtained uptake rates were judged to be only due to the uptake by macroalgae.

Batch culture experiment

Nutrient concentrations in the tanks and experimental conditions were the same as in the chemostat culture for the measurement of nutrient uptake rates in the batch culture. An aliquot of seawater (10 mL) was sampled from each tank every 10 minutes for 60-120 minutes. Duplicate tanks were set up for *Ulva*, and NO_3-N concentration was adjusted to 20 μM in the N uptake experiment. The PO_4-P concentration was adjusted to 3 μM. Ten tanks were set up for *Saccharina*, and either NO_3-N or NH_4-N concentration was adjusted to ranging from 15 μM to 40 μM in the N uptake experiment. Nutrient concentrations in these samples were measured discretely with an Auto Analyzer. Nutrient uptake rates were calculated from the decrease in nutrient for each sampling interval, and the average

nutrient concentration in each interval was used as the substrate concentration for plotting. The nutrient uptake rate was assumed to change depending on the nutrient concentration, so each uptake rate was plotted as an individual value.

Michaelis-Menten curve

The uptake rate, V, was plotted against the nutrient concentration, S, in each treatment and best fitted to the Michaelis-Menten curve according to the following equation:

$$V = \frac{V_{max} \cdot S}{K_s + S} \qquad (8)$$

where V_{max} is the maximum uptake rate (μmol g dry wt^{-1} h^{-1}); K_s is the half-saturation constant (μM) when $S = K_s$; V is half of the V_{max}; and S is the nutrient concentration (μM). The experimental results were best fitted to the Michaelis-Menten curve using a Kaleida Graph (Synergy Software, Reading, USA). In the fitting to the curve, V at S = 0 was assumed to be zero.

Furthermore, an indicator of nutrient uptake efficiency at low nutrient concentration, α (Healey, 1980), was calculated from V_{max} divided by K_s ($\alpha = V_{max} / K_s$).

RESULTS

Chemostat culture

188 Time course changes in NO_3-N and NH_4-N in the chemostat cultures using *Saccharina*
189 are shown in Fig. 2. The concentration of NO_3-N or NH_4-N continued to decrease until t
190 = 30 minutes, when the effect of the nutrient addition appeared. After controlling the
191 nutrient concentration through nutrient addition, each nutrient concentration was
192 maintained at a constant level during the experiment. The CV ranges for the
193 concentrations of NO_3-N and NH_4-N in each tank were 4.8-11.9 and 5.0-18.9%,
194 respectively. In the experiment using *Ulva*, the ranges for PO_4-P and NO_3-N were 5-13%
195 and 6-24%, respectively (Fig. 3). The averaged nutrient concentration and uptake rates
196 for *Saccharina* (Fig. 4) and *Ulva* (Fig. 5) were best fitted to the Michaelis-Menten
197 equation. V_{max} and K_S were determined from these curves (Table 1). In the experiment
198 using *Ulva*, K_s values for NO_3-N and PO_4-P were 2.39 and 1.26 μM, whereas V_{max} values
199 were 6.51 and 0.16 μmol g dry wt^{-1} h^{-1}, respectively. In the experiment using *Saccharina*,
200 K_s values for NO_3-N and NH_4-N were 5.35 and 9.29 μM, whereas V_{max} values were 22.3
201 and 23.2 μmol g dry wt^{-1} h^{-1}, respectively.
202
203 Batch culture
204 NH_4-N and NO_3-N concentrations in the batch culture using *Saccharina* decreased
205 constantly (Fig. 6). Whereas the PO_4-P concentration decreased constantly in the batch

206 culture using *Ulva*, the NO_3-N concentration decreased exponentially and was depleted
207 by t = 80 minutes (Fig. 7). The nutrient decrease rates ($(C_{t-10} - C_t)/C_t \times 100, (\%)$) for
208 each sampling interval in the experiment using *Saccharina* and in the PO_4-P uptake
209 experiment using *Ulva* were lower than 10%. In contrast, the rates in the NO_3-N uptake
210 experiment using *Ulva* were higher than 30%. The nutrient uptake rates by *Saccharina* in
211 the batch culture did not become saturated with increasing nutrient concentration but
212 rather showed a linear relationship with nutrient concentration (Fig. 8). The NO_3-N uptake
213 rate for *Ulva* showed a fit to the Michaelis-Menten curve, but no data were available at
214 higher concentrations (Fig. 9a). K_S and V_{max} for NO_3-N were 53 μM and 13.7 μmol g dry
215 wt^{-1} h^{-1}, respectively. Those for PO_4-P were 1.45 μM and 0.16 μmol g dry wt^{-1} h^{-1},
216 respectively (Fig. 9b).

217 The α value was calculated for the comparison between the results in the chemostat
218 culture and the batch culture. In the experiment using *Saccharina* in the chemostat culture,
219 the α values for NO_3-N and NH_4-N were 4.33 and 2.40, respectively (Table 1). The α
220 values in the batch culture for NO_3-N and NH_4-N were 0.83 and 1.00, respectively. The α
221 values for NO_3-N and PO_4-P in the experiment using *Ulva* in the chemostat were 66.5 and
222 3.09, respectively. Those values for the batch culture were 7.05 and 2.07, respectively.
223 The α values in the batch culture were lower than those in the chemostat culture for both

224 species.

225

226 DISCUSSION

227 It is important for the nutrient concentration in the tank to be maintained at a certain level
228 for the uptake rate calculation. However, in the batch culture, the nutrient concentration
229 decreased constantly (Figs 6 and 7), whereas the NO_3-N concentration in the *Ulva*
230 experiment decreased exponentially until it was depleted (Fig. 7a). Previous research
231 indicates that *Ulva* grows rapidly in the summer (Ono 1988), so the NO_3-N requirement
232 for *Ulva* would be very high in the summer. Harrison et al. (1989) examined several batch
233 culture methods to obtain nutrient uptake kinetic parameters. They conducted batch
234 culture experiments preparing multiple flasks with different nutrient concentrations and
235 varying incubation times (0.05, 1 and 2 hr) and a short incubation time (5 min).
236 Furthermore, they conducted a batch culture experiment with multiple sequential nutrient
237 additions. Batch culture methods provided highly variable uptake rate values with nutrient
238 concentration. This was explained by the feedback inhibition that occurs on a time scale
239 of seconds (McCarthy & Goldman 1979; Goldman & Glibert 1982).
240 On the other hand, the variation in the nutrient concentration was small in the chemostat
241 culture. The nutrient concentration in the tank decreased from the start of the experiment

until the effect of nutrient addition appeared after t = 30 minutes but was maintained at the target concentration after the nutrient addition. In the chemostat culture using *S. religiosa*, the CV in the nutrient concentration was lower than 10% (Fig. 2). The CV in the nutrient uptake rate for each sampling interval was 10-50%. The nutrient addition to maintain the nutrient concentration at the target concentration was successful, but the nutrient uptake rate fluctuated to some extent. This variation in the uptake rate might be due to a biological fluctuation, which seemed to be larger than the analytical error.

The nutrient uptake rates in the chemostat culture became saturated with increasing nutrient concentration (Figs. 4 and 5). Even though there were fewer plots than in the batch culture, each plot in the chemostat culture was averaged in value from more than five measurements and had an error bar.

No studies have compared the characteristics of nutrient uptake kinetics between a batch culture and chemostat culture using macroalgae. For chemostat cultures of *Saccharina*, V_{max} values for NO_3-N and NH_4-N were almost the same at 23.2 and 22.3 μmol g dry wt^{-1} h^{-1}, respectively (Table 1). The K_s for NO_3-N was 5.35 μM, which was lower than that for NH_4-N at 9.29 μM. On the contrary, for batch cultures of *Saccharina*, V_{max} values were 401 and 42.7 μmol g dry wt^{-1} h^{-1} for NO_3-N and NH_4-N, respectively. K_s values were 481 and 40.4 μM for NO_3-N and NH_4-N, respectively. However, those obtained by

260 the batch culture showed an order of difference between NH_4-N and NO_3-N with
261 substantially large errors. The V_{max} and K_s for PO_4-P of *Ulva* obtained by the batch culture
262 were not significantly different from those by the chemostat culture (t-test, $p>0.05$). This
263 is attributed to the scattering plots and lack of saturated values at a higher nutrient
264 concentration range in the batch culture. The reason why these parameters for PO_4-P were
265 not different was not clear in this study.
266 Harrison et al. (1986) and Subandar et al. (1993) reported V_{max} of Laminariales for NH_4-
267 N and NO_3-N ranging 10-20 μmol g dry wt^{-1} h^{-1}. This range was similar to the values
268 obtained by the chemostat culture in this study.
269 O'Brien and Wheeler (1987) reported the in situ uptake rate of NO_3-N by *Ulva* using a
270 bell jar technique. The average of 10.8 μmol g dry wt^{-1} h^{-1} was similar to values in this
271 study.
272 However, the nutrient uptake rate of NO_3-N by *Ulva* in the batch culture was less than
273 half of the chemostat culture at lower concentrations. (Figs 5 and 9). The reason for this
274 difference was not clear, but one explanation may be less data for the batch culture
275 experiment. Along the Michaelis-Menten curve, the nutrient uptake rate changes linearly
276 at lower concentrations. The nutrient concentration was kept constant at a low
277 concentration in the chemostat culture. Both methods were conducted with the same

278 experimental conditions, such as water temperature, light intensity, water mixing, and
279 amount of cultured seawater. The uptake rates in the chemostat culture were more precise
280 than in the batch culture method in the lower concentration region. The correlation
281 coefficients of the best-fitted Michaelis-Menten curves in the chemostat culture were high
282 in both samples (for *Saccharina*, NO_3-N: r = 0.99, NH_4-N: r = 0.99; for *Ulva*, NO_3-N: r =
283 0.95, PO_4-P: r = 0.93). The nutrient uptake rates in the batch culture showed a fitting to
284 the Michaelis-Menten curve but with a lower correlation (for *Saccharina*, NO_3-N: r =
285 0.63, NH_4-N: r = 0.94; for *Ulva*, NO_3-N: r = 0.98, PO_4-P: r = 0.81). Some plots did not
286 become saturated with increasing nutrients but rather showed a scattered linear
287 relationship with the nutrient concentration (Fig. 8a). This tendency was reported in a
288 previous study that measured the nutrient uptake rate of *Saccharina latissima* using a
289 batch culture (as *Laminaria groenlandica,* Harrison *et al.* 1986). Linear relationships
290 between the NH_4-N uptake rate and the NH_4-N concentration were reported in previous
291 studies using *Macrocystis sp*. (Haines & Wheeler 1978) and *Gracilaria foliifera* (D'Elia
292 & DeBoer 1978). The reason the chemostat culture method in the present study could be
293 best fitted to the Michaelis-Menten curve seemed to be that an equilibrium is attained
294 between substrate concentration and uptake rate over a longer period (~ 1 h).
295 Thomas and Harrison (1987) conducted real-time monitoring of substrate concentration

296 in seaweed incubation using an Auto Analyzer. However, they observed a time course
297 change in nutrients but did not control the substrate concentration by adding the nutrient
298 stock solution.

299 Stable isotope ^{15}N has been widely used for the uptake rate measurement of
300 phytoplankton (Glibert *et al.* 1982; Glibert & McCarthy 1984; Dugdale & Wilkerson
301 1986; Kudo *et al*. 2015). N uptake rates and uptake kinetics for seaweed and seagrass
302 have been reported using stable isotope ^{15}N (Williams & Fisher 1985; O'Brien & Wheeler
303 1987; Alexandre *et al*. 2011; Alexandre & Santos 2020). However, the application of this
304 method for macroalgae and seagrass involves the concern of a change in nutrient
305 concentration during the incubation when the uptake rate of the sample tissue is large
306 because the incubation is conducted in the batch culture. To avoid this, a preincubation
307 experiment is necessary to optimize incubation volume and time duration depending on
308 plant biomass. Additionally, the sample for stable isotope measurement must be dried and
309 pulverized; a large thallus presents difficulty for sample preparation.

310 Macroalgae and phytoplankton would suddenly show a high uptake rate to fulfill their
311 limiting nutrient pools responding to a sudden increase in nutrients (Surge uptake,
312 Lapointe 1985; Thomas & Harrison 1987; Lubsch & Timmermans 2019). Furthermore,
313 in the real ocean, nutrient concentrations remain more or less stable for different periods

of time, and are not immediately affected by the seaweed activity due to the large water volume to biomass ratio. It is ideal to apply the chemostat culture method to the nutrient uptake kinetics study. To the best of our knowledge, the method developed in this study is the first to apply a chemostat culture to measure the uptake rate of macroalgae.

The chemostat culture method is applicable to examine the response to nutrient perturbation, as well as to species-specific nutrient uptake kinetics studies. Another application of the chemostat culture method in the present study are IMTA (Integrated multi-trophic aquaculture) systems, in which species from two or more trophic levels grow in one farm and where the waste of one feeds another (Buschmann 1996; Neori et al. 2004; Cruz-Suárez et al. 2010). Fast-growing seaweed, such as *Ulva prolifera*, has been used in this system (Cruz-Suárez et al. 2010). The system has a constant flow of seawater and nutrients uptake rate may be high in the system. So there may be difficulties in measuring the uptake rate of the seaweeds correctly in IMTA systems when using the batch culture method. In contrast, the chemostat culture method would better simulate conditions and more accurately measure the uptake rate in IMTA systems.

CONCLUSIONS

In this study, we developed a new method to measure the nutrient uptake rate of

macroalgae while maintaining a constant nutrient concentration. These results demonstrate the applicability and the accuracy of measuring nutrient uptake rates. Applying this method to other macroalgal species could therefore deepen our understanding of macroalgal uptake kinetics.

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REFERENCES

Alexandre, A. and Santos, R. 2020. Competition for nitrogen between the seaweed *Caulerpa prolifera* and the seagrass *Cymodocea nodosa*. *Mar. Ecol. Prog. Ser*. **648**: 125-134.

Alexandre, A., Silva, J., Bouma, T.J. and Santos, R. 2011. Inorganic nitrogen uptake kinetics and whole-plant nitrogen budget in the seagrass *Zostera noltii*. *J. Exp. Mar. Biol. Ecol*. **401**: 7-12.

Buschmann, A.H. 1996. An introduction to integrated farming and the use of seaweeds as biofilters. *Hydrobiol.* 326: 59-60.

Button, D.R. 1985. Kinetics of nutrient-limited transport and microbial growth.

350 *Microbial. Rev*. **49**: 270-297.

351 Cruz-Suárez, L.E., León, A., Penã-Rodríguez, A., Rodríguez -Penã, G., Moll, B. and

352 Ricque-Marie, D. 2010. Shrimp/*Ulva* co-culture: a sustainable alternative to diminish

353 the need for artificial feed and improve shrimp quality. *Aquacul*. 301: 64-68.

354 D'Elia, C.F. and De Boer, J. A. 1978. Nutritional studies of two red algae. II. Kinetics

355 of ammonium and nitrate uptake. *J. Phycol*. **14**: 266-272

356 Dugdale, R. C. 1967. Nutrient limitation in the sea: dynamics, identification and

357 significance. *Limnol. Oceanogr*. **12**: 685-695.

358 Dugdale, R. C. and Wilkerson, F. P. 1986. The use of ^{15}N to measure nitrogen uptake

359 in eutrophic ocean: experimental consideration. *Limnol. Oceanogr*. **31**: 673-689.

360 Glibert, P. M., Lipschults, F., McCarthy, J. J. and Altabet, M.A. 1982. Isotope dilution

361 models of uptake and remineralization of NH_4 by marine plankton. *Limnol. Oceanogr*.

362 **27**: 639-650.

363 Glibert, P. M. and McCarthy, J. J. 1984. Uptake and assimilation of ammonium and

364 nitrate by phytoplankton; indices of nutritional status. *J. Plankton Res*. **6**: 677-697.

365 Goldman, J. C. and Glibert, P. M. 1982. Comparative rapid ammonium uptake by four

366 species of marine phytoplankton. *Limnol. Oceanogr*., **27**: 814-827.

367 Grasshoff, K., Kremling, K. and Ehrhardt, M. 1999. *Methods of sea water analysis,*

368 *Third, Completely Revised and Extended Edition,* WILEY-VCH Verlag GmbH,
369 Weinheim.
370 Haines, K.C. and Wheeler, P.A. 1978. Ammonium and nitrate uptake by the marine
371 macrophytes *Hypnea musciforms* (Rhodophyta) and *Macrocystis pyrifera*
372 (Phaeophyta). *J. Phycol*. **14**: 319-324
373 Harlin, M. and Craigie, J. 1978. Nitrate uptake by *Laminaria longicruris*
374 (Phaeophyceae). *J. Phycol*. **14**: 464-467
375 Harrison, P.J., Druehl, L.D., Lloyd, K.E. and Thompson, P.A. 1986. Nitrogen uptake
376 kinetics in three year-classes of *Laminaria groenlandica* (Laminariales : Phaeophyta).
377 *Mar. Biol*. **93**: 29-35
378 Harrison P.J., Parslow, J.S. and Conway, H.L. 1989. Determination of nutrient uptake
379 kinetic parameters: a comparison of methods. *Mar. Ecol. Prog. Ser*. **52**: 301-312.
380 Healey, F.P. 1980. Slope of the Monod equation as an indicator of advantage in
381 nutrient competition. *Microbial. Ecol.* **5**: 281-286.
382 Kudo, I., Hisatoku, T., Yoshimura, T. and Maita, Y. 2015. Primary productivity and
383 nitrogen assimilation with identifying the contribution of urea in Funka Bay, Japan.
384 *Estuar. Coast. Shelf. Sci*. **158**: 12-19.
385 Lapointe, B.E. 1985. Strategies for pulsed nutrient supply to *Gracilaria* cultures in

386 the Florida Keys: Interactions between concentration and frequency of nutrient pulses.
387 *J. Exp. Mar. Biol. Ecol*. **93**: 211-222.
388 Lubsch, A. and Timmermans, K.R. 2019. Uptake kinetics and storage capacity of
389 dissolved inorganic phosphorus and corresponding dissolved inorganic nitrate uptake
390 in *Saccharina latissima* and *Laminaria digitata* (Phaeophyceae). *J. Phycol*. **55**: 637-
391 650
392 Martinez, B., Pato, L.S. and Rico, J.M. 2012. Nutrient uptake and growth responses
393 of three intertidal macroalgae with perennial, opportunistic and summer-annual
394 strategies. *Aquat. Bot*. **96**: 14-22
395 McCarthy, J.J. and Goldman, J.C. 1979. Nitrogenous nutrition of marine
396 phytoplankton in nutrient-depleted waters. *Science* **203**: 670-672.
397 Neori, A., Chopin, T., Troell, M., Buschmann, A.H., Kraemer, G.P., Halling, C.,
398 Shpigel, M. and Yarish, C. 2004. Integrated aquaculture: rationale, evolution and state
399 of the art, emphasizing seaweed biofiltration in modern mariculture. *Aquacul*. 231:
400 361-391.
401 O'Brien, M.C. and Wheeler, P.A. 1987. Short term uptake of nutrients by
402 *Enteromorpha prolifera* (Chollophyceae). *J. Phycol*. **25**: 547-556.
403 Ono, M. 1988. Seasonal Changes of the Growth of Green Algae, *Ulva sp*. in Tosa Bay,

404 Southern Japan. *Mar. Foul*. **7**: 13-17

405 Pedersen, M.F. 1994. Transient ammonium uptake in the macroalgae *Ulva lactusa*

406 (Chlorophyta): Nature, regulation, and the consequences for choice of measuring

407 technique. *J. Phycol*. **30**: 980-986

408 Pedersen, M.F. and Borum, J. 1997. Nutrient control of estuarine macroalgae: growth

409 strategy and the balance between nitrogen requirements and uptake. *Mar. Ecol. Prog.*

410 *Ser*. **161**: 151-163

411 Subandar, A.P. and Harrison P.J. 1993. Laminaria culture for reduction of dissolved

412 inorganic nitrogen in salmon farm effluent. *J. Appl. Phycol*. **5**: 455-463.

413 Tilman, D. 1977. Resource competition between planktonic algae: An experimental

414 and theoretical approach. *Ecol.* **58**: 338-348.

415 Tilman, D. and Kilham, S.S. 1976. Phosphate and silicate growth and uptake kinetics

416 of the diatoms *Asterionella Formosa* and *Cyclotella meneghiniana* in batch and

417 semicontinuous culture. *J. Phycol*. **12**: 375-383.

418 Thomas, T.E. and Harrison, P.J. 1987. Rapid ammonium uptake and nitrogen

419 interactions in five intertidal seaweeds grown under field conditions. *J. Exp. Mar. Biol.*

420 *Ecol.* **107**, 1-8.

421 Wallentinus, I. 1984. Comparisons of nutrient uptake rates for Baltic macroalgae with

422 different thallus morphologies. *Mar. Biol*. **80**: 215-225.

423 Williams, S.L. and Fisher, T.R. 1985. Kinetics of nitrogen-15 labelled ammonium

424 uptake by *Caulerpa cupressoides* (Chlorophyta). *J. Phycol*. 21: 287-296.

425

426 Figure legends

427 Figure 1 Schematic diagram of temporal change in nutrient concentration in the
428 chemostat culture. S_x and C_x denote the timing of sampling and concentration reading
429 at time x, respectively. A_x denotes the amount of nutrient addition at time x.

430 Figure 2 The time course changes in NO_3-N (a) and NH_4-N (b) concentration in each
431 run of the chemostat culture of *Saccharina*. Horizontal lines indicate the duration of
432 the nutrient concentration control and the average value for each run.

433 Figure 3 The time course changes in NO_3-N (a) and PO_4-P (b) concentration in each
434 run of the chemostat culture of *Ulva*. Horizontal lines indicate the duration of the
435 nutrient concentration control and the average value for each run.

436 Figure 4 The best-fitted Michaelis-Menten curves for NO_3-N (a) and NH_4-N (b) in the
437 chemostat culture of *Saccharina*. Error bars indicate standard deviations (SD) of the
438 nutrient uptake rates and nutrient concentration.

439 Figure 5 The best-fitted Michaelis-Menten curves for NO_3-N (a) and PO_4-P (b) in the
440 chemostat culture of *Ulva*. Error bars indicate standard deviations (SD) of the nutrient
441 uptake rates and nutrient concentration.

442 Figure 6 The time course changes in NO_3-N (a) and NH_4-N (b) in each run of the
443 batch culture using *Saccharina*.

444 Figure 7 The time course change in NO_3-N (a) and PO_4-P (b) in the batch culture using

445 *Ulva*.

446 Figure 8 The relationship between the uptake rate and the nutrient concentration for

447 NO_3-N (a) and NH_4-N (b) in the batch culture using *Saccharina*. The line indicates the

448 best-fitted line to Michaelis-Menten curve.

449 Figure 9 The relationship between the uptake rate and the nutrient concentration for

450 NO_3-N (a) and PO_4-P (b) in the batch culture using *Ulva*. The line indicates the best-

451 fitted line to Michaelis-Menten curve

452

453

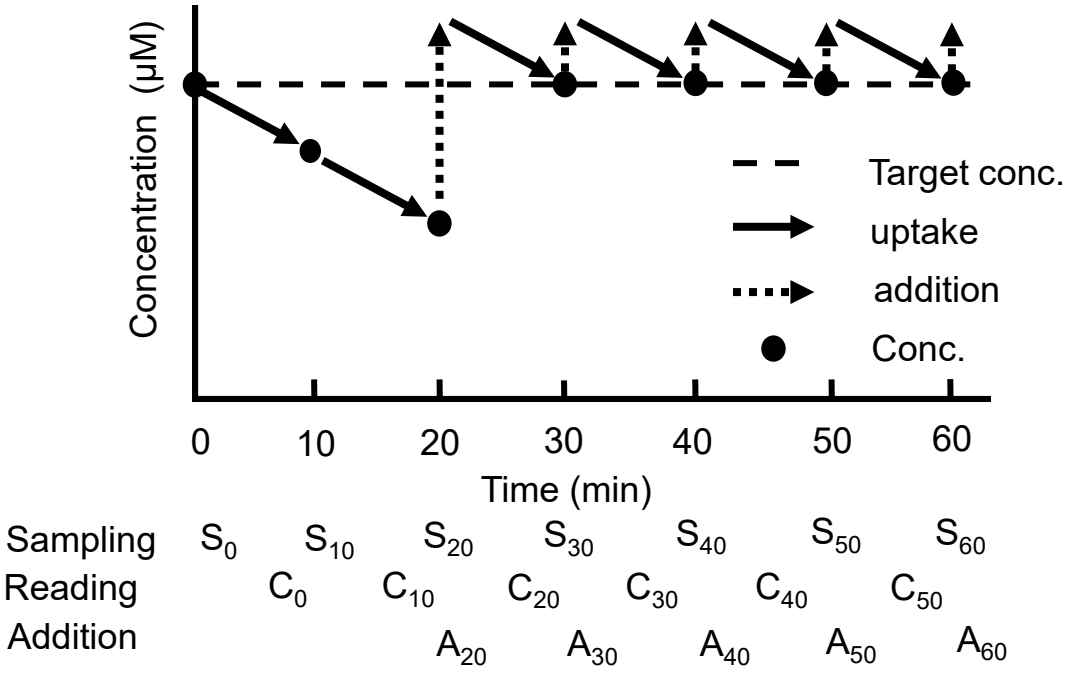
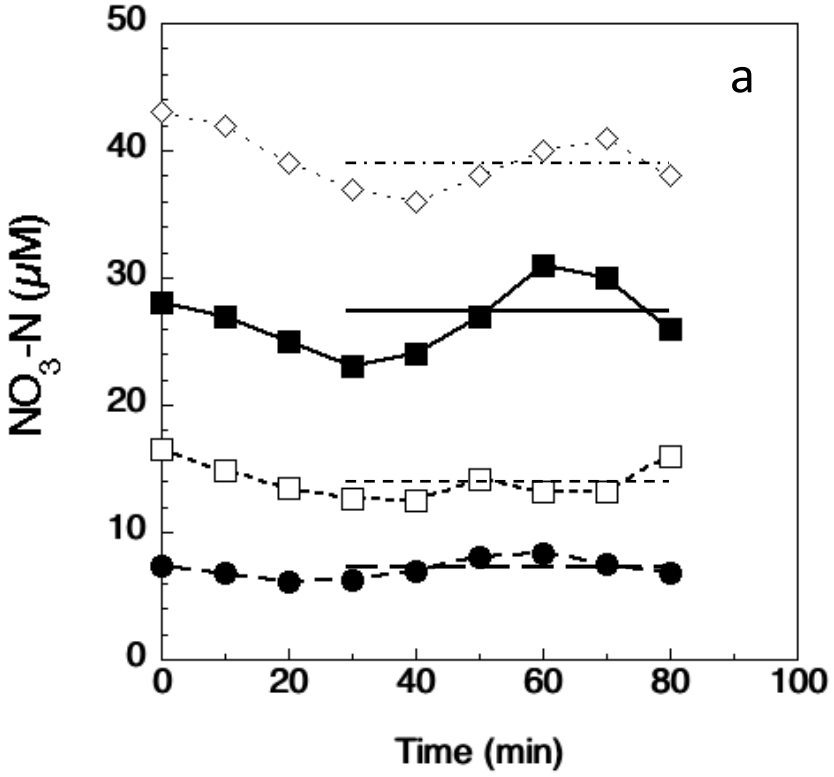

Concentration (μM)
Target conc.
uptake
addition
Conc.
0
10
20
30
40
50
60
Time (min)
Sampling S_0 S_{10} S_{20} S_{30} S_{40} S_{50} S_{60}
Reading C_0 C_{10} C_{20} C_{30} C_{40} C_{50}
Addition A_{20} A_{30} A_{40} A_{50} A_{60}

Fig. 1


a
50
40
30
20
10
0
0
20
40
60
80
100
Time (min)
NO3-N (μM)

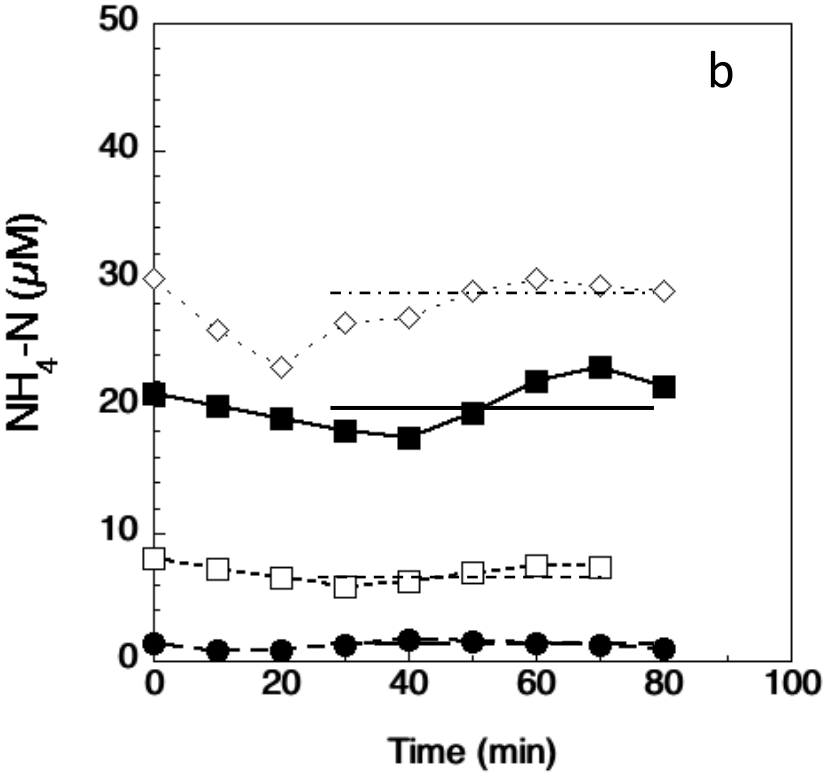

b
50
40
30
20
10
0
0
20
40
60
80
100
Time (min)
NH4-N (μM)

Fig. 2

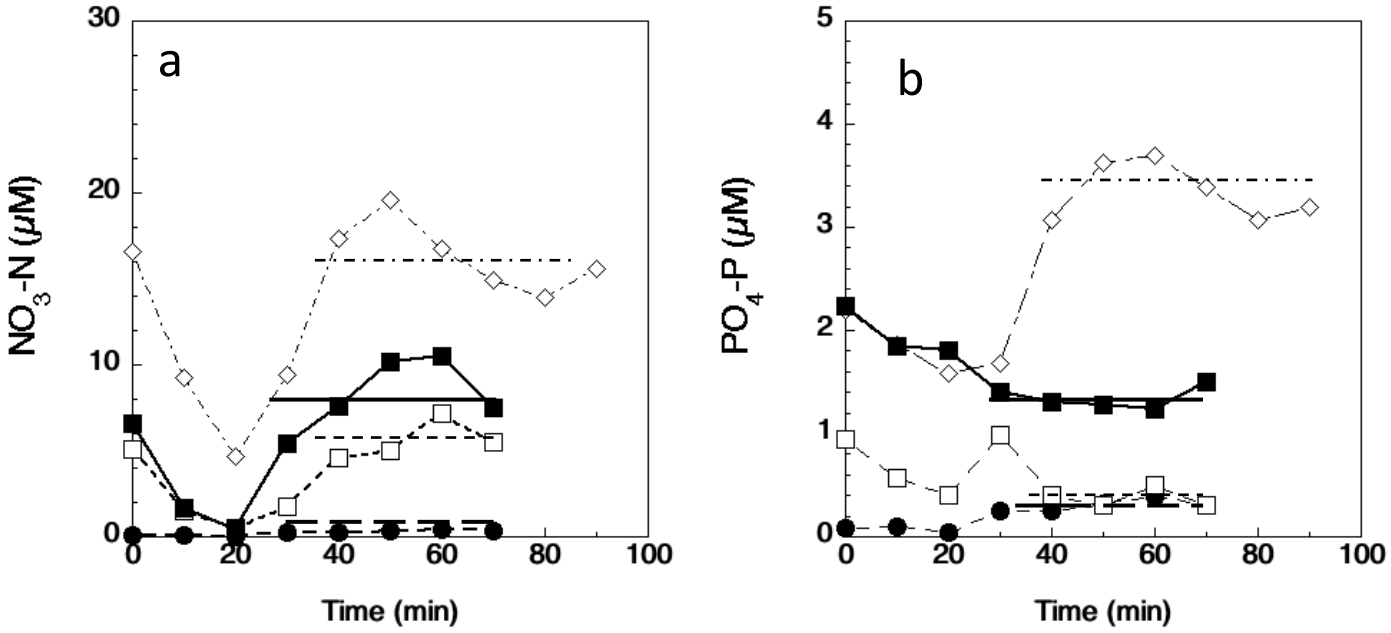
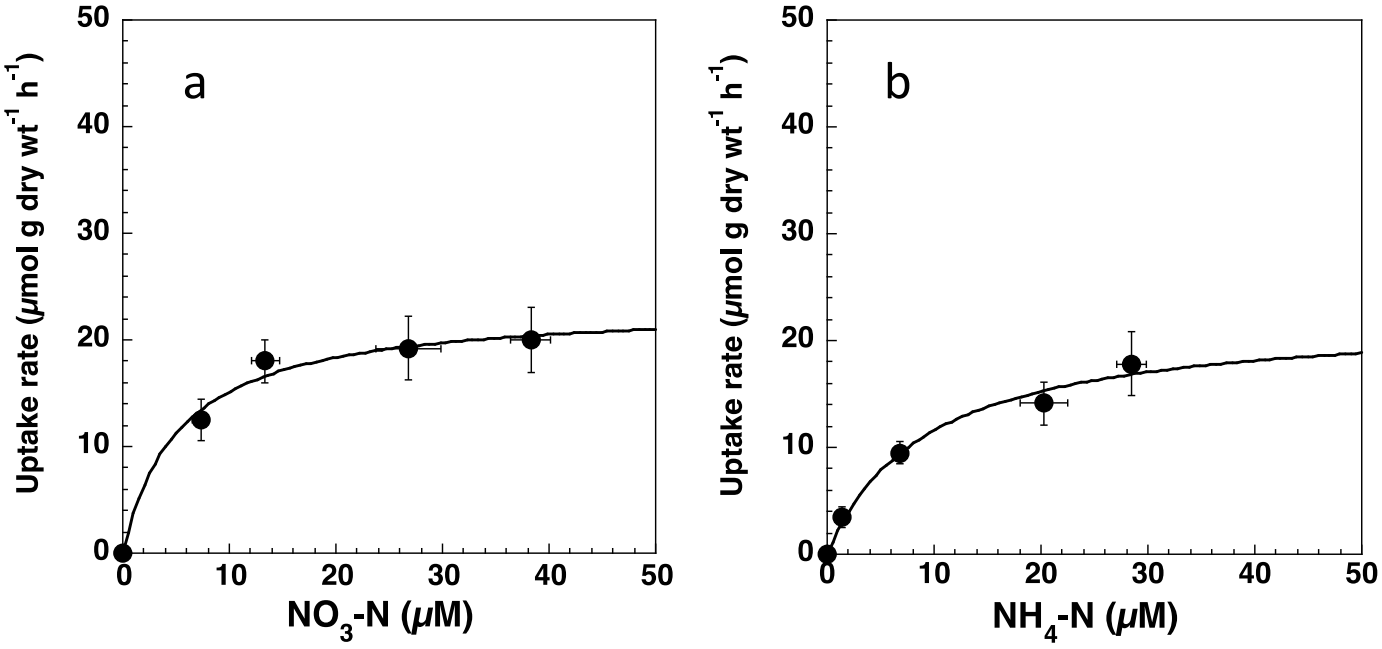

a
b
NO_3-N (μM)
PO_4-P (μM)
Time (min)
Time (min)
0
10
20
30
0
1
2
3
4
5
0
20
40
60
80
100

Fig. 3



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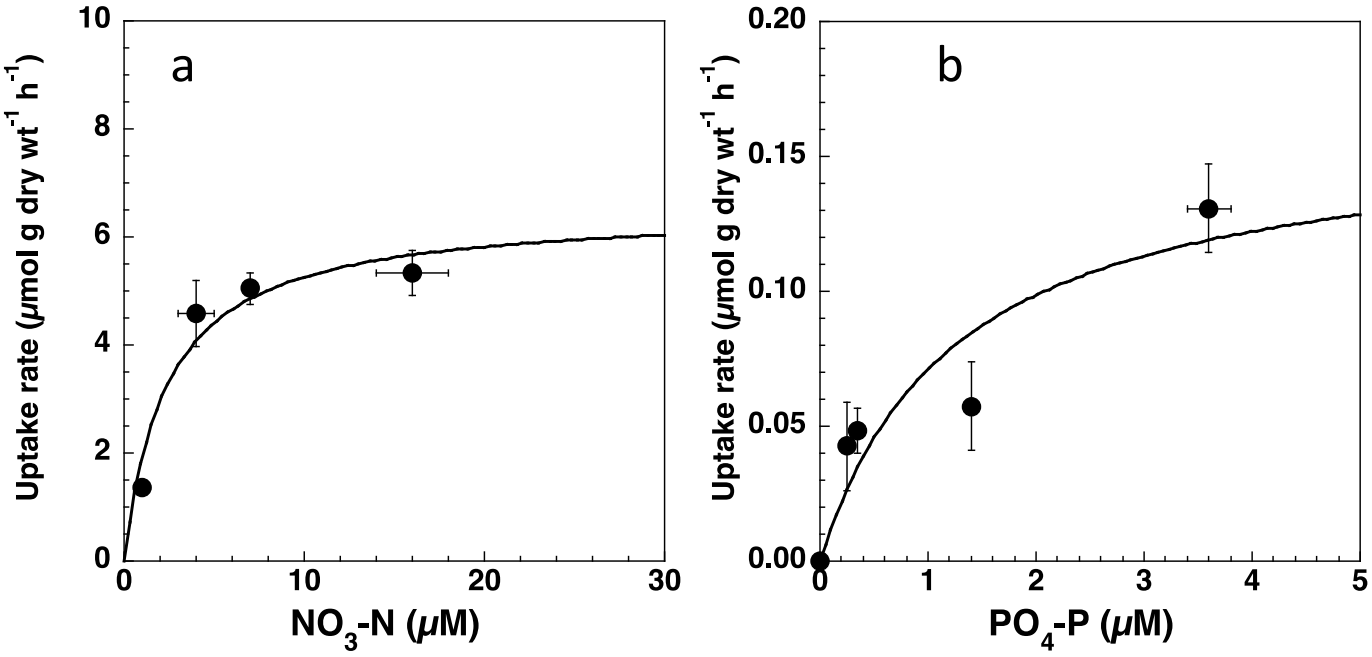
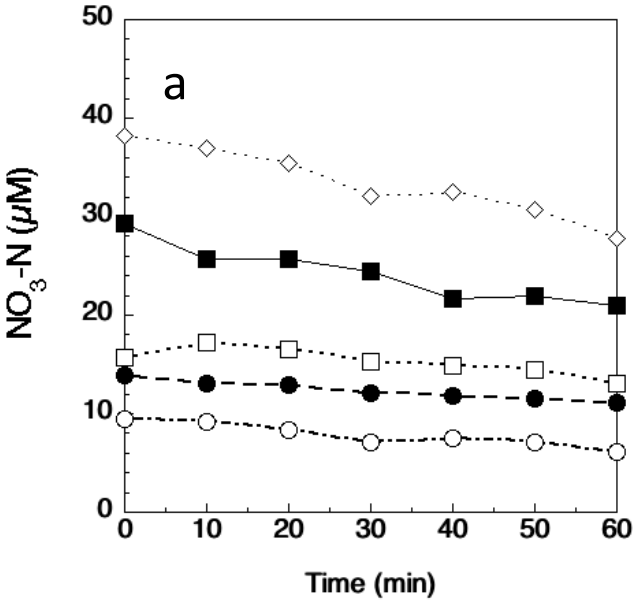

a
b
Uptake rate (μmol g dry wt-1 h-1)
NO3-N (μM)
PO4-P (μM)
0
2
4
6
8
10
0.00
0.05
0.10
0.15
0.20
0
10
20
30
1
2
3
4
5

Fig. 5


a
NO_3-N (μM)
50
40
30
20
10
0
0
10
20
30
40
50
60
Time (min)

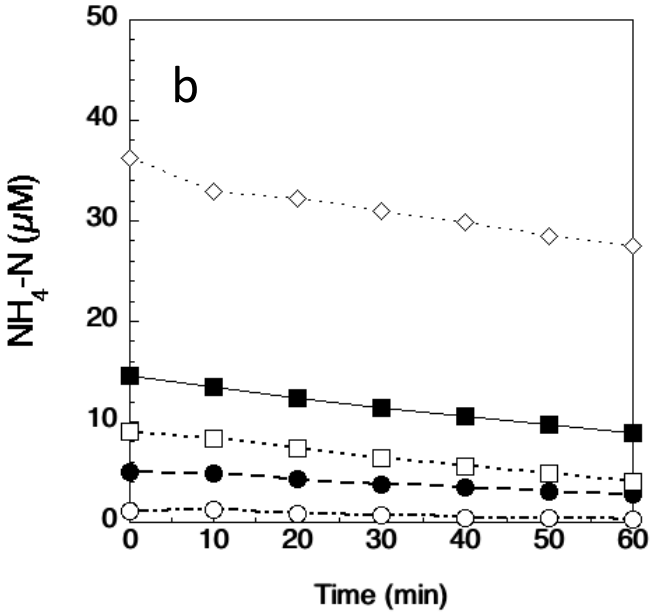

b
NH_4-N (μM)
50
40
30
20
10
0
0
10
20
30
40
50
60
Time (min)

Fig. 6

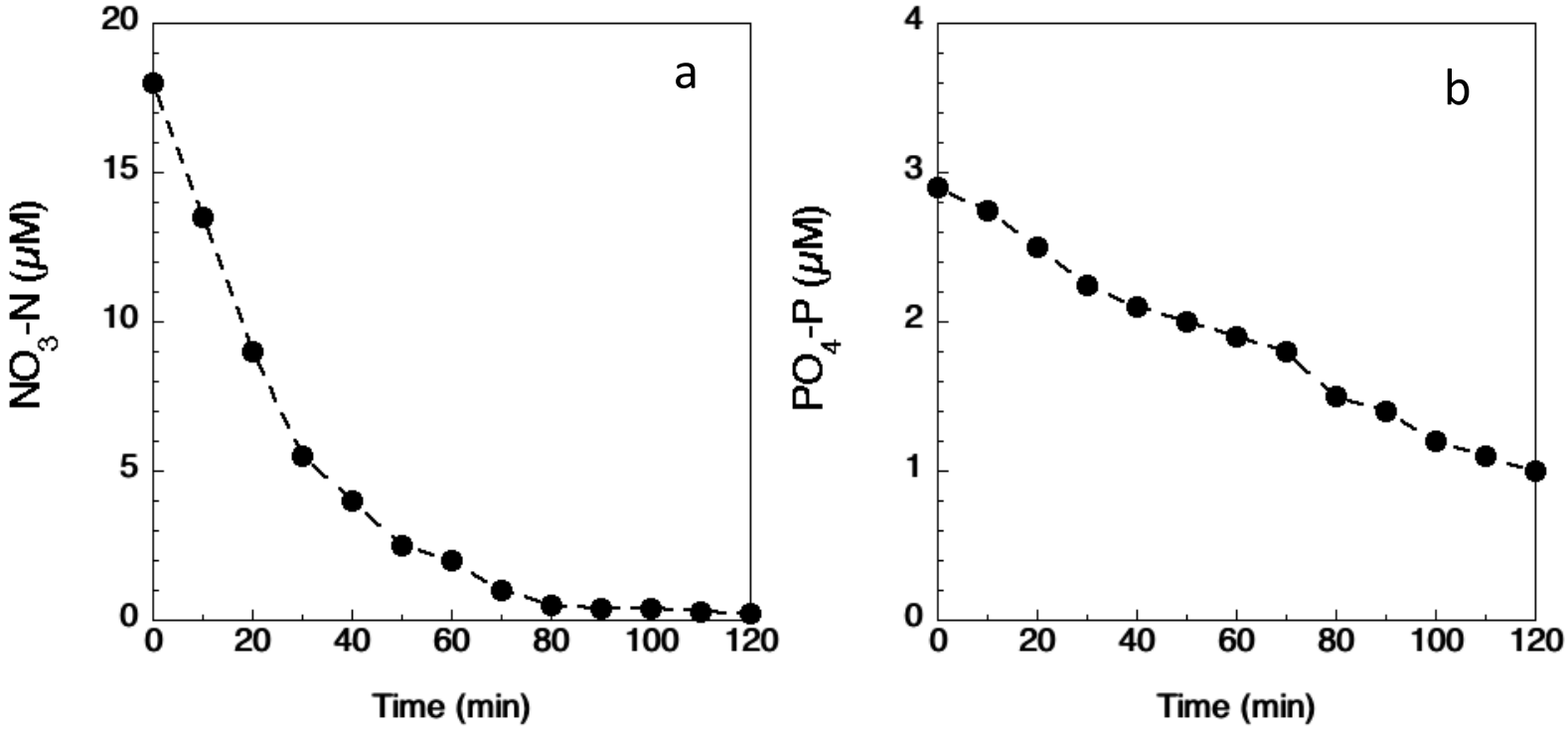

a
b
NO_3-N (μM)
PO_4-P (μM)
Time (min)
Time (min)
0
5
10
15
20
0
1
2
3
4
0
20
40
60
80
100
120

Fig. 7

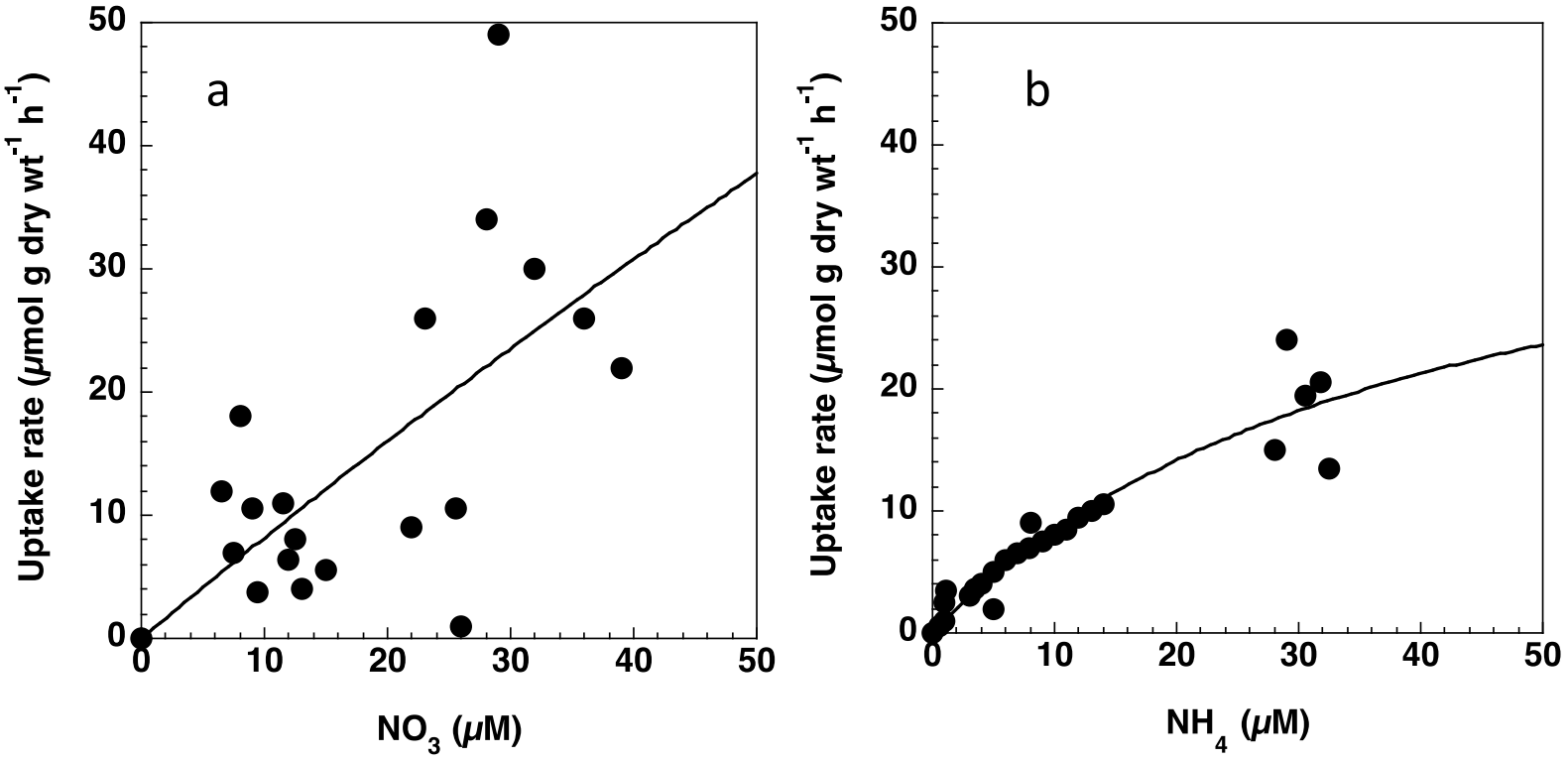
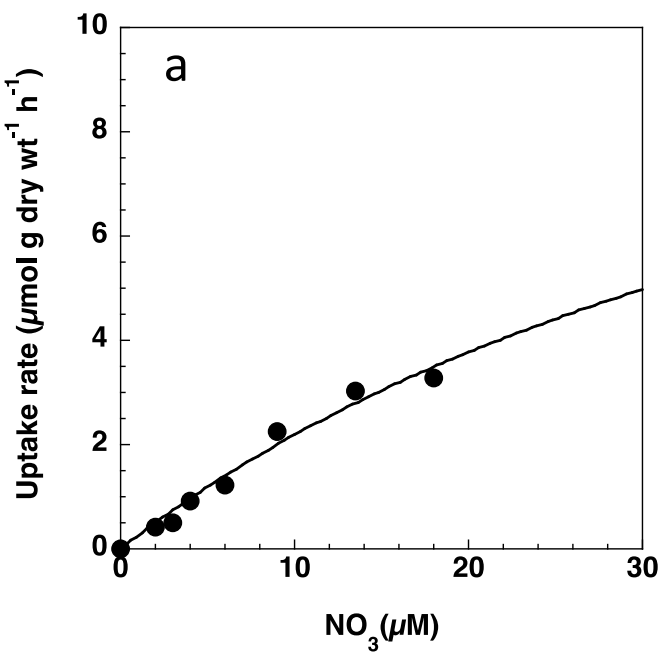


Fig. 8


a
Uptake rate (μmol g dry wt^{-1} h^{-1})
NO$_3$(μM)
0
2
4
6
8
10
0
10
20
30

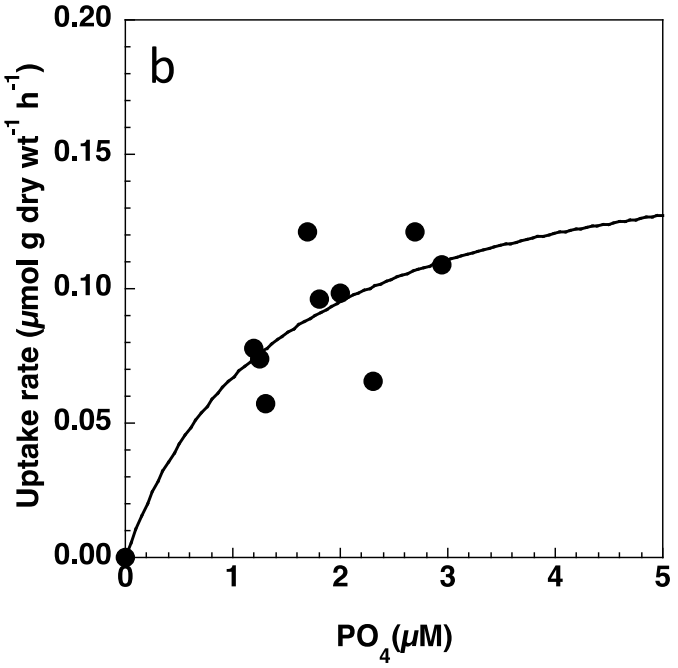

b
Uptake rate (μmol g dry wt^{-1} h^{-1})
PO$_4$(μM)
0.00
0.05
0.10
0.15
0.20
0
1
2
3
4
5

Fig. 9

Table 1　Comparison of Michaelis-Menten parameters between chemostat and batch culture.
Standard errors were given in parenthesis.

	Chemostat				Batch			
	V_{max} (μmol g dwt^{-1} h^{-1})	K_s (μM)	α	r	V_{max} (μmol g dwt^{-1} h^{-1})	K_s (μM)	α	r
S. religiosa								
NH_4	22.3	9.29	2.40	0.99	42.7	40.4	1.00	0.94
	(2.3)	(2.81)			(10.3)	(15.0)		
NO_3	23.2	5.35	4.33	0.99	401	481	0.83	0.63
	(1.5)	(1.44)			(3222)	(4091)		
	V_{max} (μmol g dwt^{-1} h^{-1})	K_s (μM)	α	r	V_{max} (μmol g dwt^{-1} h^{-1})	K_s (μM)	α	r
U. australis								
NO_3	6.51	2.39	66.5	0.98	13.7	53.0	7.05	0.98
	(0.93)	(1.19)			(7.4)	(35.6)		
PO_4	0.16	1.26	3.09	0.92	0.16	1.45	2.07	0.81
	(0.06)	(1.07)			(0.07)	(1.35)		