Research Article

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Growth rates and nitrate uptake of co-occurring red-tide dinoflagellates *Alexandrium affine* and *A. fraterculus* as a function of nitrate concentration under light-dark and continuous light conditions

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The dinoflagellate genus *Alexandrium* is known to often form harmful algal blooms causing human illness and large-scale mortality of marine organisms. Therefore, the population dynamics of *Alexandrium* species are of primary concern to scientists and aquaculture farmers. The growth rate of the *Alexandrium* species is the most important parameter in prediction models and nutrient conditions are critical parameters affecting the growth of phototrophic species. In Korean coastal waters, *Alexandrium affine* and *Alexandrium fraterculus*, of similar sizes, often form red-tide patches together. Thus, to understand bloom dynamics of *A. affine* and *A. fraterculus*, growth rates and nitrate uptake of each species as a function of nitrate (NO_3) concentration at 100 µmol photons m^{-2} s^{-1} under 14-h light : 10-h dark and continuous light conditions were determined using a nutrient repletion method. With increasing NO_3 concentration, growth rates and NO_3 uptake of *A. affine* or *A. fraterculus* increased, but became saturated. Under light : dark conditions, the maximum growth rates of *A. affine* and *A. fraterculus* were 0.45 and 0.42 d^{-1}, respectively. However, under continuous light conditions, the maximum growth rate of *A. affine* slightly increased to 0.46 d^{-1}, but that of *A. fraterculus* largely decreased. Furthermore, the maximum nitrate uptake of *A. affine* and *A. fraterculus* under light : dark conditions were 12.9 and 30.1 pM $cell^{-1}$ d^{-1}, respectively. The maximum nitrate uptake of *A. affine* under continuous light conditions was 16.4 pM $cell^{-1}$ d^{-1}. Thus, *A. affine* and *A. fraterculus* have similar maximum growth rates at the given NO_3 concentration ranges, but they have different maximum nitrate uptake rates. *A. affine* may have a higher conversion rate of NO_3 to body nitrogen than *A. fraterculus*. Moreover, a longer exposure time to the light may confer an advantage to *A. affine* over *A. fraterculus*.

Key Words: harmful algal bloom; nitrogen; nutrient; phosphate; protist; red tide

INTRODUCTION

The dinoflagellate genus *Alexandrium* often forms red tides or harmful algal blooms, causing human illness and large-scale mortality of marine organisms (Anderson et al. 2012). Therefore, the presence and population dynamics of *Alexandrium* species are primary concerns to scientists and aquaculture farmers (Dias et al. 2015, Eckford-Soper et al. 2016, Hatfield et al. 2019). The growth rate of a red-tide dinoflagellate species is the

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most important parameter in prediction models of population dynamics (Jeong et al. 2015). Among 34 officially described *Alexandrium* species, 6-7 species have been revealed to be mixotrophic species (Jacobson and Anderson 1986, Jeong et al. 2005*a*, 2005*b*, 2010, Seong et al. 2006, Yoo et al. 2009, Blossom et al. 2012, Lim et al. 2015, Lee et al. 2016). Both exclusively autotrophic and mixotrophic species need nutrients for their growth, and thus nutrient conditions are critical parameters affecting their growth (Yamamoto and Tarutani 1999, Lim et al. 2006, Maguer et al. 2007, Li et al. 2009, Jauzein et al. 2010, Lee et al. 2019). Thus, to understand the population dynamics of an *Alexandrium* species and to predict the outbreak of red tides or harmful algal blooms by them, growth rates of the species under different nutrient conditions should be determined.

In Korean coastal waters, *Alexandrium affine* and *Alexandrium fraterculus* often co-occur (Lee et al. 1998, Kim 2017, our unpublished data). Both species are known to be potentially toxic and also immobilize and / or lyse other protists (Nguyen-Ngoc 2004, Katsuo et al. 2007, Anderson et al. 2012, Basti et al. 2015, Lee et al. 2016, Kang et al. 2018). One or both of these two species usually form red-tide patches before the outbreak of red tides by the ichthyotoxic dinoflagellate *Margalefidinium* (*Cochlodinium*) *polykrikoides* (Jeong et al. 2017). Thus, the population dynamics of *A. affine* and / or *A. fraterculus* can be used for predicting the outbreak of *M. polykrikoides* red tides. Lee et al. (2016) revealed that *A. affine* and *A. fraterculus* lacked mixotrophic ability, whereas *Alexandrium andersonii* had mixotrophic ability. Therefore, nutrient conditions must be critical parameters affecting their growth. Both *A. affine* and *A. fraterculus* are known to have worldwide distributions; *A. affine* has been observed in European, North American, Asian, and Australian waters, while *A. fraterculus* has been reported to be in North and South American, Australian and New Zealand, and Japanese waters (Fraga et al. 1989, Nakanishi et al. 1996, Moita and Vilarinho 1999, Band-Schmidt et al. 2003, Hansen et al. 2003, Lagos 2003, MacKenzie et al. 2004, Nguyen-Ngoc 2004, Leaw et al. 2005, Omachi et al. 2007, Lee et al. 2009, Nagai et al. 2009, McCarthy 2013, Park et al. 2013). These waters show a wide range of nutrients such as nitrate and phosphate (Fraga et al. 1989, Nogueira et al. 1997, Kang et al. 2019). Furthermore, many phototrophic dinoflagellates can conduct vertical migration (Jeong et al. 2015); they ascend toward well-lit surface waters during the daytime, but descend toward eutrophic deep waters at night. Theoretically, using swimming speed, *A. affine* and *A. fraterculus* could be calculated to travel ca. 15-24 m (Lee et al. 2016, Jeong et al. 2017). Thus, they may experience a wide range of nutrient concentrations every day. To understand the population dynamics of *A. affine* and *A. fraterculus*, the effects of nutrient concentrations on their growth rates should be explored. The duration of day or night varies depending on latitudes and season (Van Haren and Compton 2013). The duration of light is known to affect the growth of dinoflagellates and other phytoplankton groups (e.g., Brand and Guillard 1981). Thus, it is worthwhile to explore effects of the duration of daytime or night on growth of *A. affine* and *A. fraterculus*.

In this present study, growth rates and nitrate uptake of *A. affine* and *A. fraterculus* as a function of nitrate concentration at 100 μmol photons m^{-2} s^{-1} under 14-h light : 10-h dark and continuous light conditions were determined using a nutrient repletion method (Lee et al. 2017). The results of the present study provide a basis for understanding ecophysiology of *A. affine* and *A. fraterculus* and their bloom dynamics.

MATERIALS AND METHODS

Preparation of experimental organisms

A. affine was isolated from coastal waters off Taean (western Korea) in August 2013 when the water temperature and salinity were 21.5°C and 32.2, respectively (Table 1). *A. fraterculus* was isolated from Yeosu (southern Korea) in September 2013 when the water temperature and salinity were 23.4°C and 32.8, respectively. Clonal cultures for both species were established from two serial single isolations.

A. affine was grown in enriched f/2-Si seawater medium (Guillard and Ryther 1962), while *A. fraterculus* was grown in enriched L1-Si seawater medium (Guilliard and Hargraves 1993) at 20°C under an illumination of 20 μmol photons m^{-2} s^{-1} of cool white fluorescent light on a 14-h light : 10-h dark cycle.

Growth and nitrate uptake rates under light and dark conditions

Experiments 1 and 2 were designed to investigate the growth rates and nitrate (NO_3) uptake of *A. affine* and *A. fraterculus* as a function of NO_3 concentration under light and dark conditions (Table 2). For this measurement, a nutrient repletion method was used (Lee et al. 2017).

Dense cultures of *A. affine* and *A. fraterculus*, growing in f/2-Si and L1-Si medium, respectively, were trans-

ferred to 800-mL culture flasks. The flasks were placed in a culture room at 20°C under 14-h light : 10-h dark cycle and acclimated at 100 µmol photons m^{-2} s^{-1} for 4 d. Three 1-mL aliquots were subsampled and then cells were enumerated to determine the cell concentration. Ten-milliliter aliquots were filtered through GF/F filters (Whatman Inc., Floreham Park, NJ, USA) and then concentrations of NO_3 (actually nitrate + nitrite in the Cd-coil reduction method) and phosphate (PO_4) were measured using a nutrient analyzer (QuAAtro, Seal Analytical, Norderstedt, Germany).

Cells of either species were added to triplicate 800-mL culture flasks by transferring predetermined volumes of cultures (final cell concentration = ~100 cells mL^{-1} for both species). A stock solution of NO_3 made based on the f/2 medium concentration was added for target final concentrations (NO_3 = ca. 110 µM). A sufficient amount of stock solution of PO_4 (final concentration = ca. 20 µM), also prepared based on the f/2 medium concentration, was added so that it would not be limiting before NO_3 was limiting. Trace metals and vitamins were also added plentifully with consideration of the ratio of nitrate to each chemical in an f/2 medium.

The flasks were placed in a temperature-controlled culture room and incubated at 20°C under an illumination of 100 µmol photons m^{-2} s^{-1} of cool white fluorescent light on a 14-h light : 10-h dark cycle. Thirty-milliliter aliquots were subsampled from each flask every day for 2 weeks, and 10-mL aliquots were used for the determination of cell concentration and 20-mL aliquots for the determination of NO_3 and PO_4 concentrations were filtered through GF/F filters. Cell concentrations (abundances) were determined by enumerating cells on three 1-mL Sedgwick-Rafter counting chambers. The concentrations of nutrients were measured using a nutrient analyzer.

The mean NO_3 concentration (N*) at each interval was calculated as:

$$N^* (\mu M) = [N_{t2} - N_{t1}] / [\ln(N_{t2} / N_{t1})] \quad (1)$$

, where N = NO_3 concentration at a single day, $t_2 - t_1 = 1$ d.

The specific growth rates of each *Alexandrium* species (µ, d^{-1}) at each N* were calculated as:

$$\mu = [\ln(Ct_2 / Ct_1)] / (t_2 - t_1) \quad (2)$$

, where Ct_1 and Ct_2 = cell concentrations of each *Alexandrium* species at Day t_1 and Day t_2, respectively.

The maximum growth rate (μ_{max}, d^{-1}) of each *Alexandrium* species was obtained after data were fitted to a Michaelis-Menten equation:

$$\mu = \mu_{max} [N^* / (K_{GR\text{-}NO3} + N^*)] \quad (3)$$

, where $K_{GR\text{-}NO3}$ = the NO_3 concentration sustaining $1/2\mu_{max}$.

Data were iteratively fitted to the model using DeltaGraph (SPSS Inc., Chicago, IL, USA).

The daily NO_3 uptake of each *Alexandrium* cell was determined by dividing the reduction in N* by the mean cell concentration (C*) at 1 d intervals.

$$(Nt_2\text{-}Nt_1) \text{ to } (N^*t_2\text{-}N^*t_1) \quad (4)$$

$$C^* \text{ (cells } mL^{-1}) = [Ct_2 - Ct_1] / [\ln(Ct_2 / Ct_1)] \quad (5)$$

, where $t_2 - t_1 = 1$ d.

Day 0 to 3 for *A. affine* and Day 0 to 1 for *A. fraterculus* were treated as the acclimation period, and thus data from these days were not used in calculations. The maxi-

Table 1. Conditions for the isolation of *Alexandrium affine* and *A. fraterculus* used in this study

Species	Strain name	ESD	Location	Time	T	S
Alexandrium affine	AATA1308	31.4	Taean, Korea	Aug 2013	21.5	32.2
Alexandrium fraterculus	AFYS1309	32.3	Yeosu, Korea	Sep 2013	23.4	32.8

ESD, equivalent spherical diameter (µM); T, temperature (°C); S, salinity.

Table 2. Experimental design and the light and nutrient conditions

Experiment	Species	L	L : D cycle	ICC	INO_3	IPO_4
1	*Alexandrium affine*	100	14 : 10	115	105.1	21.2
2	*Alexandrium fraterculus*	100	14 : 10	95	103.6	21.2
3	*Alexandrium affine*	100	Continuous light	105	110.7	21.2
4	*Alexandrium fraterculus*	100	Continuous light	102	104.3	21.2

L, light intensity (µmol photons m^{-2} s^{-1}); L : D cycle, light and dark cycle (hour : hour); ICC, initial cell concentration (cells mL^{-1}); INO_3, initial concentration of NO_3 (µM); IPO_4, initial concentration of PO_4 (µM).

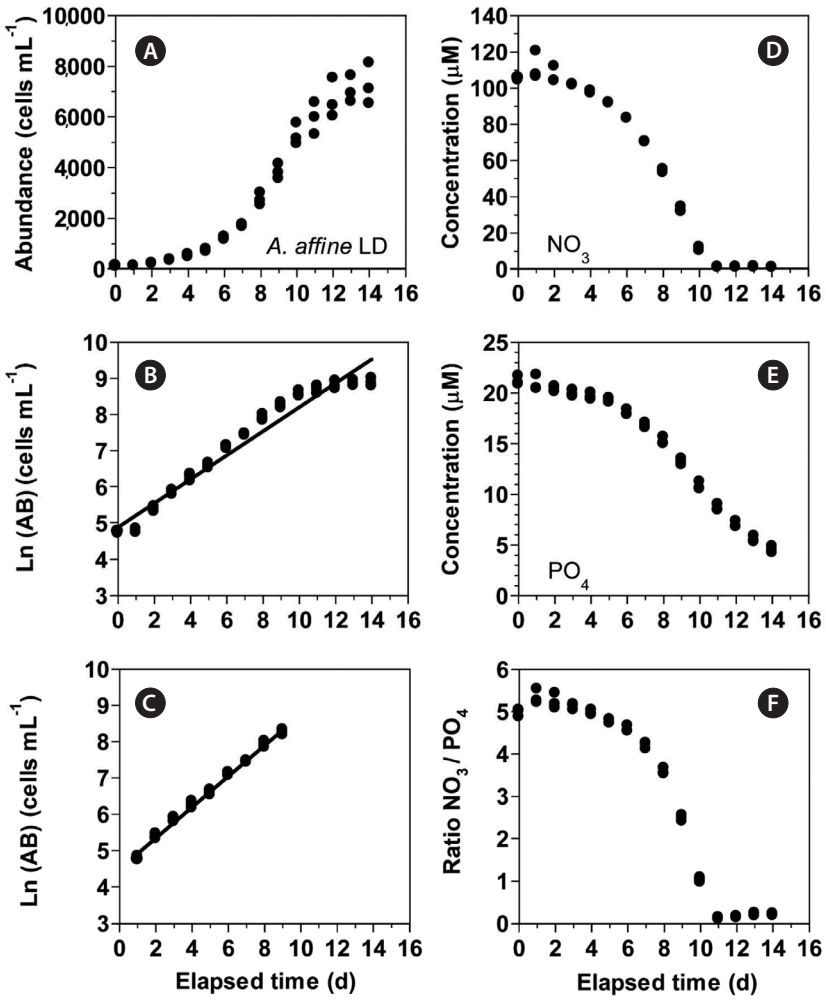

Fig. 1. (A-C) Change in the concentration (abundance, AB) of *Alexandrium affine* (cells mL^{-1}) as a function of elapsed time (d) under an illumination of 100 μmol photons m^{-2} s^{-1} on a 14-h light : 10-h dark (LD) cycle. The concentration of *A. affine* provided in the normal scale (A) and natural log (Ln) scale at Day 0 to 14 (B) and at Day 1 to 9 (C). (D-F) Change in the NO_3 (D) and PO_4 (E) concentrations and ratio of NO_3 relative to PO_4 (F) concentration as a function of elapsed time (d). Symbols represent each treatment. The curves in (B) and (C) are fitted to a linear regression using all the treatments obtained from Day 0 to 14 (B) and from Day 1 to 9 (C). (B) Ln (AB) = 0.332 (d) + 4.89, r^2 = 0.957; (C) Ln (AB) = 0.425 (d) + 4.50, r^2 = 0.993.

mum NO_3 uptake of each *Alexandrium* species (V, pM $cell^{-1}$ d^{-1}) was obtained after data were fitted to a Michaelis-Menten equation;

$$V = V_{max} [N^* / (K_{UT\text{-}NO3} + N^*)] \quad (6)$$

, where V_{max} = maximum uptake rate (pM $cell^{-1}$ d^{-1}), N* = mean NO_3 concentration (μM), and $K_{UT\text{-}NO3}$ = half saturation constant for NO_3 uptake (μM).

The mean PO_4 concentration (P*), specific growth rates of each *Alexandrium* species at each P*, and daily PO_4 uptake of each cell were calculated in the same manner.

Growth and nitrate uptake rates under continuous light conditions

Experiments 3 and 4 were designed to investigate the growth rates and NO_3 uptake of each of *A. affine* and *A. fraterculus* as a function of NO_3 concentrations under continuous light conditions (Table 2). For this measurement, a nutrient repletion method was used (Lee et al. 2017). The procedure of setting up incubating flasks, subsampling, determining cell abundances and nutrient concentrations, and incubation conditions was the same as in experiments 1 and 2 except for the light conditions.

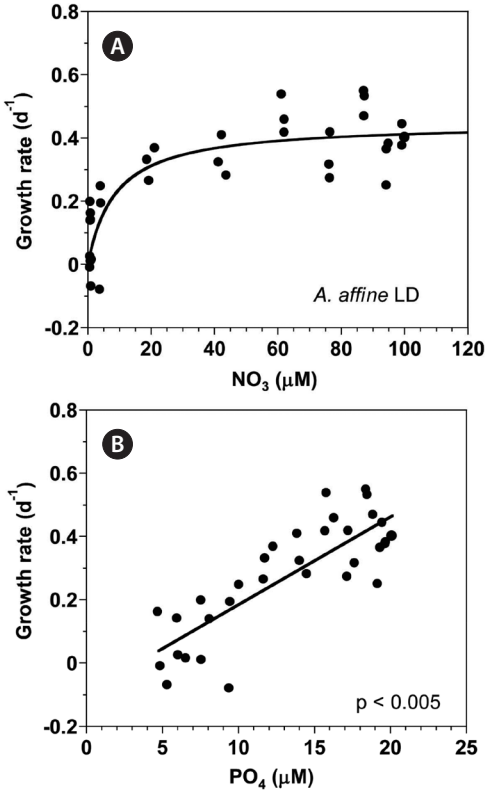

Fig. 2. Growth rates (GR, d^{-1}) of *Alexandrium affine* as a function of NO_3 (A) and PO_4 (B) concentration under an illumination of 100 μmol photons m^{-2} s^{-1} on a 14-h light : 10-h dark (LD) cycle. Symbols represent each treatment. The curve in (A) is fitted to the Michaelis-Menten equation using all the treatments obtained from Day 4 to 14 and that in (B) to a linear regression using all the treatment obtained from Day 4 to 14. (A) GR = 0.447 [NO_3 / (8.67 + NO_3)], r^2 = 0.733; (B) GR = 0.0278 (PO_4) – 0.0934, r^2 = 0.676.

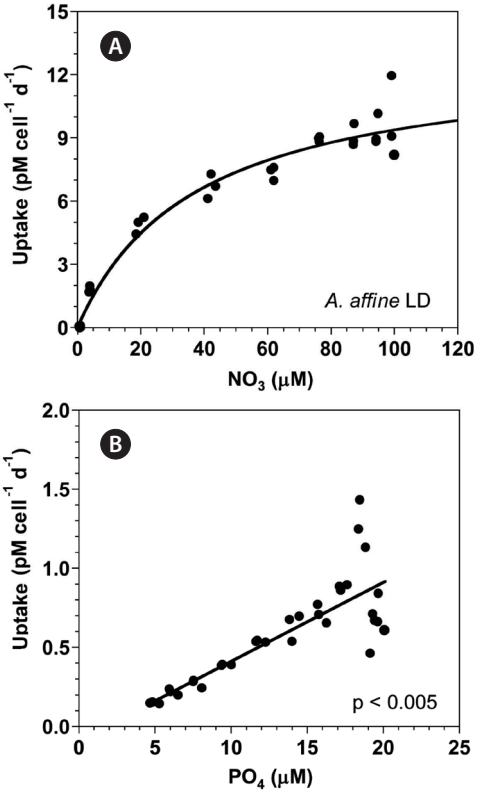

Fig. 3. (A) The NO_3 uptake (NUT, pM $cell^{-1}$ d^{-1}) of *Alexandrium affine* as a function of NO_3 concentrations (μM) under an illumination of 100 μmol photons m^{-2} s^{-1} on a 14-h light : 10-h dark (LD) cycle. (B) The PO_4 uptake (PUT, pM $cell^{-1}$ d^{-1}) of *A. affine* as a function of PO_4 concentrations (μM). Symbols represent each treatment. The curve in (A) is fitted to the Michaelis-Menten equation using all the treatments obtained from Day 4 to 14 and that in (B) to a linear regression using all the treatment obtained from Day 4 to 14. (A) NUT = 12.9 [NO_3 / (37.5 + NO_3)], r^2 = 0.971; (B) PUT = 0.05 (PO_4) – 0.0887, r^2 = 0.676.

RESULTS

Growth and nitrate uptake rates of *Alexandrium affine* under light and dark conditions

In experiment 1, with increasing incubation time, the concentration of *A. affine* rapidly increased from 115 cells mL^{-1} at Day 0 to 5,272 cells mL^{-1} at Day 10 and then continued to slightly increase (Fig. 1A). The maximum cell concentration (mean ± standard error [SE]) of *A. affine* concentrations was 7,250 ± 468 cells mL^{-1} which was achieved at Day 14, the last day of the experiment. The growth rate of *A. affine*, calculated from linear regression equation, using data between Day 1 and 9, was 0.425 d^{-1} (Fig. 1B & C). Furthermore, with increasing incubation time, the NO_3 concentration (mean ± SE) rapidly decreased from 105.1 ± 0.4 μM at Day 0 to 11.0 ± 0.6 μM at Day 10 and then became 0.9-1.2 μM at Day 11 to 14 (Fig. 1D). Moreover, with increasing incubation time, the PO_4 concentration (mean ± SE) rapidly decreased from 21.2 ± 0.3 μM at Day 0 to 4.5 ± 0.2 μM at Day 14 (Fig. 1E). With increasing elapsed incubation time, the ratio of NO_3 relative to PO_4 decreased from 5.0-5.3 at Day 0-4 to 2.5 at Day 9 and 0.1-0.2 at Day 11-14 (Fig. 1F).

With increasing NO_3 concentration, the growth rate of *A. affine* rapidly increased and then became saturated (Fig. 2A). When the data were fit to Eq. (3), the maximum growth rate (μ_{max}) of *A. affine* was 0.447 d^{-1} and the half saturation constant for growth rate ($K_{GR\text{-}NO3}$) was 8.7 μM. However, at the given range of PO_4 concentrations, the growth rate of *A. affine* linearly increased with increasing PO_4 concentration (Fig. 2B).

With increasing NO_3 concentration, the NO_3 uptake of *A. affine* rapidly increased initially, but then increased

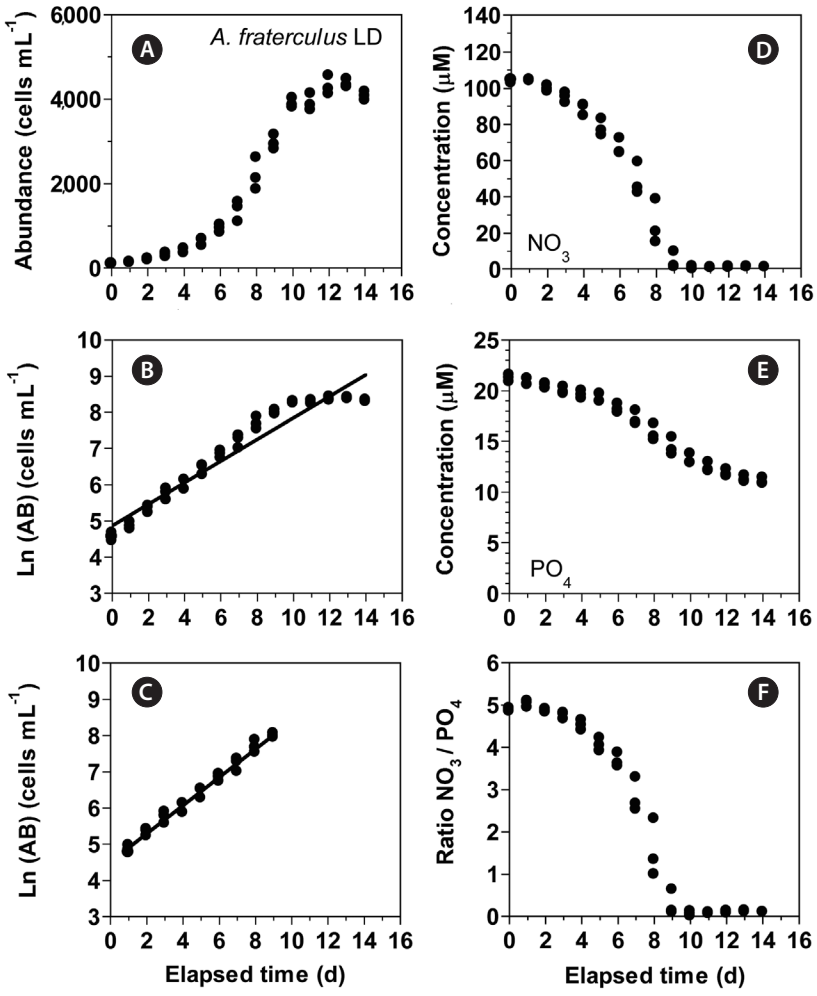

Fig. 4. (A-C) Change in the concentration of *Alexandrium fraterculus* (cells mL^{-1}) as a function of elapsed time (d) under an illumination of 100 μmol photons m^{-2} s^{-1} on a 14-h light : 10-h dark (LD) cycle. The concentration of *A. fraterculus* provided in the normal scale (A) and natural log (Ln) scale at Day 0 to 14 (B) and at Day 1 to 9 (C). (D-F) Change in the NO_3 (D) and PO_4 (E) concentrations and ratio of NO_3 relative to PO_4 (F) concentration as a function of elapsed time (d). Symbols represent each treatment. The curves in (B) and (C) are fitted to a linear regression using all the treatments obtained from Day 0 to 14 (B) and from Day 1 to 9 (C). (B) Ln (AB) = 0.298 (d) + 4.87, r^2 = 0.937; (C) Ln (AB) = 0.389 (d) + 4.52, r^2 = 0.986.

more slowly (Fig. 3A). When the data were fit to Eq. (4-6), the maximum NO_3 uptake of *A. affine* was 12.9 pM $cell^{-1}$ d^{-1} and the half saturation constant for uptake ($K_{UT\text{-}NO3}$) was 37.5 μM. However, at the given range of PO_4 concentrations, the PO_4 uptake of *A. affine* linearly increased with increasing PO_4 concentration (Fig. 3B).

Growth and nitrate uptake rates of *Alexandrium fraterculus* under light and dark conditions

In experiment 2, with increasing incubation time, the concentration of *A. fraterculus* increased from 95 cells mL^{-1} at Day 0 to ca. 4,350 cells mL^{-1} at Day 13 and then slightly decreased (Fig. 4A). The growth rate of *A. fraterculus* calculated from linear regression equation using data between Day 1 and 9 was 0.389 d^{-1} (Fig. 4B & C). Furthermore, with increasing incubation time, the NO_3 concentration rapidly decreased from 103.6 ± 0.5 μM at Day 0 to 4.2 ± 2.7 μM at Day 9 and then became 0.8-1.3 μM at Day 10 to 14 (Fig. 4D). Moreover, with increasing incubation time, the PO_4 concentration decreased from 21.2 ± 0.2 μM at Day 0 to 11.0 ± 0.2 μM at Day 14 (Fig. 4E). With increasing elapsed incubation time, the ratio of NO_3 relative to PO_4 decreased from 4.9-5.0 at Day 0-2 to 1.5 at Day 8 and 0.1-0.3 at Day 9-14 (Fig. 4F).

With increasing NO_3 concentration, the growth rate of *A. fraterculus* rapidly increased and then became saturated (Fig. 5A). When the data were fit to Eq. (3), the maximum growth rate (μ_{max}) of *A. fraterculus* was 0.422 d^{-1} and half saturation constant for growth rate ($K_{GR\text{-}NO3}$) was 5.4

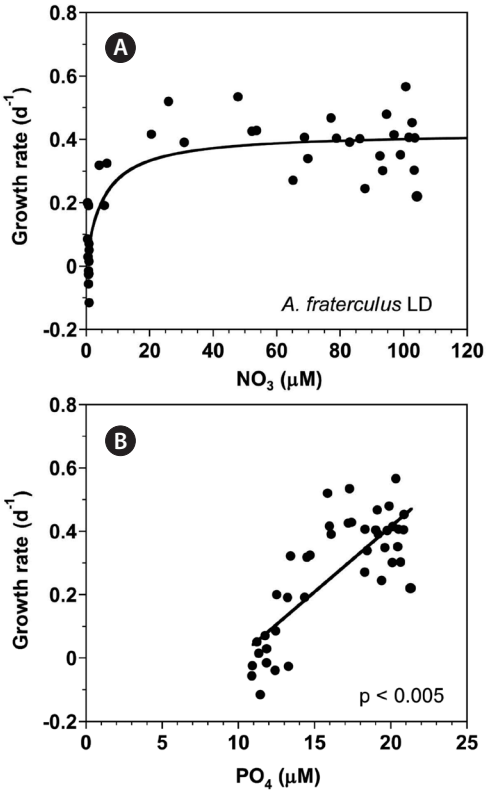

Fig. 5. Growth rates (GR, d^{-1}) of *Alexandrium fraterculus* as a function of NO_3 (A) and PO_4 (B) concentration under an illumination of 100 μmol photons m^{-2} s^{-1} on a 14-h light : 10-h dark (LD) cycle. Symbols represent each treatment. The curve in (A) is fitted to the Michaelis-Menten equation using all the treatments obtained from Day 0 to 14 and that in (B) to a linear regression using all the treatment obtained from Day 0 to 14. (A) GR = 0.422 [NO_3 / (5.38 + NO_3)], r^2 = 0.741; (B) GR = 0.0412 (PO_4) – 0.41, r^2 = 0.615.

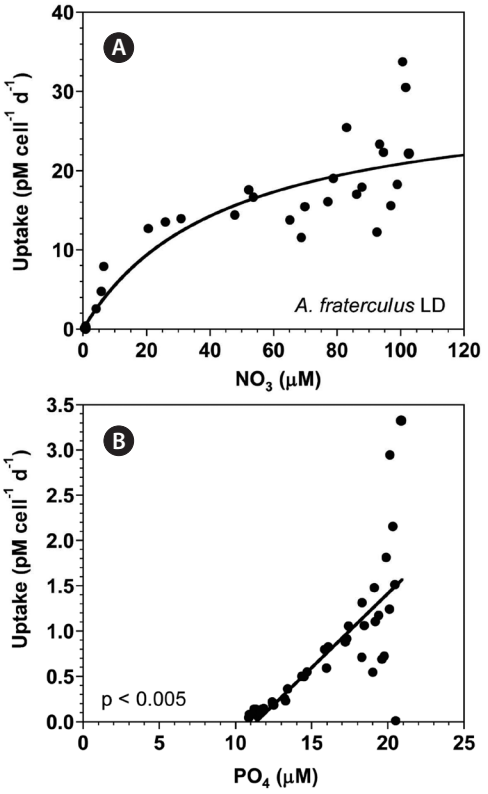

Fig. 6. (A) The NO_3 uptake (NUT, pM $cell^{-1}$ d^{-1}) of *Alexandrium fraterculus* as a function of NO_3 concentrations (μM) under an illumination of 100 μmol photons m^{-2} s^{-1} on a 14-h light : 10-h dark (LD) cycle. (B) The PO_4 uptake (PUT, pM $cell^{-1}$ d^{-1}) of *A. fraterculus* as a function of PO_4 concentrations (μM). Symbols represent each treatment. The curve in (A) is fitted to the Michaelis-Menten equation using all the treatments obtained from Day 2 to 14 and that in (B) to a linear regression using all the treatment obtained from Day 2 to 14. (A) NUT = 30.1 [NO_3 / (44.4 + NO_3)], r^2 = 0.843; (B) PUT = 0.164 (PO_4) – 1.86, r^2 = 0.548.

μM. However, at the given range of PO_4 concentrations, the growth rate of *A. fraterculus* linearly increased with increasing PO_4 concentration (Fig. 5B).

With increasing NO_3 concentration, the NO_3 uptake of *A. fraterculus* rapidly increased initially, but then increased more slowly (Fig. 6A). When the data were fit to Eq. (4-6), the maximum NO_3 uptake by *A. fraterculus* was 30.1 pM $cell^{-1}$ d^{-1} and the half saturation constant for uptake ($K_{UT\text{-}NO3}$) was 44.4 μM. However, at the given range of PO_4 concentrations, the PO_4 uptake of *A. fraterculus* linearly increased with increasing PO_4 concentration (Fig. 6B).

Growth and nitrate uptake rates of *Alexandrium affine* under the continuous light conditions

In experiment 3, with increasing incubation time, the concentration of *A. affine* rapidly increased from 105 cells mL^{-1} at Day 0 to 5,779 cells mL^{-1} at Day 12 and then became saturated (Fig. 7A). The growth rate of *A. affine*, calculated from a linear regression equation using data between Day 1 and 9, was 0.405 d^{-1} (Fig. 7B & C). Furthermore, with increasing incubation time, the NO_3 concentration rapidly decreased from 110.7 ± 6.3 μM at Day 0 to 15.7 ± 4.2 μM at Day 11 and then became 1.1-2.6 μM at Day 12 to 14 (Fig. 7D). Moreover, with increasing elapsed incubation time, the PO_4 concentration decreased from 21.2 ± 0.3 μM at Day 0 to 8.4 ± 0.5 μM at Day 14 (Fig. 7E). With increasing incubation time, the ratio of NO_3 relative to PO_4 decreased from 5.0-5.2 at Day 0-5 to 1.3 at Day 11 and 0.1-0.3 at Day 12-14 (Fig. 7F).

With increasing NO_3 concentration, the growth rate of *A. affine* rapidly increased and then became saturated (Fig. 8A). When the data were fit to Eq. (3), the maximum

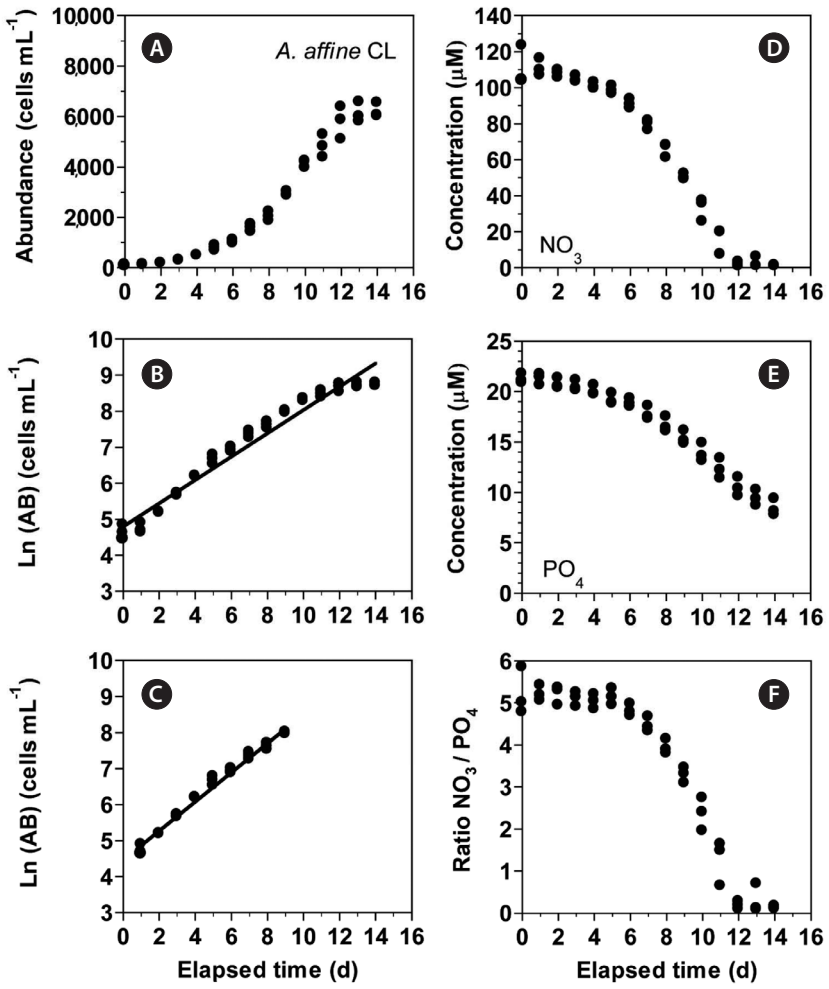

Fig. 7. (A-C) Change in the concentration of *Alexandrium affine* (cells mL^{-1}) as a function of elapsed time (d) at 100 μmol photons m^{-2} s^{-1} under continuous light conditions (CL). The concentration of *A. affine* provided in the normal scale (A) and natural log (Ln) scale at Day 0 to 14 (B) and at Day 1 to 9 (C). (D-F) Change in the NO_3 (D) and PO_4 (E) concentrations and ratio of NO_3 relative to PO_4 (F) concentration as a function of elapsed time (d). Symbols represent each treatment. The curves in (B) and (C) are fitted to a linear regression using all the treatments obtained from Day 0 to 14 (B) and from Day 1 to 9 (C). (B) Ln (AB) = 0.324 (d) + 4.79, r^2 = 0.961; (C) Ln (AB) = 0.405 (d) + 4.46, r^2 = 0.986.

growth rate (μ_{max}) of *A. affine* was 0.458 d^{-1} and the half saturation constant for growth rate ($K_{GR\text{-}NO3}$) was 21.5 μM. However, at the given range of PO_4 concentrations, the growth rate of *A. affine* linearly increased with increasing PO_4 concentration (Fig. 8B).

With increasing NO_3 concentration, the NO_3 uptake of *A. affine* rapidly increased initially, but then increased more slowly (Fig. 9A). When the data were fit to Eq. (4-6), the maximum NO_3 uptake of *A. affine* was 16.4 pM $cell^{-1}$ d^{-1} and half saturation constant for uptake ($K_{UT\text{-}NO3}$) was 83.3 μM. However, at the given range of PO_4 concentrations, the PO_4 uptake of *A. affine* linearly increased with increasing PO_4 concentration (Fig. 9B).

Growth and nitrate uptake rates of *Alexandrium fraterculus* under the continuous light conditions

In experiment 4, with increasing incubation time, the concentration of *A. fraterculus* increased from 102 cells mL^{-1} at Day 0 to 1,587 cells mL^{-1} at Day 14 (Fig. 10A). The growth rate of *A. fraterculus*, calculated from linear regression equation using data between Day 2 and 9, was 0.295 d^{-1} (Fig. 10B & C). Furthermore, with increasing incubation time, the NO_3 concentration decreased from 104.3 ± 0.3 μM at Day 0 to 49.9 ± 4.1 μM at Day 14 (Fig. 10D). Moreover, with increasing incubation time, the PO_4

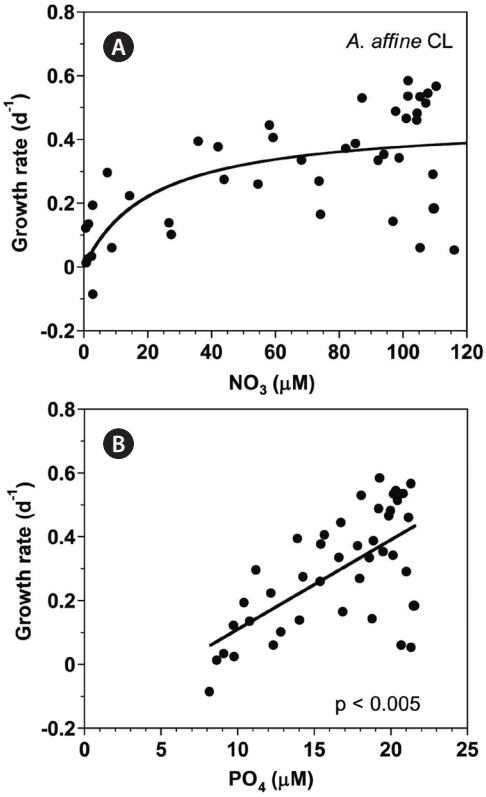

Fig. 8. Growth rates (GR, d^{-1}) of *Alexandrium affine* as a function of NO_3 (A) and PO_4 (B) concentration at 100 μmol photons m^{-2} s^{-1} under continuous light conditions (CL). Symbols represent each treatment. The curve in (A) is fitted to the Michaelis-Menten equation using all the treatments obtained from Day 0 to 14 and that in (B) to a linear regression using all the treatment obtained from Day 0 to 14. (A) GR = 0.458 [NO_3 / (21.5 + NO_3)], r^2 = 0.439; (B) GR = 0.0281 (PO_4) – 0.171, r^2 = 0.417.

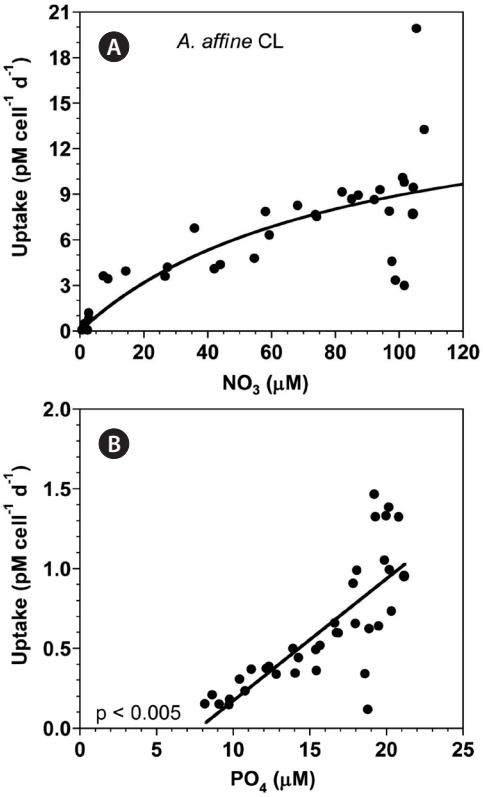

Fig. 9. (A) The NO_3 uptake (NUT, pM $cell^{-1}$ d^{-1}) of *Alexandrium affine* as a function of NO_3 concentrations (μM) at 100 μmol photons m^{-2} s^{-1} under continuous light conditions (CL). (B) The PO_4 uptake (PUT, pM $cell^{-1}$ d^{-1}) of *A. affine* as a function of PO_4 concentrations (μM). Symbols represent each treatment. The curve in (A) is fitted to the Michaelis-Menten equation using all the treatments obtained from Day 3 to 14 and that in (B) to a linear regression using all the treatment obtained from Day 3 to 14. (A) NUT = 16.4 [NO_3 / (83.3 + NO_3)], r^2 = 0.606; (B) PUT = 0.0764 (PO_4) – 0.592, r^2 = 0.603.

concentration rapidly decreased from 21.2 ± 0.3 μM at Day 0 to 17.3 ± 0.5 μM at Day 14 (Fig. 10E). With increasing elapsed incubation time, the ratio of NO_3 relative to PO_4 decreased from 4.9-5.0 at Day 0-2 to 2.9 at Day 14 (Fig. 10F).

At the given range of NO_3 concentrations, the growth rate of *A. fraterculus* linearly increased with increasing NO_3 concentration (Fig. 11A). Also, at the given range of PO_4 concentrations, the growth rate of *A. fraterculus* linearly increased with increasing PO_4 concentration (Fig. 11B).

At the given range of NO_3 concentrations, the NO_3 uptake of *A. fraterculus* linearly increased with increasing NO_3 concentration (Fig. 12A). Furthermore, at the given range of PO_4 concentrations, the PO_4 uptake of *A. fraterculus* linearly increased with increasing PO_4 concentration (Fig. 12B).

DISCUSSION

The growth rate of *A. affine* at 64 μM, which was the mean NO_3 concentration between Day 1 and 9, calculated using the Michaelis-Menten type equation in Fig. 2A (0.394 d^{-1}), is only 8% different from that calculated using the linear regression equation in Fig. 1C (0.425 d^{-1}). Similarly, the growth rate of *A. fraterculus* at 31 μM, which was the mean NO_3 concentration between Day 1 and 9, calculated using the Michaelis-Menten type equation in Fig. 5A (0.360 d^{-1}), is also only 8% different from that calculated using the linear regression equation in Fig. 4C (0.389 d^{-1}). Thus, the nutrient repletion method is a reasonable tool for determining growth rates of *A. affine* and *A. fraterculus* as a function of NO_3 concentration.

The maximum growth rate of *A. affine* obtained in the present study is comparable to that reported in Nguyen-

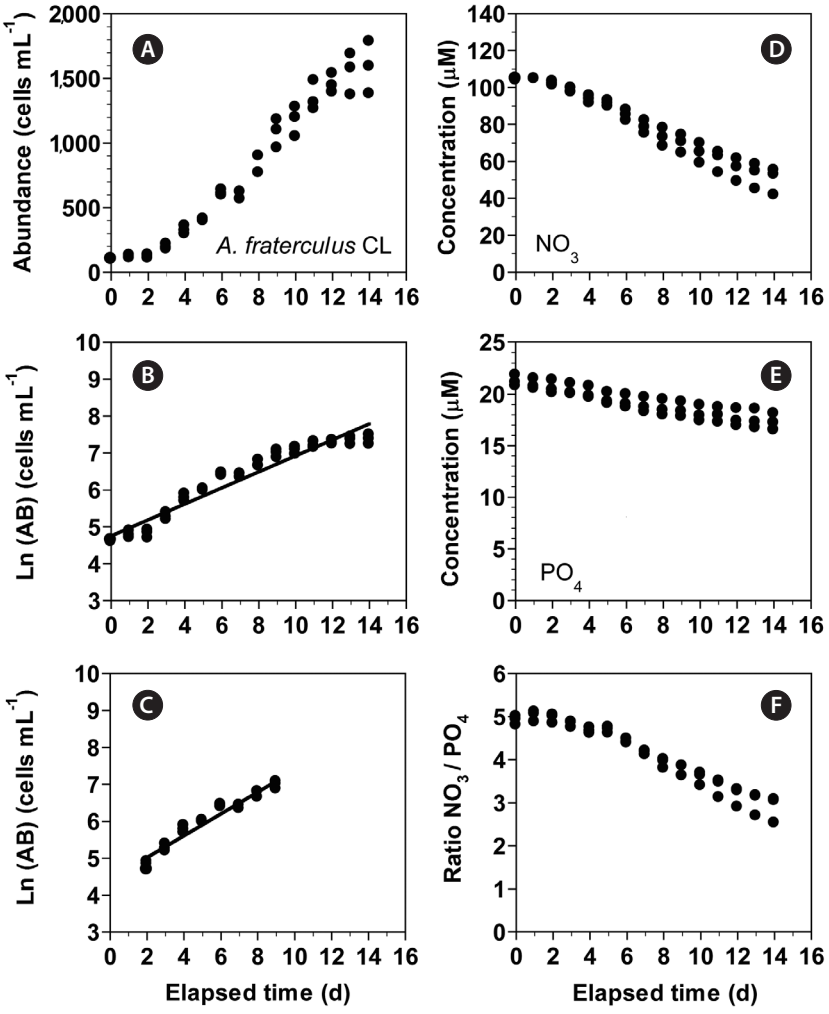

Fig. 10. (A-C) Change in the concentration of *Alexandrium fraterculus* (cells mL^{-1}) as a function of elapsed time (d) at 100 μmol photons m^{-2} s^{-1} under continuous light conditions (CL). The concentration of *A. fraterculus* provided in the normal scale (A) and natural log (Ln) scale at Day 0 to 14 (B) and at Day 2 to 9 (C). (D-F) Change in the NO_3 (D) and PO_4 (E) concentrations and ratio of NO_3 relative to PO_4 (F) concentration as a function of elapsed time (d). Symbols represent each treatment. The curves in (B) and (C) are fitted to a linear regression using all the treatments obtained from Day 0 to 14 (B) and from Day 2 to 9 (C). (B) Ln (AB) = 0.217 (d) + 4.75, r^2 = 0.936; (C) Ln (AB) = 0.295 (d) + 4.43, r^2 = 0.951.

Ngoc (2004) and Lim et al. (2019), while the maximum growth rate of *A. fraterculus* obtained in the present study is slightly higher than Lim et al. (2007) (Table 3). The present study reports for the first time the half saturation constant for growth rate (K_{GR-NO3}) and for NO_3 uptake (K_{UT-NO3}) of *A. affine* and *A. fraterculus* (Table 3). The values of K_{GR-NO3} and K_{UT-NO3} are important in understanding competition among red-tide or harmful algal bloom species, because those with lower K_{GR-NO3} and K_{UT-NO3} can grow and uptake NO_3 rapidly at lower NO_3 concentrations. Furthermore, growth rates and NO_3 uptake of the species largely change when NO_3 concentrations change near K_{GR-NO3} and K_{UT-NO3}. The range of NO_3 concentrations in the waters, collected from April 2015 to October 2018, from Taean (West Sea of Korea) and Yeosu (South Sea of Korea) from which *A. affine* and *A. fraterculus* were isolated, were 1.6-10.7 and 0.8-26.4 μM, respectively (Kang et al. 2019). Furthermore, the range of NO_3 concentrations in Junk Bay, Hong Kong and Ria de Vigo, northwest Spain, from which *A. affine* was found were 0-15.0 and 0-30.0 μM, respectively (Fraga et al. 1989, Nogueira et al. 1997, Hodgkiss and Lu 2004, Lee et al. 2009). The K_{GR-NO3} of *A. affine* (8.7 μM) and *A. fraterculus* (5.4 μM) fall in the ranges of the NO_3 concentrations in the waters off Taean and Yeosu, Korea, Junk Bay, Hong Kong, and Ria de Vigo, Spain. Therefore, a small change in NO_3 concentrations in these waters may sometimes cause a large change in growth rates of *A. affine* and *A. fraterculus*.

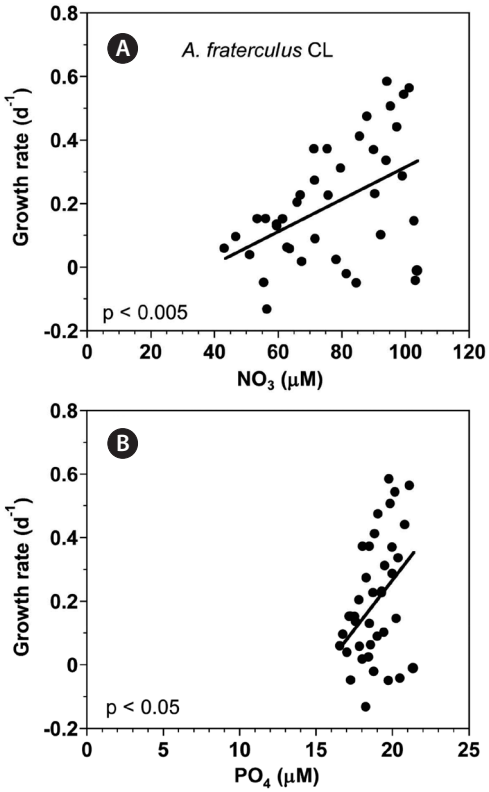

Fig. 11. Growth rates (GR, d^{-1}) of *Alexandrium fraterculus* as a function of NO_3 (A) and PO_4 (B) concentration at 100 μmol photons m^{-2} s^{-1} under continuous light conditions (CL). Symbols represent each treatment. The curve in (A) is fitted to a linear regression using all the treatments obtained from Day 2 to 14 and that in (B) from Day 2 to 14. (A) GR = 0.00508 (NO_3) – 0.193, r^2 = 0.224; (B) GR = 0.0623 (PO_4) – 0.98, r^2 = 0.165.

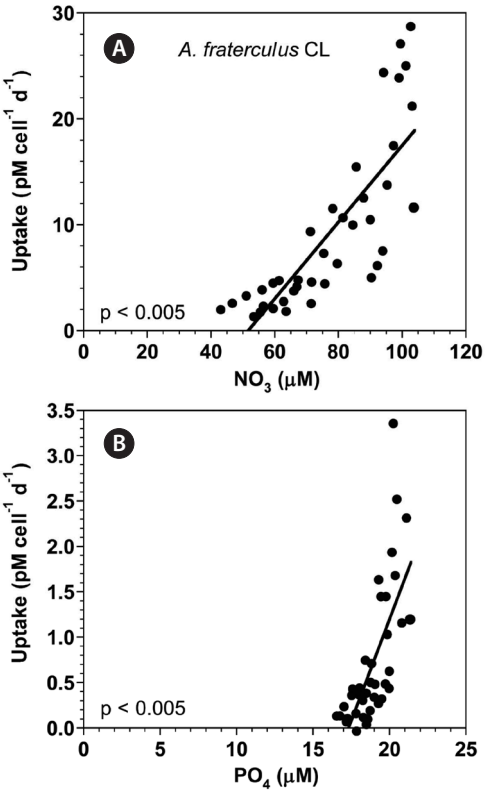

Fig. 12. (A) The NO_3 uptake (NUT, pM $cell^{-1}$ d^{-1}) of *Alexandrium fraterculus* as a function of NO_3 concentrations (μM) at 100 μmol photons m^{-2} s^{-1} under continuous light conditions (CL). (B) The PO_4 uptake (PUT, pM $cell^{-1}$ d^{-1}) of *A. fraterculus* as a function of PO_4 concentrations (μM). Symbols represent each treatment. The curve in (A) is fitted to a linear regression using all the treatments obtained from Day 2 to 14 and that in (B) from Day 2 to 14. (A) NUT = 0.362 (NO_3) – 18.7, r^2 = 0.656; (B) PUT = 0.447 (PO_4) – 7.74, r^2 = 0.502.

Table 3. Maximum growth rates (μ_{max}, d^{-1}) of *Alexandrium affine* and *A. fraterculus*, half saturation constant for growth rate as a function of nitrate concentration ($K_{GR\text{-}NO3}$, μM), nitrate maximum uptake ($V_{max\text{-}NO3}$, pM $cell^{-1}$ d^{-1}), half saturation constant for the nitrate uptake as a function of nitrate concentration ($K_{UT\text{-}NO3}$, μM) and conditions for growth

Species / ESD	μ_{max}	$K_{GR\text{-}NO3}$	$V_{max\text{-}NO3}$	$K_{UT\text{-}NO3}$	T	S	L	L : D cycle	NO_3	Reference
A. affine										
29.7	0.60	-	-	-	25	-	-	-	-	Jeong et al. (2010)
31.4[a]	0.34	-	-	-	5-30	-	230	10 : 14	-	Band-Schmidt et al. (2003)
31.4[a]	0.37	-	-	-	15-25	5-30	150	15 : 9	-	Lim et al. (2007)
31.4[a]	0.43	-	-	-	15-30	20-32	100	12 : 12	-	Lim et al. (2019)
31.4	0.45	8.7	12.9	37.5	20	-	100	14 : 10	105	This study
31.4	0.46	21.5	16.4	83.3	20	-	100	CL	111	This study
31.4[a]	0.90	-	-	-	22	-	120	16 : 8	5-400	Lee et al. (2009)
35.0	0.49	-	-	-	21-27	10-35	25	12 : 12	-	Nguyen-Ngoc (2004)
A. fraterculus										
32.3[a]	0.35	-	-	-	15-25	5-30	150	15 : 9	-	Lim et al. (2007)
32.3	0.42	5.4	30.1	44.4	20	-	100	14 : 10	104	This study

ESD, equivalent spherical diameter (μm); T, temperature (°C); S, salinity; L, light intensity (μmol photons m^{-2} s^{-1}); L : D cycle, light and dark cycle (hour : hour); NO_3, range of concentration of nitrate used in experiment (μM); CL, continuous light.
[a]Data from Kang et al. (2018).

The range of NO_3 concentrations in the waters, collected from April 2015 to October 2018, from 9 stations located in South Sea of Korea in which *A. affine* and *A. fraterculus* have caused red tides or harmful algal blooms, was 0.4-102.9 μM (Kang et al. 2019). Moreover, the range of NO_3 concentrations in Seto Inland Sea, Japan in which *A. affine* and *A. fraterculus* were found was 5.4-70.0 μM (Nakanishi et al. 1996, Montani et al. 1998, Nagai et al. 2009). The $K_{UT\text{-}NO3}$ of *A. affine* (37.5 μM) and *A. fraterculus* (44.4 μM) fall in the ranges of the NO_3 concentrations in South Sea of Korea and Seto Inland Sea. Therefore, a small change in NO_3 concentrations in South Sea of Korea and Seto Inland Sea may sometimes cause a large change in the NO_3 uptake of *A. affine* and *A. fraterculus*. In addition, the $K_{UT\text{-}NO3}$ of *A. affine* was lower than that of *A. fraterculus*, while the $K_{GR\text{-}NO3}$ of *A. fraterculus* was lower than that of *A. affine*. Therefore, *A. affine* rapidly uptakes NO_3 at lower NO_3 concentrations than *A. fraterculus*, but *A. fraterculus* rapidly grows at lower NO_3 concentrations than *A. affine*.

Interestingly, the $K_{GR\text{-}NO3}$ of *A. affine* and *A. fraterculus* were much lower than their $K_{UT\text{-}NO3}$. That is, the growth rates of *A. affine* and *A. fraterculus* became saturated at the NO_3 concentrations at which NO_3 uptakes increased. For a cell to divide, the cell should uptake a certain amount of NO_3. Thus, NO_3 acquired by the cell may not have been high enough to be used for cell division. Similar patterns are often observed in feeding by mixotrophic and heterotrophic protists; growth rate of a mixotrophic or heterotrophic protist on algal prey become saturated at prey concentrations at which its ingestion rates increase (Jeong et al. 2007, 2014, 2018*a*, 2018*b*, Lim et al. 2014). In conclusion, the values of $K_{GR\text{-}NO3}$ and $K_{UT\text{-}NO3}$ of a species should be determined separately because they can be different from each other. The nutrient repletion method can be a useful tool for determining $K_{GR\text{-}NO3}$ and $K_{UT\text{-}NO3}$ of the species as a function of NO_3 concentration simultaneously.

The maximum NO_3 uptake of *A. affine* (ca. 13-16 pM cell^{-1} d^{-1}) is comparable to that of *Alexandrium minutum* (4.3-16.8 pM cell^{-1} d^{-1}) and *Alexandrium tamiyavanichii* (ca. 10-20 pM cell^{-1} d^{-1}), but the maximum NO_3 uptake of *A. fraterculus* (ca. 30 pM cell^{-1} d^{-1}) is greater than that of these *Alexandrium* species (Lim et al. 2006, Maguer et al. 2007). The size of *A. fraterculus* (32.3 μm) is larger than that of *A. minimum* (20.5 μm), comparable to that of *A. affine* (31.4 μm), but smaller than that of *A. tamiyavanichii* (38.6 μm). The maximum NO_3 uptake of these *Alexandrium* species was not significantly correlated with their size ($p > 0.1$, linear regression ANOVA). Thus, the maximum NO_3 uptake of an *Alexandrium* species should be measured because it may not be calculated using the equation of a regression between the maximum NO_3 uptake and size of *Alexandrium* species.

The maximum growth rate of *A. fraterculus* under continuous light conditions was much lower than that under light and dark conditions, whereas the maximum growth rate of *A. affine* grown under continuous light conditions was slightly higher than that under light and dark conditions. This evidence suggests that growth rate of *A. fraterculus* may be inhibited by continuous light. One scenario is that *A. fraterculus* has a strong circadian rhythm and a continuous light condition disturbs this rhythm. There have been many dinoflagellates species showing circadian rhythms (Prézelin et al. 1977, Knaust et al. 1998, Van Dolah et al. 2007, Dapena et al. 2015); *A. minutum* showed nuclear and morphological changes during cell cycle and growth, indicating a strong circadian rhythm (Dapena et al. 2015). In contrast, to our best knowledge, there has only been one dinoflagellate species identified as showing inhibition of growth under a continuous light condition; the growth rate of *Tripos* (*Ceratium*) *ranipes* under a continuous light of 320 μmol photons m^{-2} s^{-1} was much lower than that under light and dark cycle (Brand and Guillard 1981). Thus, the present study adds *A. fraterculus* to a few dinoflagellate species showing inhibition of growth under a continuous light condition. Another scenario is that an excessive amount of photons inhibits the operation of photosystems of *A. fraterculus* (Richardson et al. 1983, Krause 1988). The amount of photons produced by a continuous light is ca. 40% greater than that by 14-h illumination. Thus, the amount of photons produced by a continuous light at 100 μmol photons m^{-2} s^{-1} is equivalent to that at 140 μmol photons m^{-2} s^{-1} under 14-h light : 10-h dark cycle. The autotrophic growth of the mixotrophic dinoflagellate *Takayama helix* is known to be inhibited at 115 μmol photons m^{-2} s^{-1} under 14-h light : 10-h dark cycle (Ok et al. 2019). Therefore, it is worthwhile to explore possible inhibition of growth of *A. fraterculus* at high light intensities under 14-h light : 10-h dark cycle to test this hypothesis.

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