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Effects of Iron, Chelators and Nitrate Concentration on *in vivo* Fluorescence and Nitrate Reductase of the Red Tide Organism *Amphidinium carterae*

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A red tide organism, *Amphidinium carterae* was incubated under different iron/chelator and nitrate concentrations to investigate the factors controlling the growth. The chelation capacity played a critical role in regulating the nitrate reductase (NR) activity and *in vivo* fluorescence of this organism. However, there was a significant difference between the NR activity and *in vivo* fluorescence in response to trace metals and chelator treatments. *In vivo* fluorescence was the highest in FeEDTA 10 μM treatments and the lowest in DTPA 10 μM treatments. This indicates that the availability of the trace metal is important in regulating the *in vivo* fluorescence of this photosynthetic microalgae. In contrast, NR activity showed the highest values in trace metal enriched treatments, and trace metal+DTPA treatments showed fairly high NR activities. This suggests that DTPA treatment did not hinder the NR activity as much as it did *in vivo* fluorescence. *In vivo* fluorescence and NR activity increased with nitrate concentration of up to 50 μM and remained relatively constant or the rate of increase decreased above that concentration, indicating that initial nitrate concentration of higher than a certain level would not accelerate the growth of *A. carterae*. Further investigation is needed to elucidate the reason for the difference in timing sequence between the NR and *in vivo* fluorescence in response to different metal treatments and chelation capacity.

INTRODUCTION

The frequency of the red tide, or harmful algal bloom (HAB), is increasing worldwide due to the increasing degree of anthropogenic eutrophication (Smayda, 1997; and references therein). In coastal areas of Korea, it occurred frequently since 1970s and it became more widespread and with longer durations (Hahn, 1998). The culprit for the formation of HAB was usually the pollutant load (especially inorganic nutrients including N and P) from terrestrial origin and self pollution from aquaculture farms (Park, 1991), and it has been claimed that the red tide can be prevented if these pollutant loads can be reduced substantially.

Nutrients are essential to maintain the material cycling of ecosystems, and N is usually the limiting factor for primary production in marine environments (Ryther and Dunstan, 1971). In coastal areas, especially where a sufficient supply of nutrients from terregeneous origin exists, nutrients are usually abundant leading to eutrophication (Nixon, 1995). However, this does not always lead to the outbreak of HAB. The presence of nutrient is one of the necessary conditions for HAB but does not appear to be the sufficient condition. Accordingly, we can infer that factors other than nurtients should act as triggers or co-limiting factors for the formation of HAB.

Silicate is essential for the growth of diatoms which are major photosynthetic organisms in the marine ecosystem. When silicate is abundant, diatoms tend to predominate and inhibits the growth of dinoflagellates to some extent. Also the stability of water column affects the development of red tide. The red tide usually outbreaks after the spring diatom bloom when silicate is depleted and after the water column is stabilized. Most of the red tide organisms have flagella and are vulnerable to high turbulence (Thomas and Gibson, 1990; Thomas *et*

al., 1995), not like diatoms which need a certain degree of vertical mixing to remain in the euphotic zone for light and to acquire nutrients through passive sinking. There are reports that continued agitation prevented cell duplication and even led to a complete cell disintegration of dinoflagellates (Berdalet and Estrada, 1993).

Light is one of the necessary conditions for the red tide to occur, which is also one of the classical conditions needed for the spring bloom formation. However, even if there is enough sunlight at the surface, high turbidity (*e.g.*, coastal areas of the Yellow Sea) or intense vertical mixing due to typhoons or tidal activity will hinder the formation of the red tide. Temperature is usually related to the light intensity. High temperature is favorable for the growth of red tide organisms (approximately 15—30°C; usually above 21—25°C in coastal areas of Korea; Honjo, 1993; Qi *et al.*, 1993).

In addition to the classical physico-chemical factors mentioned above, the chelation capacity could act as one of the important factors for the red tide by determining the availability of trace metals, even though needed in tiny amounts, which are essential for the growth of phytoplankton (Harvey, 1937; Boyer *et al.*, 1987). The iron in aqueous solution mostly occurs as ferric (Fe^{3+}) forms under the oxic conditions. The solubility of ferric iron (Fe^{3+}) is extremely low ($K_{sp}=10^{-32}$), and most of the iron in the sea water is biologically unavailable (Anderson and Morel, 1982; Sulzberger *et al.*, 1989). Consequently, there is a large difference between the concentration and the bio-availability of trace metals in sea water. Some marine microorganisms including eucaryotic phytoplankton and cyanobacteria excrete highly species-specific iron chelators (siderophores), and monopolize iron from competing species (Trick *et al.*, 1983; Murphy *et al.*, 1984; Kerry *et al.*, 1988). The secretion of siderophore plays an important role in species succession in marine environments (Murphy *et al.*, 1976). Also there is a difference in the requirement for the trace metals of different phytoplankton species; coastal species have higher requirements for essential trace metals than purely oceanic species (Brand *et al.*, 1983).

There are many articles on the role of iron in the phytoplankton metabolism (Raven 1988; Sunda, 1989; Morel *et al.*, 1991). The nitrate reductase (NR) which catalyzes the first step in nitrate utilizing enzyme systems, needs iron as a cofactor. Iron is also essential for cytochromes and ferredoxin which are redox enzymes of the electron transport system in mitochondria and chloroplasts (Raven, 1988), and for the synthesis of chlorophyll (Devlin and Witham, 1983). In this way, the availability of iron could exert a great influence on the metabolism and growth of phytoplankton. If red tides can be controlled by reducing iron and trace metal availability not by reducing N or P which exists in huge amounts and costly to control, it would be an economically more beneficial and ecologically more sound solution.

MATERIALS AND METHODS

Amphidinium carterae was grown in culture media at L:D=16:8 cycles and the changes in NR activity and fluorescence were monitored under different metal, chelation and nitrate conditions. NR was measured according to Davidson and Stewart (1984) and Hochman *et al.* (1986). Aliquots of 10 ml phytoplankton culture stock were dispensed into 20 ml glass scintillation vials and up to 1000 μl of *n*-propanol were added with final concentration of 10 mM KNO_3. The addition of *n*-propanol makes the phytoplankton cells permeable to nitrite, and nitrite produced through nitrate reduction is excreted to the media. The vials were put into a rotating shaker for certain period of time (up to 4 hours) and the reaction was terminated by adding 20 μl of TCA (trichloroacetate). Nitrate reduction was determined by measuring nitrite, which was excreted into the media after nitrate reduction by phytoplankton cells, according to Parsons *et al.* (1984). *In vivo* fluorescence was measured using a spectrofluorometer (Jasco FP-920) to monitor the changes in cell concentration (Samuelson and Öquist, 1976; Vincent, 1980; Krause and Weis, 1991). Chlorophyll *a* concentrations were measured for some experiments according to Parsons *et al.* (1984).

The preparation of *A. carterae* culture for iron chelation experiments was done as follows. The culture was maintained in complete f/2 media at L:D=16:8 cycle using an array of white fluorescence lamps (light intensity=60 μE $m^{-2} \cdot s^{-1}$) and harvested by centrifuging at ×150 g for 15 min. The incubation bottles were 250 ml polycarbonate centrifuge bottles and used for both as growth chambers and as centrifugation vessels. The centrifuged pellets were resuspended with metal free (also FeEDTA free) f/2 media and centrifuged twice

to completely remove trace metals from the media. Final pellets were resuspended with FeEDTA free media and dispensed into 250 ml PC bottles for metal and/or chelator treatments. No pre-incubation was done to deplete the internal storage of trace elements in the algae.

RESULTS

Preliminary experiments

Determination of the optimum *n*-propanol concentration: To determine the optimum *n*-propanol concentration for NR activity measurements, 0, 50, 250, 500 800, 1000 µl aliquots of *n*-propanol was added to each 10 ml cultures in 20 ml glass scintillation vials and incubated for 2 hours. Fig. 1a shows that there was no significant difference among treatments except for 250 µl additions. To determine the minimum concentration needed, more detailed assay was conducted between 0 and 300 µl of *n*-propanol additions (Fig. 1b). The optimum amount was between 40 and 60 µl. 50 µl *n*-propanol was chosen for later analyses, otherwise indicated.

Determination of the optimum time duration: After the proper *n*-propanol concentration was determined, time course experiments (up to 4 hours) were conducted to determine the optimum duration for the NR reaction. For 2-hour incubation, the reaction rate has increased at 2 hour after the 30 minutes to 1 hour lag period (Fig. 2a). 2-hour incubation was not long enough to determine the optimum condition for the experiment. Fig. 2b shows 3-hour incubation and the reaction rate seemed to level off after 2 hours. Fig. 2c shows 4-hour incubations with different concentrations of algae. The absorbance was normalized for the

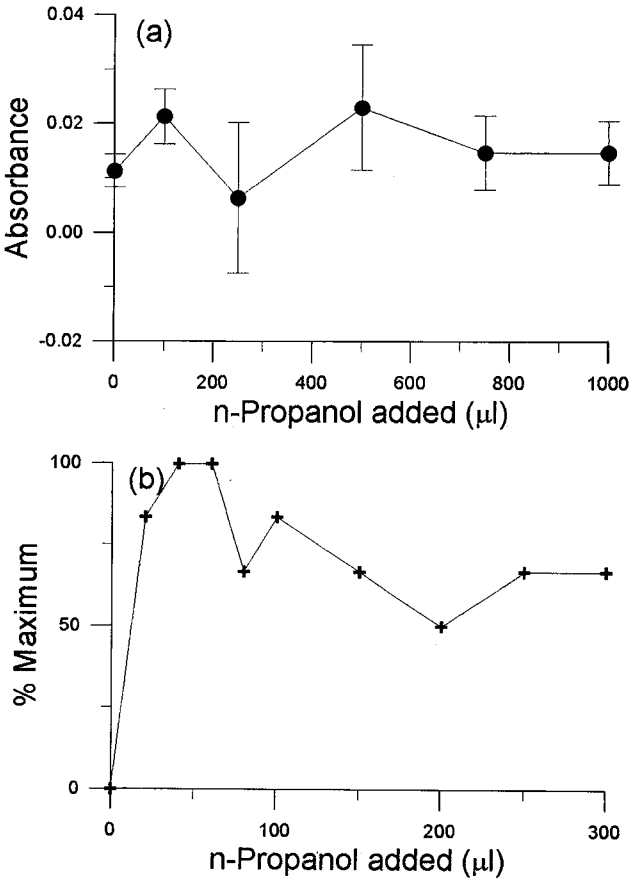

Fig. 1. Nitrate reductase activity at different *n*-propanol concentrations. (**a**) Circles indicate the mean of triplicates at each *n*-propanol concentration and error bars indicate the standard deviation of the mean. (**b**) Represented as the percentage of the maximum activity.

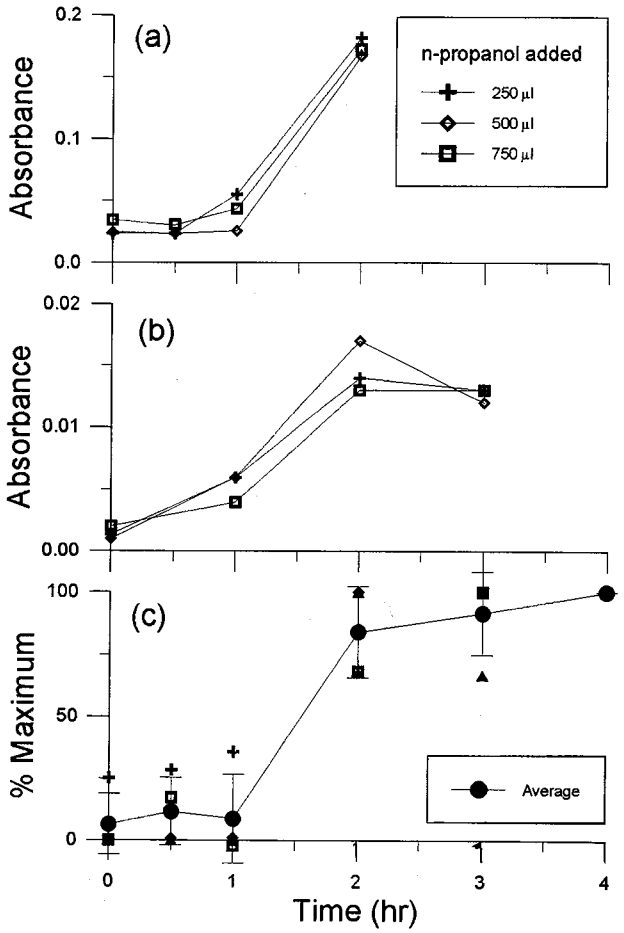

Fig. 2. Time course experiments of nitrate reductase activity. (**a**) At different *n*-propanol concentrations. (**b**) Triplicates with 250 µl *n*-propanol additions. (**c**) 50 µl *n*-propanol with different concentrations of algae. Absorbance was normalized to the maximum.

maximum absorbance of each group for comparison. The reaction seemed to be stable or the increase rate was small after 2 hours. The optimum incubation time of 3 hour was adopted for later analysis.

Experiment 1: the effect of Fe and chelation capacity

Iron/chelation experiment was conducted under 8 different conditions of iron and chelator treatments including the control (Figs. 3 and 4). The control was aged and filtered seawater taken from the East Sea surface water, with low nitrate (<220.5 μM) and other trace metals. T1 was enriched with nitrate and phosphate 100 μM and 10 μM, respectively. T2-T7 treatments were all enriched with nitrate and phosphate as in T1 in addition to metal/chelator treatments. T2 was enriched with FeEDTA 2 μM, T3 FeEDTA 10 μM, T4 DTPA (diethylene-triamine-pentaacetic acid) 10 μM, T5 with metals according

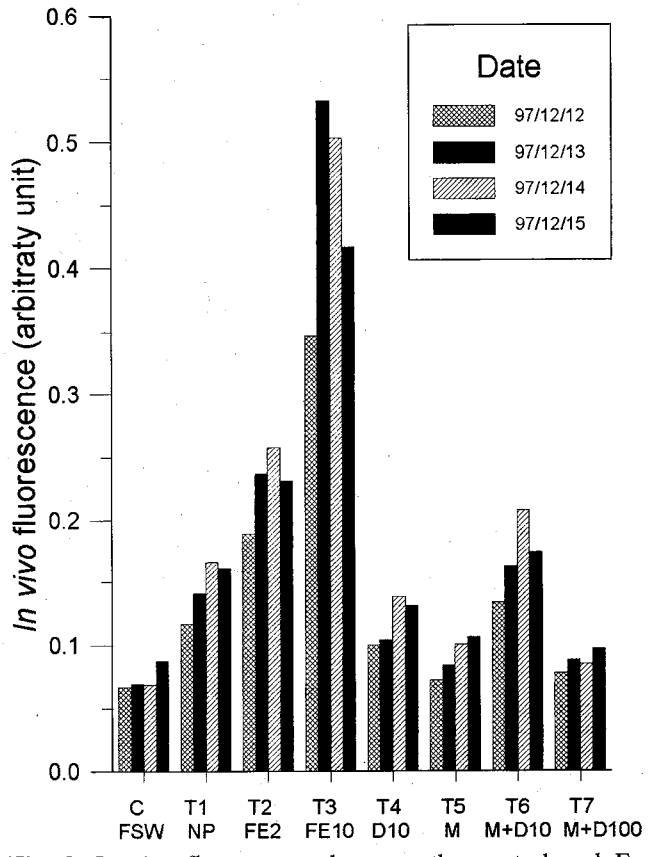

Fig. 3. *In vivo* fluorescence between the control and Fe, EDTA, and DTPA treatments between December 12—15, 1997 (Experiment 1). FSW=filtered seawater, NP=nitrate and phosphate adddition, FE=FeEDTA, D=DTPA, M=metal. The number after each identification character is the final concentration added in μM. NP was added to all treatments except the control.

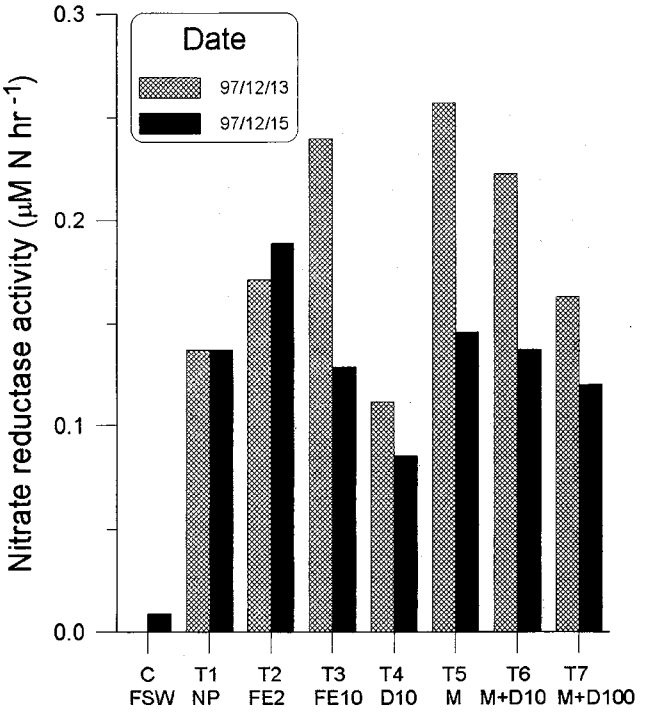

Fig. 4. Nitrate reductase activity of experiment 1.

to f/2 media, T6 metal plus DTPA 10 μM, T7 metal plus DTPA 100 μM in addition to T1. DTPA, which is a strong chelator, was added to scavenge metals which may present in trace concentrations after the centrifugation and resuspension in metal free media.

Figure 3 shows the *in vivo* fluorescence values for each treatment during four consecutive days. The control, which did not have any N, P or trace metal enrichment, showed the lowest fluorescence values. T1, with only N and P enrichment, showed higher fluorescence than the control. T3, with 10 μM FeEDTA enrichment (T3) showed the highest fluorescence, which 2 μM FeEDTA (T2) enrichment followed. T5 (DTPA 10 μM), T6 (metal), T7 (metal +100 μM DTPA), all showed lower fluorescence than only N+P treatment (T1). It is reasonable that DTPA 10 μM addition (T4) and metal with excess amount of strong chelator DTPA (T7) showed lower fluorescence than T1, because ambient and enriched trace metal could have been scavenged by DTPA and became biologically unavailable. The low fluorescence with metal enrichment could be explained by the lack of chelation capacity. Trace metals in solution lacking chelators will not be available for phytoplankton due to low solubility, and heavy metal toxicity can not be ameliorated without proper chelation capacity. However, the NR activity showed somewhat different pattern from fluorescence (Fig. 4).

It was almost negligible without N enrichment (control). Nutrients (especially nitrate) could have been depleted in the media and phytoplankton did not show any NR activity, even though fluorescence showed significantly positive values. In contrast to fluorescence, the highest NR appeared in metal enriched samples. The second highest NR activity was observed in FeEDTA 10 μM enriched samples. Metals with DTPA 10 μM and DTPA 100 μM samples also showed fairly high NR activity. DTPA appears to act as an inhibiting agent for *in vivo* fluorescence, but not for the NR activity. The reason for this unexpected result deserves further investigation. Productivity measurements using ^{14}C-$NaHCO_3$ and other biochemical assays (nitrate uptake rate, chlorophyll concentration, and cell composition) need to be examined in the subsequent experiments.

Experiment 2: the effect of different nitrate concentrations

Two batches of experiments were performed at different nitrate additions of up to 200 μM for batch 1 and 320 μM for batch 2. Cultures were maintained under the same condition as in Experiment 1. After the harvest with centrifugation, cells were resuspended in complete f/2 media with different initial nitrate concentrations.

For the first batch of experiment, nitrates added were 0 (C), 10 (T1), 20 (T2), 30 (T3), 40 (T4), 50 (T5), 100 (T6), and 200 (T7) μM for final concentrations. Fig. 5 shows the changes in *in vivo* fluorescence of 6 days during the 11-day incubation period. Fluorescence was highest on January 5, which was the 6th day of the incubation. It was highest in 50 μM NO_3 enriched samples. At the beginning (Fig. 5a), there were no significant differences among different nitrate additions. On January 5 (Fig. 5b), fluorescence (arbitrary unit), increased significantly (up to three-fold) compared to the beginning. The increase was sigmoidal up to 50 μM nitrate addition and decreased a little at 100 and 200 μM nitrate additions. On next day (Fig. 5c), the fluorescence decreased but the trend was the same as the previous day. After that, the maximum of *in vivo* fluorescence occurred at lower nitrate additions but the magnitude was much smaller than previous days (Figs. 5d, 5e and 5f). NR activities measured between 9 and 12, January are shown in Fig. 6. The slopes were steeper than those of *in vivo* fluorescence, and reached a maximum at lower

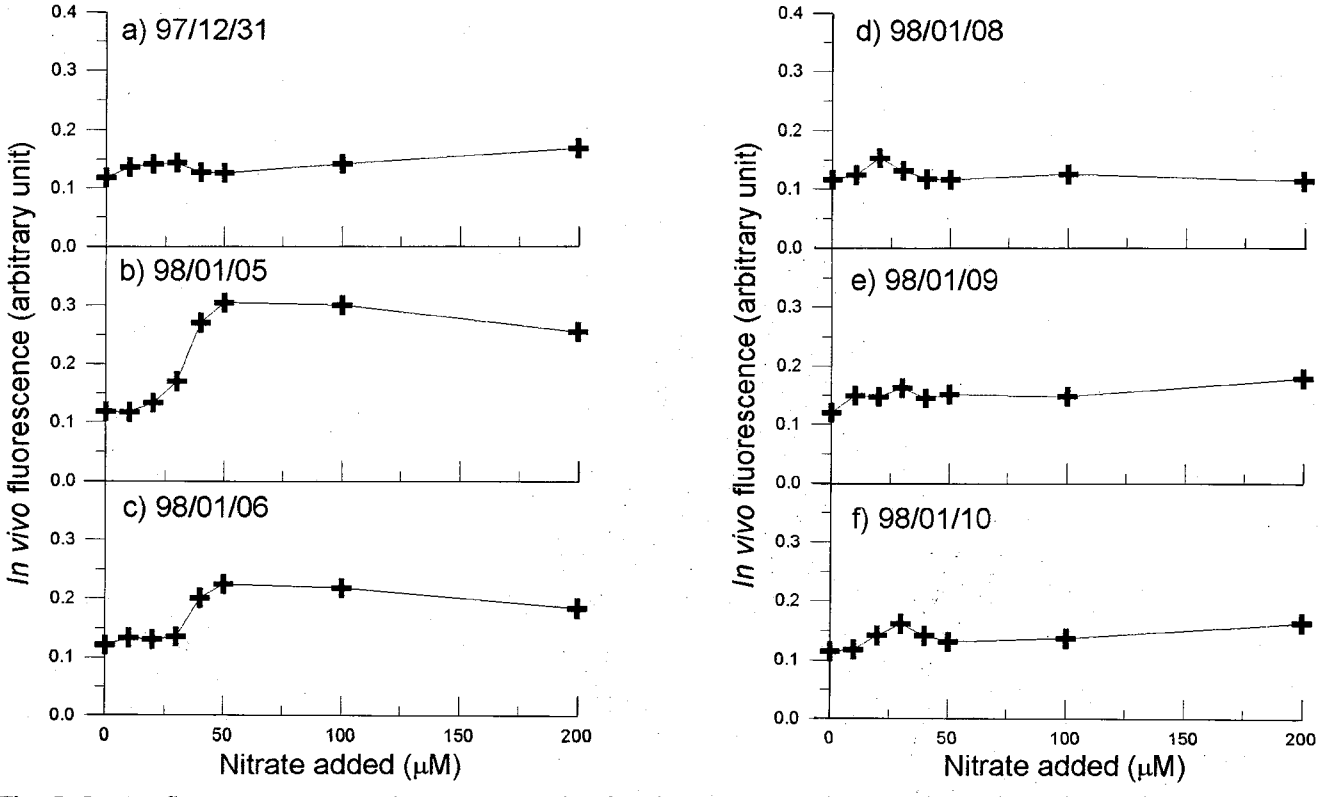

Fig. 5. *In vivo* fluorescence versus nitrate concentration for nitrate concentration experiment (Experiment 2).

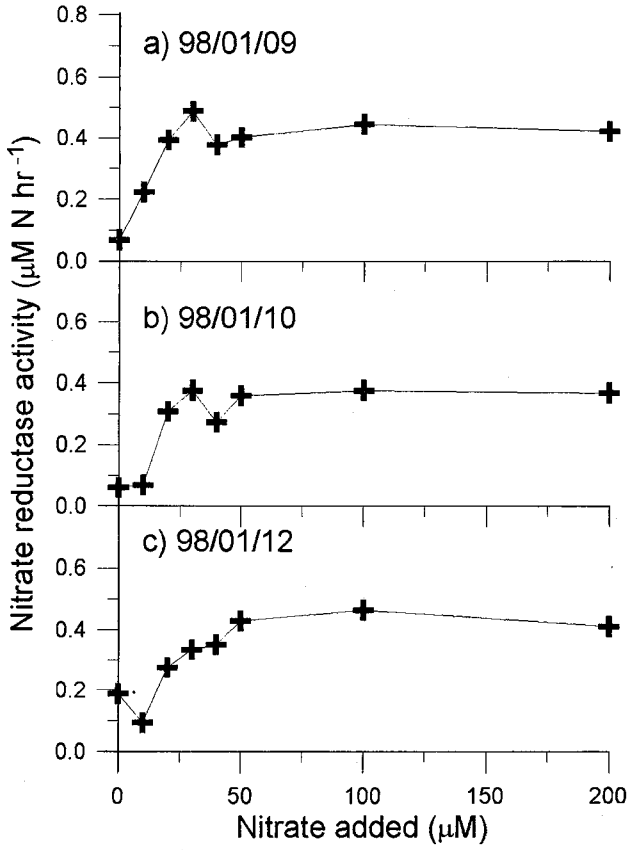

Fig. 6. Nitrate reductase activity versus nitrate concentration on (**a**) January 9, (**b**) January 10 and (**c**) January 12, 1998 for Experiment 2.

concentrations of nitrate added. It appears that NR activity has higher affinity (low KM) for the substrate concentration than fluorescence. NR activity showed fairly high values even though fluorescence has declined after day 8. This mismatch between the fluorescence and NR will be discussed later.

Figure 7 shows the time course of *in vivo* fluorescence at different initial nitrate concentrations of 0, 10, 20, 30, 40, 50, 100 and 200 μM additions. When initial nitrate concentration was higher than 40 μM, *in vivo* fluorescence increased about two-fold at day 5 of the incubation, decreased to a minimum at day 8, and increased again afterwards. In contrast, there was no significant increase until day 9 at nitrate concentration of lower than 30 μM. The pattern was similar to that of ^{15}N nitrate uptake experiments (Yang, 1997), in which nitrate uptake reached a maximum after 3—4 days of the incubation, reached a minimum after a week, and then increased again when nitrate became depleted.

DISCUSSION

The measurement of *in vivo* fluorescence is an easy way to monitor the changes in the growth of photosynthetic organisms and can be used to monitor the possible outbreak of red tides (Yamamoto and Fujisaki, 1989). Also the method of NR assay proved to be a useful tool assessing the capability of phytoplankton in nitrogen metabolism. However, there seems to be a time lag and/or some discrepancy between the *in vivo* fluorescence and

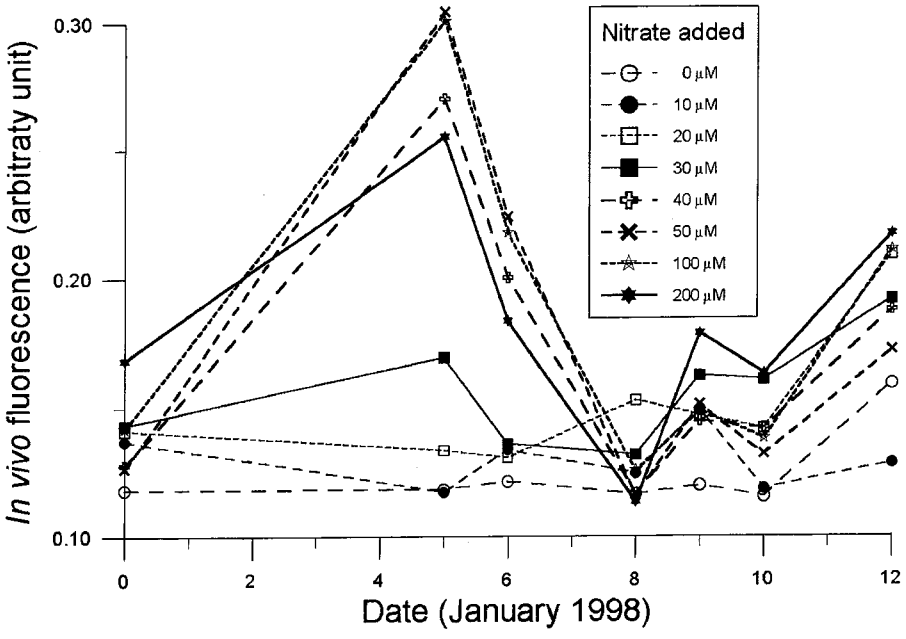

Fig. 7. Time course of *in vivo* fluorescence at different concentrations of initial nitrate between December 31, 1997 and January 12, 1998 for Experiment 2.

NR activity. NR could be a good indicator of the nitrate incorporation rate under steady state conditions (Berges and Harrison, 1995a). Some non-steady states invoke special regulatory features of NR (Solomonson and Barber, 1990) that may lead to an uncoupling between *in vitro* and *in vivo* activity of the enzyme. There are reports of transient increases in NR activity when nitrogen is depleted (Watt *et al.*, 1992). More experiment is needed to elucidate the timing sequence of the NR activity under transient conditions.

From metal/chelation experiments, the chelation capacity was found to be a plausible candidate for controlling the development of red tide. When FeEDTA was added, there were significant increases in both the *in vivo* fluorescence and NR activity. When strong chelator (DTPA) was added in addition to nutrients, there was a significant decrease in *in vivo* fluorescence compared to samples with nutrients enrichment only. Metal treatments showed the lowest *in vivo* fluorescence, indicating that addition of metals into poorly chelated seawater could result in the impairment of physiology due to metal toxicity. When 10 μM DTPA was added in addition to metal, there was a significant increase in fluorescence probably due to the decrease in heavy metal toxicity by chelation. When DTPA was added in excess amounts (100 μM), fluorescence was depressed significantly. To the contrary, the addition of metal or DTPA did not inhibit NR as much as *in vivo* fluorescence. The activity of NR showed the highest value with metal enrichment. It appears that metal enhanced NR activity despite of the improper chelation. This phenomenon needs to be examined further in later experiments.

Timmermans *et al.* (1994) reported that the addition of iron significantly stimulated the NR acitivity in uni-algal cultures and in natural samples. They also showed that even though iron deficiency impairs NR activity, iron depletion did not cease NR activity completely. This matches well with the result from this experiment. Berges *et al.* (1995) reported that NR activity was poorly correlated with changes in cell size or biomass, falling rapidly as the stationary phase was approached. This was comparable to declines in rates of nitrate incorporation and nutrient depletion. It is well known that the uptake rate of nitrogen compounds by algae increases after a period of nitrogen deprivation (Syrett, 1981), and Berges and Harrison (1995a, 1995b) speculated that this could be the adaptation which facilitates the efficient utilization of available nitrogen.

Graneli *et al.* (1986), from metals and chelator bioassays, reported that trace metals and natural chelators are important factors behind shifts in phytoplankton species composition, and Yamochi (1984) suggested that the chelated iron is one of the crucial factors triggering the red tide outbreaks in case of eutrophic waters. The application of yellow loess to the waters with red tide has been common practice in Korea for several years (Bae *et al.*, 1998). However, the effect of yellow loess on the physiology of the red tide organism is poorly understood and its secondary effect, especially on the benthic community of coastal areas, is not clearly elucidated. If the practice of applying yellow loess into red tide plagued areas is repeated every year, the side effect may nullify the beneficial effect by desolating the benthic ecosystem. If the control of trace metal availability can be applied to inhibit the development of red tide in the field, this could reduce the cost and harmful side effect of other red tide control operations.

The effect of nitrate concentration on *in vivo* fluorescence and NR showed a similar timing sequence to the shift-up (Schaechter, 1968) of nitrate uptake using a green alga *Dunaliella tertiolecta* under simulated upwelling conditions (Yang, 1997). The threshold concentration of nitrate for the shift-up of *A. carterae* was approximately 30 μM. This concentration is not unusual in eutrophicated coastal areas where the occurrence of red tide is common. Hersey and Swift (1976) reported that K_s (half-saturation constant) of NR in *A. carterae* was approximately 2.0 μM. About 15 fold difference between the threshold concentration of shift-up and K_s of NR of *A. carterae* is similar for the difference of *D. tertiolecta* with 20 μM (Yang, 1997) and 1.4 μM (Eppley *et al.*, 1969) for threshold concentration of shift-up and K_s of NR, respectively. This could be partly due to the difference in culture conditions but mainly due to the differences in the nature of the parameters.

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