Research Article

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Nitrate uptake of the red tide dinoflagellate *Prorocentrum micans* measured using a nutrient repletion method: effect of light intensity

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The ability of a red tide species to take up nutrients is a critical factor affecting its red tide dynamics and species competition. Nutrient uptake by red tide species has been conventionally measured by incubating nutrient-depleted cells for a short period at 1 or 2 light intensities. This method may be applicable to certain conditions under which cells remain in oligotrophic water for a long time and high nutrients are suddenly introduced. Thus, a new method should be developed that can be applicable to the conditions under which cells are maintained in eutrophicated waters in healthy conditions and experience light and dark cycles and different light intensities during vertical migration. In this study, a new repletion method reflecting these conditions was developed. The nitrate uptake rates of the red tide dinoflagellate *Prorocentrum micans* originally maintained in nitrate repletion and depletion conditions as a function of nitrate concentration were measured. With increasing light intensity from 10 to 100 μE m^{-2} s^{-1}, the maximum nitrate uptake rate (V_{max}) of *P. micans* increased from 3.6 to 10.8 pM $cell^{-1}$ d^{-1} and the half saturation constant ($K_{s\text{-}NO3}$) increased from 4.1 to 6.9 μM. At 20 μE m^{-2} s^{-1}, the V_{max} and $K_{s\text{-}NO3}$ of *P. micans* originally maintained in a nitrate repletion condition were similar to those maintained in a nitrate depletion condition. Thus, differences in cells under nutrient repletion and depletion conditions may not affect $K_{s\text{-}NO3}$ and V_{max}. Moreover, different light intensities may cause differences in the nitrate uptake of migratory phototrophic dinoflagellates.

Key Words: dinoflagellate; half saturation constant; harmful algal bloom; nitrate; nutrient; red tide; uptake rate

INTRODUCTION

Red tides or harmful algal blooms, discolorations of the sea surface due to plankton blooms, sometimes cause large-scale mortalities of fish and shellfish (Glibert et al. 2014, Hu et al. 2014, Jeong et al. 2015, Lee et al. 2016). Furthermore, they cause great losses to the aquaculture and tourism industries of many countries (Anderson et al. 2012, Fu et al. 2012, Park et al. 2013*b*). Therefore, minimizing losses due to red tides is a critical concern to people in the aquaculture industry, scientists, and government officials. To minimize the losses, it is necessary to understand and predict the outbreak and spread processes of red tides caused by certain red tide causative species.

Phototrophic diatoms, flagellates, and dinoflagellates are the major red tide causative species, and they compete strongly with each other (Anderson et al. 2002, Glibert et al. 2012, Jeong et al. 2013, Lim et al. 2014). Furthermore, many species within each major group also compete with each other. In these competitions, the relative nutrient

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acquisition and growth rates are very important parameters. In general, diatoms grow faster by taking up nutrients at lower nutrient concentrations than flagellates or dinoflagellates having similar volumes (Jeong et al. 2015). However, flagellates and dinoflagellates outcompete diatoms by growing under low nutrient conditions through conducting mixotrophy (i.e., feeding on prey) or vertical migration between well-lit oligotrophic surface water and dim-lit eutrophicated deep water (Smayda 1997, Ji and Franks 2007, Jeong et al. 2015). Therefore, there have been many efforts to obtain data on the nutrient uptake and growth rates of red tide species. However, so far, among the ~300 reported red tide species, the nutrient uptake of only 20-30 species has been reported (Smayda 1997, Kudela et al. 2010, Jeong et al. 2015). In particular, data on the nutrient uptake rates, nutrient half saturation constant, or growth rates of many major red tide organisms are still lacking (Jeong et al. 2015).

Eppley and Coatsworth (1968) first measured the nutrient uptake rate of microalgae; they measured the nitrate uptake of the diatom *Ditylum brightwellii* after incubating nitrate depleted cells for 2-3 h. Later, many studies measuring the nutrient uptake (i.e., uptake of nitrate, nitrite, ammonia, and phosphate) of red tide species used this method or partially modified methods in which nutrient depleted cells were incubated for a short period (usually <3 h). There are 2 major methods of rendering the nutrient concentration in the stock of a target red tide species negligible: 1) wait until cells in a culture utilize all nutrients; and 2) harvest cells growing in nutrient repletion conditions and wash them several times with nutrient-free medium (Cochlan and Harrison 1991, Qi and Zhu 1994, Lomas and Glibert 2000, Fan et al. 2003, Herndon and Cochlan 2007, Sinclair et al. 2009, Hu et al. 2014, Killberg-Thoreson et al. 2014). However, these methods render cells unhealthy and require that nutrient concentrations in the stock be measured several times to make sure that the concentrations are negligible. Indeed, these conventional methods may be applied to certain conditions under which cells stay in oligotrophic water for a long time and high nutrients are suddenly introduced. Furthermore, these conventional methods do not reflect the fact that red tide species experience a light-dark cycle and most flagellates and dinoflagellates experience different light intensities during diel vertical migration (Jeong et al. 2015). Thus, a new method should be developed that can be applied to the conditions under which cells are maintained in eutrophicated waters in healthy conditions and experience a light and dark cycle and different light intensities during diurnal vertical migration.

In this study, a new repletion method reflecting these conditions that many red tide cells experience was developed. In this nutrient repletion method, 1) cells growing exponentially in nutrient repletion conditions were used; 2) two different initial nutrient concentrations (i.e., high and medium concentrations) were provided to cover a wide range of nutrient concentrations; 3) a light and dark cycle was established to reflect the biological clock; 4) cell abundance and nutrient concentrations were measured on a daily basis for 5-10 d; 5) the daily uptake rate of a red tide species was calculated using the differences in cell abundance and nutrient concentrations at a daily interval; 6) daily uptake rates were plotted as a function of the nutrient concentration after discounting rates in acclimating periods and when they dropped below a certain level; 7) the maximum uptake rate and half saturation constant were obtained using Michaelis-Menten equations.

This study used this new method to measure the nitrate uptake of *Prorocentrum micans*, which is one of the most frequent red tide forming species in the coastal waters of many countries (Uchida 1981, Shumway 1990, Zheng-fang et al. 1995, Peña-Manjarrez et al. 2005, Park et al. 2013*a*). To compare the effects of the maintenance conditions of cells, the nitrate uptake of *P. micans* originally maintained in nitrate depletion conditions was also measured by incubating cells for >6 d in a light and dark cycle. These results were also compared with the results of Qi and Zhu (1994) in which the nitrate uptake of *P. micans* was measured by using cells in nitrate depletion conditions for a short period without a light and dark cycle.

Many phototrophic dinoflagellates, including *P. micans*, experience wide ranges of light intensities because they conduct diel vertical migration (Jeong et al. 2015). Thus, the effects of light intensity on the nutrient uptake of a red tide species should be explored. Therefore, the nitrate uptake of *P. micans* was measured under 6 different light intensities of 0-200 μE m^{-2} s^{-1} (i.e., 0, 10, 20, 50, 100, and 200 μE m^{-2} s^{-1}).

Nutrient concentration and light intensity are 2 of the most critical factors affecting photosynthesis and, in turn, the growth of phototrophic red tide organisms. Thus, the results of this study provide a basis for understanding the nitrate kinetics of red tide dinoflagellates and light effects and eventually red tide dynamics. Moreover, this study provides a new method of easily measuring the nitrate uptake of phytoplankton using healthy cells.

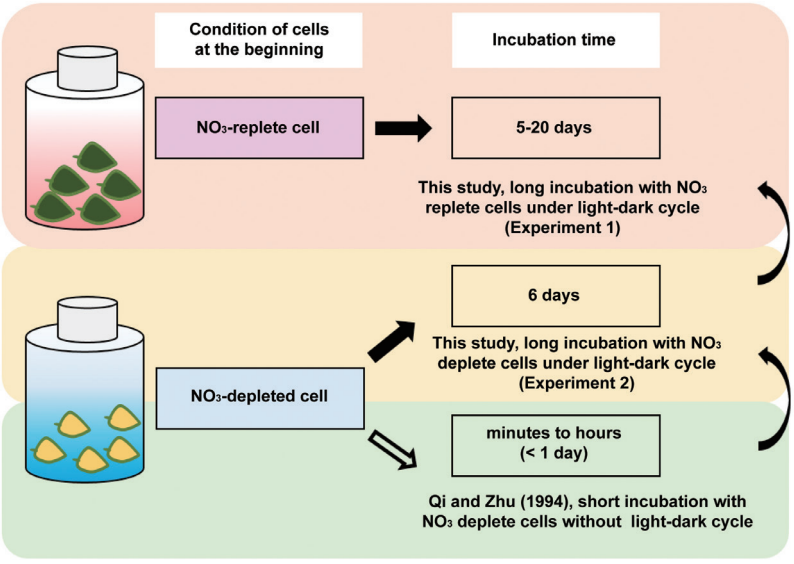

Fig. 1. Diagram of the 3 different methods of measuring nitrate (NO_3) uptake rates. The NO_3 conditions of cells at the beginning of the experiments, acclimation period, and incubation time were compared. Closed arrows and open arrow indicate this study and Qi and Zhu (1994), respecetively.

MATERIALS AND METHODS

Preparation of experimental organisms

P. micans used in this study was originally isolated from a plankton water sample collected from Shiwha Bay, Korea, in October 2009. Phototrophic cells were grown in enriched f/2-Si seawater media (Guillard and Ryther 1962, Guillard 1975) at 20°C under an illumination of 20 μE m^{-2} s^{-1} of cool white fluorescent light on a 14 : 10 h light-dark cycle.

Nitrate uptake as a function of nitrate concentrations under different light intensities using the N repletion method

Experiment 1 was designed to investigate the nitrate uptake of *P. micans* as a function of nitrate concentration at each of the 6 different light intensities when cells were originally maintained under nitrate (NO_3) repletion conditions (Table 1, Fig. 1).

Dense cultures of *P. micans* growing photosynthetically in f/2-Si medium were transferred to 1-L polycarbonate (PC) bottles and the bottles were placed on a shelf in a culture room at 20°C under a 14 : 10 h light : dark cycle and acclimated at the target light intensity for 4 d. Three

Table 1. Experimental design and comparison of the 3 different methods of measuring the nitrate (NO_3) uptake of *Prorocentrum micans*

Method	Initial cells condition	T (°C)	LI (μE m^{-2} s^{-1})	Measured component	HNC (μM)	IT
Experiment 1: long incubation with NO_3 replete cells under light-dark cycle	N replete	20	0, 10, 20, 50, 100, 200	V_{max}, $K_{s\text{-}NO3}$, MGR	120-130	1-3 wk
Experiment 2: long incubation with NO_3 deplete cells under light-dark cycle	N deplete	20	20	V_{max}, $K_{s\text{-}NO3}$	100	6 d
Qi and Zhu (1994): short incubation with NO_3 deplete cells without light-dark cycle	N deplete	20	56-63	V_{max}, $K_{s\text{-}NO3}$	10	Likely short[a]

T, temperature; LI, light intensity; HNC, highest NO_3 concentration tested; IT, incubation time; V_{max}, the maximum uptake rate; $K_{s\text{-}NO3}$, half saturation constant; MGR, the maximum growth rate.

[a]Qi and Zhu (1994) did not provide information on incubation time, light : dark cycle, and initial cell abundance and only indicated that they used a method similar to Eppley (1973), in which the incubation time was short.

1-mL aliquots were subsampled and then cells were enumerated to determine the cell concentration.

P. micans cells were added to triplicate 800 mL culture flasks by transferring predetermined volumes of cultures (final cell concentration = ~1,000 cells mL^{-1}, but ~500 cells mL^{-1} for 200 μE m^{-2} s^{-1} and medium initial nutrient concentrations) (Table 1). A stock solution of nitrate made based on the f/2-Si medium concentration was added to the 2 different final concentrations (NO_3 = 20-30 μM for medium initial concentrations and 100-130 μM for high initial concentrations) (Table 1). A sufficient amount of stock solution of phosphate (PO_4) also prepared based on the f/2-Si 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-Si medium.

The flasks were placed on shelves in 3 temperature-controlled culture rooms. By adjusting distances between light source and flasks, target light intensities of 0 (complete darkness), 10, 20, 50, 100, and 200 μE m^{-2} s^{-1} were established (Table 1). Triplicate culture flasks for target light intensity (14 : 10 h light : dark cycle) were incubated at 20°C. The duration of the incubation was ca. 1 week for medium nitrate concentrations (20-30 μM), but 2-3 weeks for higher concentrations (100-130 μM) (Table 1).

Twenty-mL aliquots were subsampled from each flask every day, and 6-mL aliquots were used for the determination of cell concentration and 14-mL aliquots for the determination of nutrient concentrations.

Cell concentration was determined by enumerating cells on three 1-mL Sedgwick-Rafter counting chambers. Fourteen-mL aliquots were filtered through GF/F filters and then concentrations of nitrate (actually nitrate + nitrite in the Cd-coil reduction method) and phosphate were measured using a nutrient analyzer (QuAAtro; Seal Analytical, Norderstedt, Germany).

Nitrate uptake using the N depletion method

Experiment 2 was designed to investigate the nitrate uptake of *P. micans* at a single light intensity by cells originally maintained under nitrate depletion conditions (Table 1, Fig. 1).

Cells in a dense culture of *P. micans* growing photosynthetically in f/2-Si medium were concentrated using a 20 μm mesh filter after nitrate was depleted and immediately transferred to 1-L PC bottles containing oligotrophic oceanic waters (NO_3 concentration = 0.67 μM, PO_4 = 0.12 μM). The bottles were placed on a shelf in a culture room at 20°C under a 14 : 10 h light : dark cycle and then maintained for 9 d. The final concentration of nitrate was 0.35 μM. Three 1-mL aliquots were subsampled and then cells were enumerated to determine the cell concentration.

P. micans cells were added to triplicate 250-mL culture flasks by transferring predetermined volumes of cultures (final concentration = ~1,000 cells mL^{-1}). A stock solution of NO_3 based on f/2-Si medium concentrations was added to the 7 different final concentrations (1, 2.5, 5, 10, 25, 50, and 100 μM) (Table 1). Stock solutions of PO_4, trace metals, and vitamins based on the f/2-Si medium were added sufficiently so as to not be limiting.

The flasks were placed on shelves in a temperature-controlled culture room at 20°C at 20 μE m^{-2} s^{-1} with a 14 : 10 h light : dark cycle (Table 1). Triplicate culture flasks were also set up. The duration of the incubation was 6 d.

Fifteen-mL aliquots were subsampled from each flask every day and 5-mL aliquots were used for the determination of cell concentration and 10-mL aliquots for the determination of nutrient concentrations. Cell and nitrate concentrations were determined as described above.

Calculation of nitrate uptake rates and half saturation constant in the N repletion method

In experiment 1, the nitrate uptake rate of a *P. micans* cell was determined by dividing the reduction in nitrate concentration (N) by the mean cell concentration at 1 or 2 d intervals;

$$\text{Reduction in the nutrient concentration in a day } (\mu M\ d^{-1}) = (N_{t2} - N_{t1}) / (t_2 - t_1) \quad (1)$$

, where $t_2 - t_1$ = 1 d, but 2 d for the high nitrate concentration at 10 μE m^{-2} s^{-1}.

$$\text{Mean cell concentration } (\text{cells } mL^{-1}) = [Ct_2 - Ct_1] / [\ln(Ct_2 / Ct_1)] \quad (2)$$

, where $t_2 - t_1$ = 1 d, but 2 d for the high nitrate concentration at 10 μE m^{-2} s^{-1}.

Day 0 to 1 or day 2 were treated as the acclimation period, and thus data from these days were not used in calculation. Data on daily nitrate uptake rates from day 2 or 3 to the day before the growth rate of *P. micans* exceeded half the maximum growth rate in each experiment were plotted by the Michaelis-Menten equation;

$$V = V_{max} [N^* / (K_{s\text{-}NO3} + N^*)] \quad (3)$$

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

$$\text{Mean nitrate concentration } (\mu M) = [N_{t2} - N_{t1}] / [\ln(N_{t2} / N_{t1})] \quad (4)$$

, where N = nitrate concentration at a single day, $t_2 - t_1 = 1$ d, but 2 d for the high nitrate concentration at 10 μE m^{-2} s^{-1}.

The specific growth rate of *P. micans* (μ, d^{-1}) was calculated as:

$$\mu = [\ln(Ct_2 / Ct_1)] / (t_2 - t_1) \quad (5)$$

The maximum growth rate (μ_{max}) of *P. micans* was obtained after data were fitted to a Michaelis-Menten equation:

$$\mu = \mu_{max} [N^* / (K_{GR} + N^*)] \quad (6)$$

, where N* = mean nitrate concentration (μM), K_{GR} = the nutrient concentration sustaining $1/2\mu_{max}$.

Data were iteratively fitted to the model using DeltaGraph (SPSS Inc., Chicago, IL, USA), and statistical analyses were conducted using IBM SPSS Statistics version 21 (IBM Corp., Armonk, NY, USA).

Calculation of nitrate uptake rates and half saturation constant in the N depletion method

In experiment 2, the nitrate uptake rate of a *P. micans* cell was also determined by dividing the reduction in nitrate concentration (N) by the mean cell concentration at 1 d intervals; the reduction in the nitrate concentration, mean nitrate concentration, and mean cell concentration were obtained using Eqs. (1), (2), and (3), respectively.

Day 0 to 1 was treated as an acclimation period, and thus data obtained in this period were not used. The data on daily nitrate uptake rates from day 1 or 3 were plotted to Eq. (3) and (4) as the repletion method and iteratively fitted to the model using DeltaGraph (SPSS Inc.).

RESULTS

Daily variations in nitrate and cell concentrations and growth rate in the N repletion method

In experiment 1, with increasing elapsed incubation time, the *P. micans* concentration increased and then became saturated, while the nitrate concentration rapidly decreased and then became depleted (Figs 2 & 3).

With increasing light intensity from 10 to 200 μE m^{-2} s^{-1}, the time for nitrate to be depleted (TND; <1.5 μM) and time for the growth rate of *P. micans* to reach a lag phase (cell concentrations >7,000 cells mL^{-1}) decreased (Figs 2 & 3). When the initial nitrate concentrations were 100-130 μM and the initial *P. micans* concentrations were ~1,000 cells mL^{-1}, the nitrate concentrations became depleted and the growth rate of *P. micans* concentrations reached a lag phase after 14 d at 10 μE m^{-2} s^{-1}, but at 6-8 d at 100-200 μE m^{-2} s^{-1}. However, in darkness, *P. micans* continuously decreased to <100 cells mL^{-1} at 18 d and nitrate concentrations did not clearly change. In all light intensities except for darkness, *P. micans* cells eventually reached ca. 10,000 to 12,000 cells mL^{-1} at the end of the experiment. With increasing light intensity, the highest growth rate of *P. micans* at each light intensity increased from 0.17 d^{-1} at 10 μE m^{-2} s^{-1} to 0.36 d^{-1} at 100 μE m^{-2} s^{-1}, but decreased to 0.30 d^{-1} at 200 μE m^{-2} s^{-1}.

When the initial nitrate concentrations were ca. 20-30 μM and initial *P. micans* concentrations were 1,000 cells mL^{-1} (but ~500 cells mL^{-1} for 200 μE m^{-2} s^{-1}), the TND were 7 d at 10 μE m^{-2} s^{-1}, but 3-5 d at 100-200 μE m^{-2} s^{-1} (Figs 2 & 3). With increasing light intensity, the highest growth rate of *P. micans* at each light intensity continuously increased from 0.19 d^{-1} at 10 μE m^{-2} s^{-1} to 0.42 d^{-1} at 200 μE m^{-2} s^{-1}.

Nitrate uptake rate measured using the N repletion method

In experiment 1, *P. micans* did not clearly take up nitrate in darkness (Fig. 4A). However, with increasing mean nitrate concentrations, the uptake rate of nitrate by *P. micans* at 10-100 μE m^{-2} s^{-1} rapidly increased at NO_3 concentrations of <20 μM, but slowed and then became saturated at higher NO_3 concentrations (Fig. 4B-E), while at 200 μE m^{-2} s^{-1} it became saturated at NO_3 concentrations of <10 μM (Fig. 4F).

When data were fitted to Eq. (3), with increasing light intensity from 10 to 100 μE m^{-2} s^{-1}, the maximum uptake rate of nitrate (V_{max}) by *P. micans* also increased from 3.6 pM $cell^{-1}$ d^{-1} to 10.8 pM $cell^{-1}$ d^{-1}, but decreased to 6.1 pM $cell^{-1}$ d^{-1} at 200 μE m^{-2} s^{-1} (Table 2, Fig. 4B-F). Similarly, with increasing light intensity from 10-20 to 100 μE m^{-2} s^{-1}, the half saturation constant for nitrate uptake ($K_{s\text{-}NO3}$) increased from 4.1-4.2 to 6.9 μM, but decreased to 3.5 μM at 200 μE m^{-2} s^{-1} (Table 2, Fig. 4B-F).

Nitrate uptake rate measured using the N depletion method

In experiment 2, with increasing mean nitrate concentrations, the nitrate uptake rate of *P. micans* at 20 μE m^{-2} s^{-1} rapidly increased at NO_3 concentrations of <10 μM, but

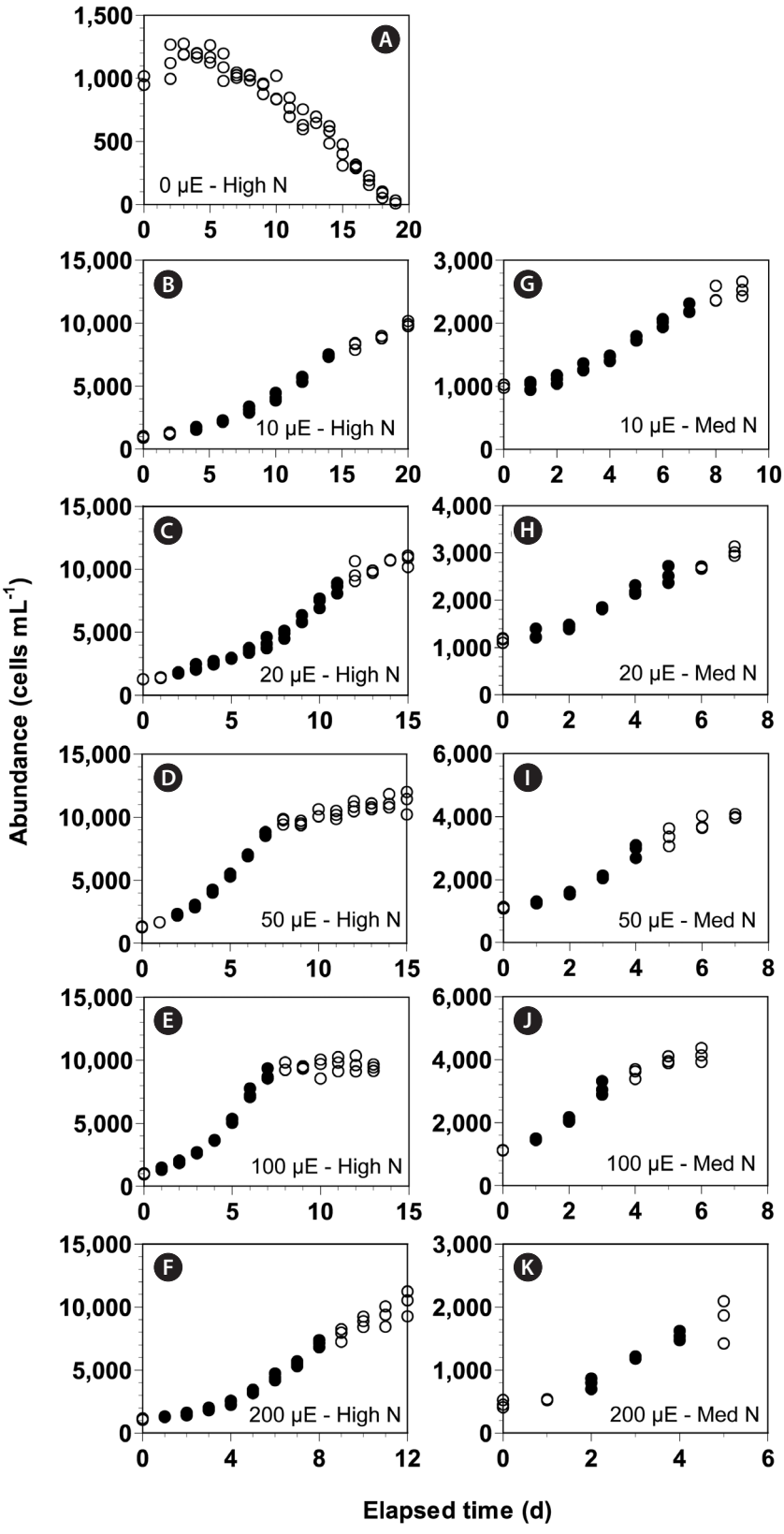

Fig. 2. Change in the concentration of *Prorocentrum micans* (cells mL^{-1}) as a function of elapsed time (d) at 0, 10, 20, 50, 100, and 200 μE m^{-2} s^{-1} when high (A-F) and medium (G-K) initial NO_3 concentrations were provided. Data points indicated by closed circles were used for calculating NO_3 uptake and growth rates, while those indicated by the open circles were not used.

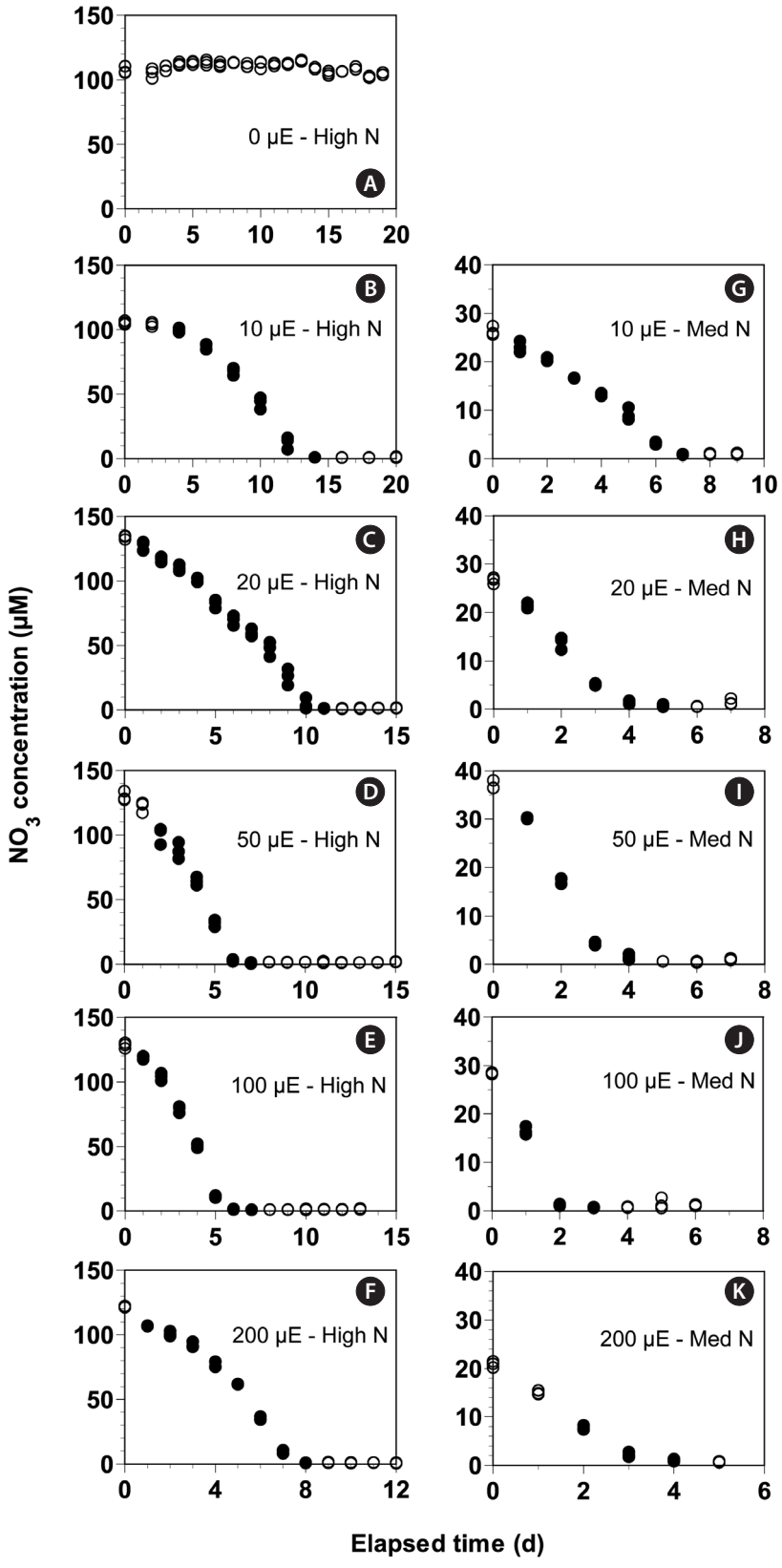

Fig. 3. Change in the concentration of nitrate plus nitrite (NO_3, μM) as a function of elapsed time (d) at 0, 10, 20, 50, 100, and 200 μE m^{-2} s^{-1} when high (A-F) and medium (G-K) initial NO_3 concentrations were provided. Closed circles were used for calculating NO_3 uptake rates, while those indicated by the open circles were not used.

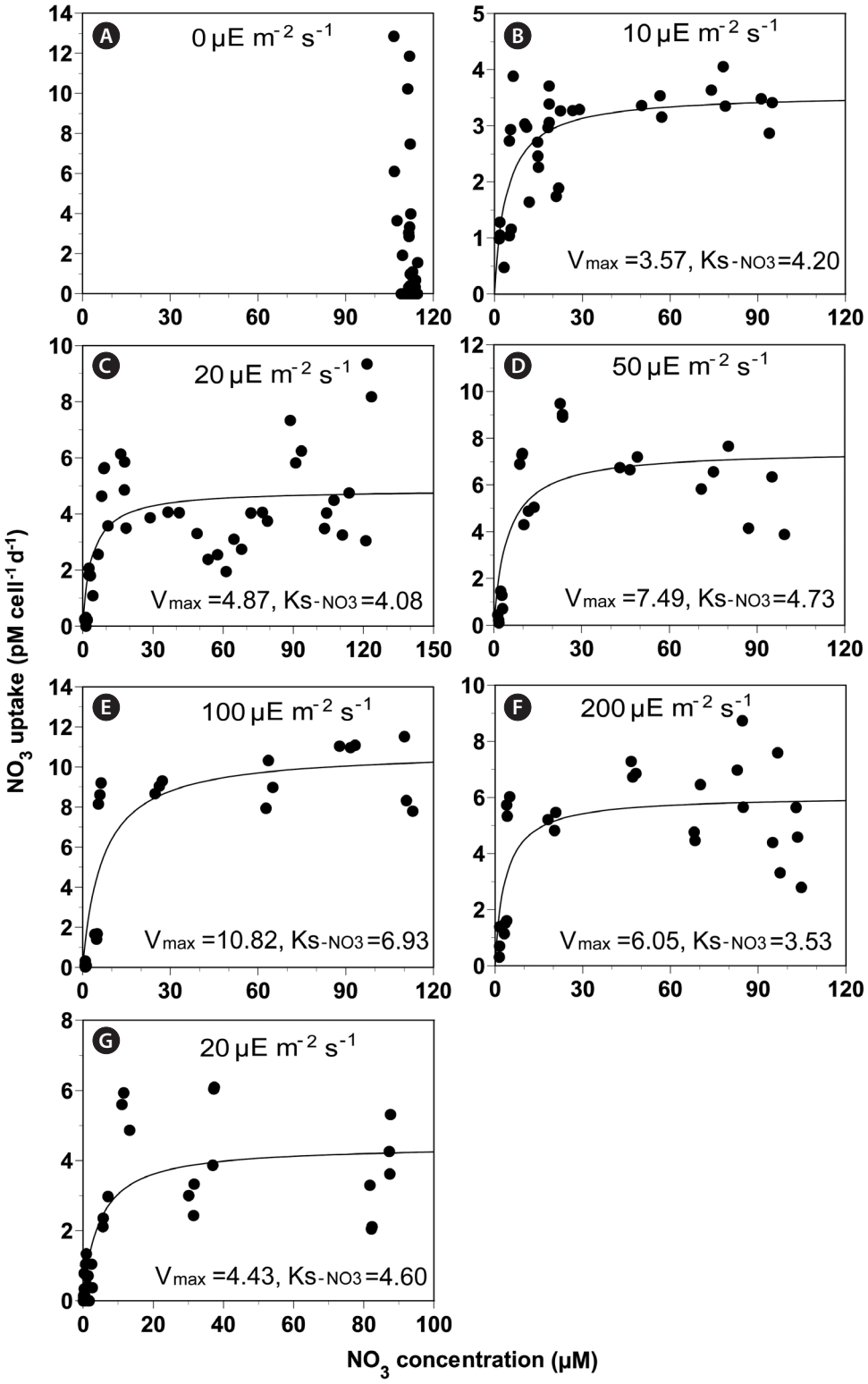

Fig. 4. Maximum NO_3 uptake rates (V_{max}, pM $cell^{-1}$ d^{-1}) as a function of concentrations of NO_3 (μM). 0, 10, 20, 50, 100, and 200 μE m^{-2} s^{-1} using the NO_3 replete (A-F) and 20 μE m^{-2} s^{-1} using the NO_3 depleted (G) long incubation method. The unit of the half saturation constant for uptake rates ($K_{s\text{-}NO3}$) is μM.

Table 2. The maximum uptake rate (V_{max}) or the volume specific maximum uptake rate ($V_{max\text{-}s}$), and half saturation constant ($K_{s\text{-}NO3}$) for the uptake rates of red tide dinoflagellates measured using cultures

Species	ESD[a] (μm)	LI (μE m^{-2} s^{-1})	V_{max} (pM $cell^{-1}$ h^{-1})	$V_{max\text{-}s}$ ($\times10^{-3}$ h^{-1})	$K_{s\text{-}NO3}$ (μM)	μ_{max} (d^{-1})	MSS[a] (μm s^{-1})	Reference
Comparison among *Prorocentrum* species								
Prorocentrum minimum	12.7	40-120	0.10	-	5.00	-	194	Lomas and Glibert (2000)
	-	120	0.12	-	5.18	-	-	Fan et al. (2003)
	-	60	-	-	-	0.51		Hu et al. (2011)
Prorocentrum donghaiense	13.3	60	-	34.4	1.30	0.74	280	Hu et al. (2014)
Prorocentrum micans	28.1	10[b]	0.15	10.1	4.20	0.15	380	This study
	-	20[b]	0.20	13.7	4.08	0.20	-	
	-	50[b]	0.31	21.1	4.73	0.29	-	
	-	100[b]	0.45	30.4	6.93	0.35	-	
	-	200[b]	0.25	17.0	3.53	0.27	-	
	-	20[c]	0.18	12.4	4.60	-	-	
	-	56-63	4.72	319	1.55	-	-	Qi and Zhu (1994)
Comparison with other dinoflagellate species								
Alexandrium minutum	-	200	0.29-0.70	-	0.22-0.28	0.6	-	Maguer et al. (2007)
Karenia brevis	23.0	30	0.41-0.85	-	0.19-1.32 (Diu), 0.05-0.16 (Noc)[c]	0.12-0.13	417	Sinclair et al. (2009)
Prorocentrum micans	28.1	10-200[b]	0.15-0.45	10.1-30.4	4.08-6.93	0.15-0.35	380	This study
	-	56-63	4.72	319.2	1.55	-	-	Qi and Zhu (1994)
Lingulodinium polyedrum (=*Gonyaulax polyedra*)	38.2	120	-	-	8.6-10.3	0.18[a]	510	Eppley et al. (1969)
Alexandrium catenella	30.0	100-150	-	3.0-47.0	0.6-28.1	0.5[a]	175	Collos et al. (2004)
Alexandrium tamarense (=*Protogonyaulax tamarensis*)	32.6	-	-	-	1.5-2.8	-	406	MacIsaac et al. (1979) cited by Kudela et al. (2008*b*)
Gymnodinium catenatum	34.1	300	-	207.1	7.60	0.16[a]	247	Yamamoto et al. (2004)
Akashiwo sanguinea (=*Gymnodinium splenders*)	42.2	120	-	-	3.80	-	300	Eppley et al. (1969)

ESD, equivalent spherical diameter; LI, light intensity; μ_{max}, maximum growth rate; MSS, maximum swimming speeds of red tide dinoflagellates; Diu, diurnal uptake; Noc, nocturnal uptake.

[a]Data from Jeong et al. (2015).

[b]Data were acquired from long incubations with NO_3 replete cells under light-dark cycle.

[c]Data were acquired from long incubations with NO_3 deplete cells.

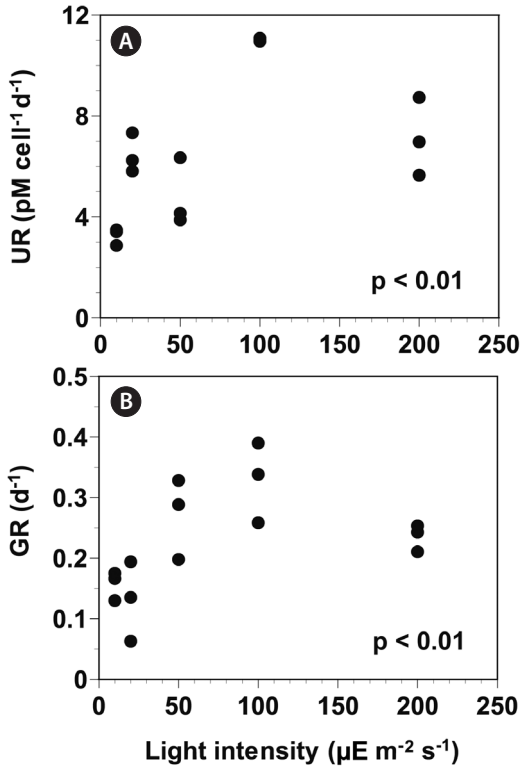

Fig. 5. Nitrate uptake rate (UR, pM cell^{-1} d^{-1}) (A) and growth rate (GR, d^{-1}) (B) of *Prorocentrum micans* as a function of light intensity (μE m^{-2} s^{-1}) when UR and GR at each light intensity were saturated.

slowed and then became saturated at higher NO_3 concentrations (Fig. 4G). When data were fitted to Eq. (3), the V_{max} by *P. micans* was 4.4 pM cell^{-1} d^{-1} and the $K_{s\text{-}NO3}$ was 4.6 μM (Fig. 4G).

Maximum growth rate in the N repletion method

In experiment 1, *P. micans* did not grow obviously in darkness. However, when data were fitted to Eq. (6), with increasing light intensity from 10 to 100 μE m^{-2} s^{-1}, the maximum growth rate of *P. micans* also increased from 0.15 to 0.35 d^{-1}, but decreased to 0.27 d^{-1} at 200 μE m^{-2} s^{-1} (Table 2).

Effects of light intensity on the maximum nitrate uptake rate and maximum growth rate

In experiment 1, the nitrate uptake rates of *P. micans* at a mean NO_3 concentration of 80-100 μM were significantly affected by a light intensity of 10 to 200 μE m^{-2} s^{-1} ($p < 0.01$, ANOVA) (Fig. 5A). Similarly, the maximum growth rates of *P. micans* were also significantly affected by light intensity ($p < 0.01$, ANOVA) (Fig. 5B).

DISCUSSION

Comparison of the results from the 3 different methods

This study shows that the maximum nitrate uptake rates (V_{max}) and half saturation constants for nitrate uptake ($K_{s\text{-}NO3}$) of *P. micans* at 20 μE m^{-2} s^{-1} measured using the nutrient repletion method are similar to those measured using the nutrient depletion method. Thus, the new repletion method of measuring the nitrate uptake rate and $K_{s\text{-}NO3}$ using cells maintained under a nitrate repletion condition gives results similar to that using cells maintained under a nitrate depletion condition. However, the V_{max} of *P. micans* originally maintained under nitrate repletion at 50 μE m^{-2} s^{-1} obtained in this study (7.49 pM cell^{-1} d^{-1}, equivalent to 21 × 10^{-3} h^{-1}) is much lower than that at 56-63 μE m^{-2} s^{-1} under nutrient depletion (113 pM cell^{-1} d^{-1}, equivalent to 319 × 10^{-3} h^{-1}) as observed by Qi and Zhu (1994), while the $K_{s\text{-}NO3}$ of *P. micans* originally under nitrate repletion at 50 μE m^{-2} s^{-1} in this study was greater than that at 56-63 μE m^{-2} s^{-1}. Qi and Zhu (1994) did not provide information on incubation time, light : dark cycle, and initial cell abundance, but only indicated that they used a modification of the method of Eppley (1973). Therefore, it is difficult to know the exact incubation time. However, the conventional and partially modified methods usually incubated cells for <3 h without a light : dark cycle in their nitrate uptake experiments (Table 3), and thus the incubation time in the study of Qi and Zhu (1994) was likely to be shorter than that in our experiment and may not have considered the light and dark cycle. Cells maintained in a nitrate depletion condition may take up nitrate rapidly and with a short incubation time regardless of the light and dark cycle and may have a relatively high maximum uptake rate. However, the physiology and behaviors of red tide dinoflagellates, such as nutrient acquisition, cell division, and vertical migration, are known to be affected by the light and dark cycle or circadian rhythm (Suzuki and Johnson 2001, Van Dolah et al. 2007). Therefore, the nutrient depletion (long and short incubation time) method may apply to the conditions under which red tide causative cells are introduced from oligotrophic offshore surface waters to eutrophic coastal waters or when highly concentrated nutrients due to heavy rains are discharged from rivers to coastal waters after nutrient depletion conditions persist (Fig. 6A). However, this new nutrient repletion method may apply to the conditions under which nutrients are added to eutrophic waters, and high nutrient conditions in surface waters per-

Table 3. The red tide species, taxonomical group (TG), nitrate concentration range tested (NCR), incubation time (IT), light intensity (LI), and temperature (T) in previous studies measuring NO_3 uptake in the laboratory or field and this study

Species	TG	NCR (μM)	IT	LI (μE m^{-2} s^{-1})	T (°C)	Reference
Ditylum Brightwellii	DIA	<30	2-3 h	~160	18 or 20	Eppley and Coatsworth (1968)
Chaetoceros gracilis, Astrionella japonica	DIA	1-20	15-90 min	~111	18	Eppley and Thomas (1969)
16 marine planktons	DIA, DN, FLA	0-10	50% uptake of nitrate at low concentration and 2 μM uptake in higher levels	~120	18	Eppley et al. (1969)
Cytonella nana, Fragilaria pinnata, Bellerochia sp.	DIA	0-30	5-30 min	86	20	Carpenter and Guillard (1971)
Chatonella antiqua	RA	1, 2, 5, 10, 20	80 min	127	25	Nakamura and Watanabe (1983)
Micromona pusilla[a]	PF	0-15	6 h	120	17	Cochlan and Harrison (1991)
Thalassiosira pseudonana	DIA	0.1-5	1-9 min	100-120	18	Dortch et al. (1991)
Prorocentrum micans, Chaetoceros lorenzianus	DN, DIA	0-10	<50% of the added nitrate was taken up	56-63	20	Qi and Zhu (1994)
Chaetoceros sp., *Skeletonema costatum, Thalassiosira weissflogii, Dunaliella tertiolecta, Pavlova lutheri, Prorocentrum minimum*[a]	DIA, DN	0.01-200	20 min	40-120	20	Lomas and Glibert (2000)
Lingulodinium polyedrum[a]	DN	0-36	75 min	~2,300 (ambient sunlight)	14	Kudela and Cochlan (2000)
Prorocentrum minimum[a]	DN	0-30 (field, lab)	30 min (field, lab)	On deck (field), 120 (lab)	20 (lab)	Fan et al. (2003)
Gymnodinium catenatum	DN	2.5-50	120 min	300	25	Yamamoto et al. (2004)
Alexandrium catenella	DN	0-100	1 h	100-150	20	Collos et al. (2004)
Heterosigma akashiwo	RA	0.1-12	10 min	40, 110	15	Herdon and Cochlan (2007)
Alexandrium minutum[a]	DN	0.1-30	1 h	200	18	Maguer et al. (2007)
Akashiwo sanguinea[a]	DN	0.14-14.49, 0.20-20.20	30 min	180-240	15-16	Kudela et al. (2008*a*)
Cochlodinium sp.[a]	DN	0-5	30 min	240	15-16	Kudela et al. (2008*b*)
Pseudo-nitzschia spp., *Alexandrium catenella, Dinophysis acuminata*	DIA, DN	0.19-20.16	3 h	-	12-16	Seeyave et al. (2009)
Karenia brevis[a]	DN	0-50	30 min	30	22	Sinclair et al. (2009)
Karenia mikimotoi[a]	DN	1-50	30 min	On deck	-	Li et al. (2010)
Prorocentrum donghaiense[a]	DN	0.1-50	<1 h	60	23	Hu et al. (2014)
Karenia brevis[a]	DN	0-200	0.5-1 h	On deck (field), 36 (lab)	22 (lab)	Killberg-Thoreson et al. (2014)
Prorocentrum micans	DN	0-130	1-3 wk	0, 10, 20, 50, 100, 200	20	This study (long incubation with NO_3 replete cells under light-dark cycle)
Prorocentrum micans	DN	0-100	6 d	20	20	This study (long incubation with NO_3 deplete cells under light-dark cycle)

DIA, diatoms; DN, dinoflagellates; FLA, flagellates; RA, raphidophytes; PF, picoflagellate.

[a]Measured with isotope labelled nutrient.

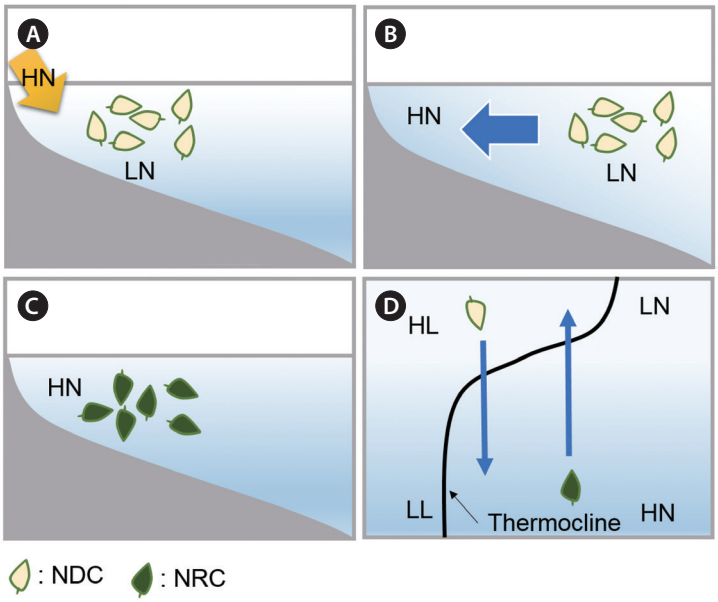

Fig. 6. Diagrams of *Prorocentrum micans* cells under N repletion and depletion conditions and then experiencing high and low nutrient waters. (A) *P. micans* cells experiencing high N after being maintained in N depleted waters. (B) *P. micans* cells are transported from N depleted waters offshore to N replete waters near the shore. (C) *P. micans* cells continuously maintained in N replete waters near the shore. (D) *P. micans* cells migrating between well-lit oligotrophic surface waters and dim eutrophicated deep waters. NDC, nitrogen depleted cell; NRC, nitrogen replete cell; HN, high N concentration water; LN, low N concentration water; HL, high light intensity; LL, low light intensity.

sist for a long time (Fig. 6B). In addition, this new method can be applied to red tide species that inhabit deep, but partially lit, eutrophic waters below the thermocline (Fig. 6C & D). In natural environments, red tide species are exposed to nutrient repletion or depletion conditions, and both conditions alternate. Therefore, the results of both experiments obtained by using nutrient replete and depleted cells can be applied to certain waters where both nutrient replete and depleted conditions occur.

Effects of light intensity on the maximum nitrate uptake rate and half saturation constant for nitrate uptake

Prior to this study, there have been no studies on the effects of light intensity on the $K_{s\text{-}NO3}$ and V_{max} of nitrate of red tide organisms. Eppley et al. (1969) measured the NO_3 uptake of phytoplankton with the assumption that $K_{s\text{-}NO3}$ is not influenced by irradiance. Later, most studies measured NO_3 uptake at 1 light intensity. Sinclair et al. (2006, 2009) measured NO_3 uptake at 2 different light intensities in each study and showed different rates between 2 light intensities. However, this study explored the effects of 6 different light intensities. The results of this study clearly show that the uptake rate of *P. micans* is affected by light intensity. The maximum swimming speed of *P. micans* is 380 μm s^{-1}, so it is theoretically able to descend to 14 m during 10 h of travel (Jeong et al. 2015). *P. micans* usually ascends at sunrise, but descends at sunset (Hasle 1950). Therefore, it is likely to experience a wide range of light intensity. Thus, it may take up nitrate at different rates at different depths when vertically migrating through the water column. In particular, the NO_3 uptake rates of *P. micans* at darkness were almost negligible. Therefore, it may not take up NO_3 much after sunlight completely disappears from the water column.

The light intensity at which the highest $K_{s\text{-}NO3}$ and V_{max} are achieved is the same as that at which the highest growth rate is achieved (i.e., 100 μE m^{-2} s^{-1} for *P. micans*). Thus, it is reasonable to measure the $K_{s\text{-}NO3}$ and V_{max} of nitrate at the light intensity after the optimal light intensity for growth is discovered because it is generally easier to measure growth rates than nutrient uptake rates.

Comparison of the maximum nitrate uptake rate and half saturation constant for the nitrate uptake of red tide dinoflagellates

The $K_{s\text{-}NO3}$ of 10 red tide dinoflagellate species measured using cultures has been reported so far, and either the V_{max} or volume-specific maximum uptake rates of nitrate ($V_{max\text{-}s}$) of 7 red tide dinoflagellate species have

been reported (Table 2). Within the genus *Prorocentrum*, the V_{max} of the Korean strain of *P. micans* at 100 μE m^{-2} s^{-1} obtained in this study is 380% higher than that of *P. minimum* at a similar light intensity, but the volume specific maximum uptake rate of the Korean strain of *P. micans* was lower than that of *Prorocentrum donghaiense* (Table 2). The larger size of *P. micans* may be partially responsible for the higher V_{max} and lower volume-specific maximum uptake rate compared to those of *P. minimum* and *P. donghaiense*. The $K_{s\text{-}NO3}$ of the Korean strain of *P. micans* at 100 μE m^{-2} s^{-1} is slightly greater than that of *P. minimum*, but much greater than that of *P. donghaiense* (Table 2). Therefore, when nitrate concentrations are <4 μM, *P. donghaiense* may take up nitrate and grow rapidly to form red tide patches, while *P. micans* may not do so. Therefore, *P. donghaiense* may inhibit *P. micans* red tide outbreaks by causing nitrate depletion or by maintaining a low nitrate concentration. However, even when nitrate concentrations are >4 μM, the abundance of *P. donghaiense* is expected to be greater than that of *P. micans* because the growth rate of *P. donghaiense* is twice the maximum growth rate of *P. micans*. The maximum swimming speed of *P. micans* and, in turn, the depth it reaches through 10 h travel (i.e., 380 μm s^{-1} and 14 m) are greater than those of *P. donghaiense* (i.e., 280 μm s^{-1} and 10 m). Thus, *P. micans* is likely to outgrow *P. donghaiense* when the thermocline depth is deeper than ~10 m.

Implications for red tide dynamics

P. micans is a common red tide species in the waters of many countries (Allen 1941, Uchida 1981, Pybus 1990, Shumway 1990, Zheng-fang et al. 1995, Shankle et al. 2004, Peña-Manjarrez et al. 2005, Jeong et al. 2013, Kang et al. 2013, Park et al. 2013*a*); it is 1 of 3 major red tide dinoflagellates in the waters of southern California (i.e., *Akashiwo sanguinea*, *Lingulodinium polyedrum*, and *P. micans*) (Allen 1941, Cullen and Horrigan 1981, Shankle et al. 2004). It is likely to compete severally with many phototrophic species to form red tides. However, it may have difficulty in forming red tide patches in eutrophicated coastal waters due to lower growth rates, lower V_{max}, and higher $K_{s\text{-}NO3}$ than co-occurring red tide species. Its abilities to conduct mixotrophy and produce allelopathic materials over competitors may enable it to form red tides (Jeong et al. 2005, Ji et al. 2011); it is known to be able to feed on many algal prey species and inhibit the growth of the diatom *Skeletonema costatum* and the dinoflagellate *Karenia mikimotoi* using allelopathic materials (Jeong et al. 2005, Ji et al. 2011). Therefore, to understand the red tide dynamics of *P. micans* in natural environments, both NO_3 uptake from ambient waters through photosynthesis and organic nitrogen through mixotrophy should be taken into consideration.

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