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Response of phytoplankton to nitrogen addition in the Taiwan strait upwelling region: Nitrate reductase and glutamine synthetase activities

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ABSTRACT

This study investigated the activities of two nitrogen assimilation enzymes, nitrate reductase (NR) and glutamine synthetase (GS) in phytoplankton in relation to sample, as well as the nutrient levels and phytoplankton biomass (Chl-*a* concentrations) during an upwelling event in the southern Taiwan Strait during an upwelling period from 6 to 12 July, 2005. The results showed that high NR activity (NRA) was always found with low nitrate and high Chl-*a* concentrations, while GS activity (GSA) exhibited positive correlations with ammonium and Chl-*a* concentration. Both NRA and GSA varied with the time and stage of upwelling: high NRA and GSA were observed initially at the subsurface layers in the early stage of upwelling, accompanied by the consumption of nutrients and the increase of Chl-*a* concentration; and then at the surface with high Chl-*a* concentrations in the middle and late stages of upwelling. Results from *in situ* enzyme bioassays on water samples along the tracing of upwelling track and on board mesocosm experiments on board the ship showed that there was a time-lag between nitrate addition and NRA and GSA, but. However, no time-lag was found between ammonium addition and GSA. The present results indicated that both NRA and GSA reflect the status of ambient nitrogen levels and the assimilation process of the phytoplankton, and could be used as effective parameters for the analysis of the physiological response of the phytoplankton to nitrogen variations during upwelling periods. Measurements of NRA and GSA in the phytoplankton in newly upwelled water appeared to provide ecophysiological indicators of phytoplankton, which will make it possible to trace during the sequence of upwelling events (such as nutrient supplementation) leading to enhanced productivity.

1. Introduction

A coastal upwelling system is described as a conveyer belt of nutrients and carbon (Wilkerson and Dugdale, 1987), which exhibits rapid nutrient turnover rates and high biological production (Botas et al., 1990; Fernández and Bode, 1991; Fernández et al., 1991). Early studies in upwelling regions have shown that a series of increased physiological rates occur along the conveyor (MacIsaac et al., 1985; Wilkerson and Dugdale, 1987; Dugdale and Wilkerson, 1990; Bode et al., 1997). Phytoplankton cells transported to the surface undergo an upward energy shift in the high irradiance and high nutrient surface environment, and rates of nutrient-uptake increase along the upwelling plume associated with an increase in phytoplankton biomass. An optimal environmental window for large cell phytoplankton (e. g. diatoms) has been reported in a previous study (Legendre and Le Fever, 1989). During this window, upwelled phytoplankton must be able to "shift-up" their rate of physiological processes in response to maximize nutrient uptake and growth processes (Hutchings et al., 1995; Kudela and Dugdale, 2000). A hypothesis that environmental shifts resulting from improved conditions induce a suite of molecular and physiological responses that lead to a higher nutrient uptake and growth rate of the phytoplankton has also been postulated (Schaefer et al., 1999). When N deficient cells are exposed to the improved growth conditions, the rate of induction of nitrate metabolism, in particular, may control the dynamics of the phytoplankton bloom (Smith et al., 1992).

Nitrate and ammonium are the two major inorganic nitrogen species that support new production (based on nitrate) and regenerated production (based on ammonium) in coastal upwelling systems (Dugdale and Goering, 1967). It is known that the utilization of nitrogen in phytoplankton cells involves complex processes including membrane transport, assimilation and incorporation of external dissolved nitrogen into biochemical compounds inside the cell via several enzymatic systems (Syreet, 1981; Falkowski, 1983; Dortch and Postel, 1989). Among these enzymes, nitrate reductase (NR; EC 1.6.6.1) and glutamine synthetase (GS; EC 6.3.1.2) are the two important enzymes involved in nitrogen assimilation.

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The former, catalyzing the reduction of nitrate to nitrite, is the first enzyme involved in nitrogen assimilation and controls the process of nitrate metabolism in cells (Touchette and Burkholder, 2001), while the latter, catalyzing the formation of glutamine from ammonium and glutamate in the presence of ATP, plays an important role in regulating nitrogen metabolism, not only as the center for both nitrate and ammonium assimilation, but also as a key enzyme linking carbon to nitrogen metabolism by incorporating inorganic nitrogen into organic nitrogen via GS/glutamate synthase (GOGAT). NR activity (NRA) and GS activity (GSA) have been used as qualitative indicators and/or as quantitative measures of nitrate/ammonium assimilation in phytoplankton (Eppley et al., 1969; Collos and Slawyk, 1977; Berges and Harrison, 1995; El Alaoui et al., 2001), and show high environmental regulation in phytoplankton cells (Smith et al., 1992; Maurin and Gal, 1997). It is reported that nitrate uptake rate and NRA are enhanced under simulated upwelling conditions, and that modulation of NR gene expression and enzyme activity by environment factors affect the time scales of nitrate utilization and bloom formation in the sea (Smith et al., 1992).

The Taiwan Strait, a shallow shelf-channel linking the South China Sea (SCS) with the East China Sea (ECS), is characterized by highly dynamic seasonal and year-round upwelling events (Chen et al., 1982; Xiao, 1988; Hong et al., 1991; Tang et al., 2002; Shang et al., 2005). In the southern Taiwan Strait, phytoplankton blooms and the highest Chl-*a* concentration are generally observed in summer due to coastal upwelling induced by the southwest monsoon (Chen et al., 1992; Zhang et al., 1997; Tang et al., 2002; Shang et al., 2004). Many studies have shown that primary production, phytoplankton biomass, composition and size-fraction structure in the upwelling area vary significantly and rapidly in response to environmental shifts (Hong et al., 1991; Li and Wang., 1991; Yang et al., 1991; Wang et al., 2002), but the response mechanism of phytoplankton to the upwelling events, such as upwelled nutrients, is poorly understood. Nitrogen is regarded as one of the limiting factors to phytoplankton growth in the southern Taiwan Strait during the non-upwelling period (Wang et al., 1997; Zhang et al., 1997). In light of this, nitrogen supplementation and utilization dynamics in phytoplankton cells might control the successional process and phytoplankton bloom formation during the upwelling period.

In the present study, both the activities of two nitrogen assimilation enzymes, NR and GS transferase in phytoplankton samples, and the nutrient levels and phytoplankton biomass (Chl-*a* concentration) were investigated in the southern Taiwan Strait during an upwelling period from 6 to 12 July, 2005. The purpose of this study was to examine the nitrogen assimilation process of the phytoplankton *in situ* so as to obtain a better understanding of the cellular regulatory response to elevated nutrients, and hence provide insight both into the response of phytoplankton to ambient nutrient variation and the bloom formation mechanism.

2. Materials and methods

First, the temperature and salinity were investigated around the southern Taiwan Strait and a low temperature and high salinity center was found around a nearshore station, Stn. B0. Then Stn. B0 was selected for tracing the upwelling event during 6 to 12 July, 2005. Samples from the 5 depths (0, 5, 10, 15 and 25 m) at Stn. B0 were collected using a CTD-rosette system (SeaBird), equipped with 12 Go-Flo bottles (8 l each). NRA and GSA of the water samples as well as concentrations of nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+) and Chl-*a* were measured. To avoid the influence of irradiance, all samples were collected at the same time (around 10:00 am) each day at Stn. B0. The surface water samples were also collected at 16 stations in the southern Taiwan Strait (Fig. 1) and the same parameters were measured as Stn. B0 for mapping distribution.

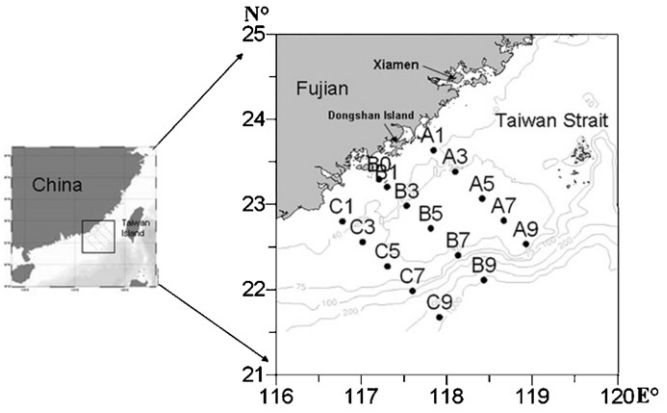

Fig. 1. Location of the study area in the Taiwan Strait.

Besides, a mesocosm culture experiment simulating the upwelling conditions at Stn. B0 was conducted on board in three column bags, the total volume of each bag (with a diameter of 1.0 m and depth of 1.5 m) was 700 l. The seawater was pre-filtrated using a 200 μm filter to remove the large grazers before the experiments were started. Seawater conditions at Stn. B0 were as follows: temperature 22.1 °C, salinity 34.0, Chl-*a* concentration 1.27 μg l^{-1}, and the concentrations of nitrate, phosphate and silicon were 0.70, 0.03 and 6.0 μmol l^{-1}, respectively. Nitrate, phosphate and silicon were added to the experimental bags on 6 July. Three mesocosm cultures were designed: M.1 was the control with no nutrient addition; M. 2 was the low nutrient treatment with the addition of 12 μmol l^{-1} SiO_3–Si (Na_2SiO_3), 0.5 μmol l^{-1} PO_4–P ($NaH_2PO_4 \cdot H_2O$) and 12 μmol l^{-1} NO_3–N ($NaNO_3$); and M. 3 was the high nutrient treatment with the addition of 48 μmol l^{-1} SiO_3–Si, 2 μmol l^{-1} PO_4–P and 48 μmol l^{-1} NO_3–N. The N/P ratio was designed according to the N/P ratio of the surface water of Stn. B0 on 6 July. Study on the seawater used for mesocosm showed that nitrogen and phosphate were both limiting factors for phytoplankton growth and silicon was not a limiting factor (Wang et al., 2008). Samples were collected at 8:00 am each day from 6 to 12 July, and NRA, GSA, NO_3^- and Chl-*a* data were analyzed. Before sampling, the seawater was mixed evenly using an oar and surface seawater was collected from each experimental bag. The mesocosm culture was under nature solar radiation and temperature was about 21–26 °C.

Concentrations of nitrate, nitrite and ammonium were measured immediately according to the procedures of Parsons et al. (1984) after being filtered using cellulose acetate membranes (Whatman, 0.45 μm). Chl-*a* concentration was determined using a fluorescence spectrophotometric method after filtration on GF/F membranes (Whatman) (Yentsch and Menzel, 1963). The fluorimeter was calibrated against a standard made from pure Chl-*a* (Sigma Chemical. Co.). The phytoplankton samples were filtered using cellulose acetate membranes (Whatman, 0.45 μm) for NRA and GSA detection.

NRA was determined according to the methods of Berges and Harrison (1995) and Joseph et al. (1997) with a small modification. Duplicate samples were collected on the cellulose acetate membranes (Whatman, 0.45 μm) and broken up in 2.0 ml extraction buffer with 200 mmol l^{-1} phosphate buffer, pH 7.9, 0.03% (wt/vol) dithiothreitol (DTT), 0.3% (wt/vol) polyvinyl pyrrolidone (PVP), 0.1% Triton X-100 (vol/vol), 5 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA), and 3% (wt/vol) BSA using a sonicator. Homogenates

were centrifugated at 750 g for 5 min at 4 °C. The supernatants were collected for NRA assay. 100 μl of the crude enzyme extract, 100 μl flavin adenine dinucleotide (FAD, 2 mmol l^{-1}), 100 μl nicotinamide adenine dinucleotide (NADH, 0.5 mmol l^{-1}) and 350 μl of 200 mmol l^{-1} phosphate buffer (pH 7.9) were added to a tube and the reaction was initiated by adding 250 μl of 200 mmol l^{-1} KNO_3. To a second tube, the same buffer was added as a time zero control, and the third tube contained the same buffer without the addition of crude enzyme extract as a reagent blank. All tubes were incubated at 23 °C for 45 min and then 2.0 ml of 550 mmol l^{-1} zinc acetate was added to the first and the third tube to stop the reaction. In the second tube, zinc acetate was added immediately after the reaction started. The tubes were centrifuged and the excess NADH was oxidized by adding 20 μl of 125 μmol l^{-1} phenazine methosulphate (PMS). The nitrite produced was measured colorimetrically with sulfanilamide and N-(1-napthyl)-ethylenediamine 2 HCl solutions. The activity was expressed in terms of absolute rates: one unit (U) of enzyme activity meant that 1 μmol of substrate was converted to nitrite per hour per liter seawater.

For GS transferase activity, cell-free extracts were prepared according to the methods of García-Fernández et al. (1997) and Humanes et al. (1995) with a small modification. Duplicate samples were collected on the cellulose acetate membranes (Whatman, 0.45 μm) and broken up in 2.0 ml extraction buffer with 50 mmol l^{-1} Tris-HCl buffer, pH 7.5; 2 mmol l^{-1} DTT; 1 mmol l^{-1} EDTA and 2.5 mmol l^{-1} $MgCl_2$ using a sonicator. In this study, we used an ultrafiltration (Microcon, YM-100) method instead of ultracentrifugation during extraction. Filtrates with molecular weight less than 100 kD were collected and 100 mmol l^{-1} streptomycin (pH 7.0) was added to them to remove pigments. After continuous stirring for 15 min, the filtrate was centrifuged again and filtrates with molecular weight less than 100 kD were collected. All procedures described above were carried out at 4 °C.

GS transferase activity was measured according to Bressler and Ahmed's method (1984). Briefly, to one tube, 100 μl of the crude enzyme extract, 960 μl of 1.0 mol l^{-1} imidazole–HCl buffer, 600 μl of 0.1 mol l^{-1} glutamine (pH 7.3), 60 μl of 0.01 mol l^{-1} $MnCl_2$, 80 μl of 0.01 mol l^{-1} ADP (pH 7.3), 40 μl of 1.0 mol l^{-1} K-arsenate and 60 μl of 2.0 mol l^{-1} hydroxylamine were added. To the second tube, the same solutions were added as a time zero control. The third tube contained the same solutions but without the addition of crude enzyme extract as a reagent blank. The reactions were conducted at 37 °C for 30 min and stopped by the addition of 2.0 ml of a mixture of 4.0 ml of 10% $FeCl_3$, 1.0 ml of 24% trichloroacetic acid, 0.5 ml of 6 mol l^{-1} HCl and 6.5 ml Milli-Q water. In the second tube, the reaction was stopped immediately after the solution was added. After stopping the reaction, the absorbance was measured at 540 nm. For GS activity, a unit (U) was determined when 1 μmol of product was formed per hour per liter seawater.

3. Results

3.1. Distribution of NRA and GSA in the southern Taiwan Strait

Contours of seawater surface temperature (SST) and salinity of the southern Taiwan Strait as well as the wind data during the study period are shown in Figs. 2a, b and 3. The wind data on 8 July, 2005 (Fig. 2) showed that the southwest monsoon was strong during the survey period which caused the upwelling of this area. Two regions with low SST and high salinity were observed: one was around Stn. B0 with low SST and high salinity. The lowest SST (22.1 °C) and the highest salinity (34.0) on Stn. B0 exclude the possibility of coastal freshwater advection

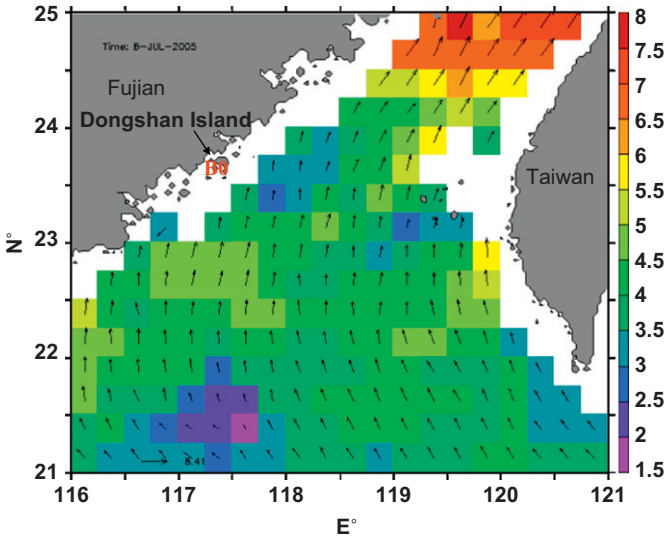

Fig. 2. Sea surface winds on 8 July, 2005 in the Taiwan Strait.

indicating that Stn. B0 was the center of the coastal upwelling; and the other was located to the southwest of Stn. A1. The high SST (30.2 °C) and low salinity (27.3) was found around Stn. C3 which indicated the advection of coastal water and here the Han River are the mainly advection sources.

The distribution of nutrients, Chl-*a* concentration, NRA and GSA in the surface seawater are shown in Fig. 3 c–h. During the survey period, high Chl-*a* concentrations were found at Stn. A3, A7, B0 and C1 with the highest value at Stn. B0, up to 2.7 μg l^{-1} (Fig. 3c), whereas high concentrations of nitrate and nitrite both appeared at Stn. C3 with the highest values of 10.2 μmol l^{-1} for nitrate and 0.4 μmol l^{-1} for nitrite, respectively (Fig. 3d, e). The highest nitrate and nitrite on Stn. C3 were also indicative of the advection of coastal water. There were two high value areas of ammonium, one was around Stn. A3 and B3, and the other was around Stn. A9 and B9. The highest value was found at Stn. B3, up to 1.6 μmol l^{-1} (Fig. 3f).

NRAs at Stn. A1, A3, B0, B1 and C1 were higher than those at other stations, and the highest NRA was found around Stn. B0, up to 0.341U (Fig. 3g). This coincided with the highest Chl-*a* concentration at the same location, indicating that NRA was dependent on Chl-*a* concentration, but no relationship was found between NRA and nitrate concentrations. High GSAs were observed in two areas, one was along the coastal region, Stn. A1, B1 and C1 with high Chl-*a* and/or ammonium concentrations with the highest value of 1.866U at Stn. B1, and the other was around Stn. B9 with high ammonium concentration (Fig. 3h).

3.2. Variations of NRA and GSA during the upwelling

Variations of seawater temperature (ST) and salinity in the euphotic layer (<25 m) at Stn. B0 from 6 July to 12 July are shown in Fig. 4a, b. The upwelling at Stn. B0 exhibited a pulse input: an input of cold water with low temperature and high salinity was observed on 6 July at the surface, following a brief relaxation; and then a weak upwelling occurred on 8 July with another relaxation on 10 July. At the end of the tracing, there was a strong input of cold water which reached the surface on 12 July.

Results of NRA, GSA and concentrations of Chl-*a*, nitrate and ammonium during the upwelling period are shown in Fig. 4c–g. In the first two days (6 and 7 July) Chl-*a* concentrations were low in all layers. From 8 July, the Chl-*a* concentration of the upper-layer (≤ 10 m) increased rapidly and reached its highest concentration

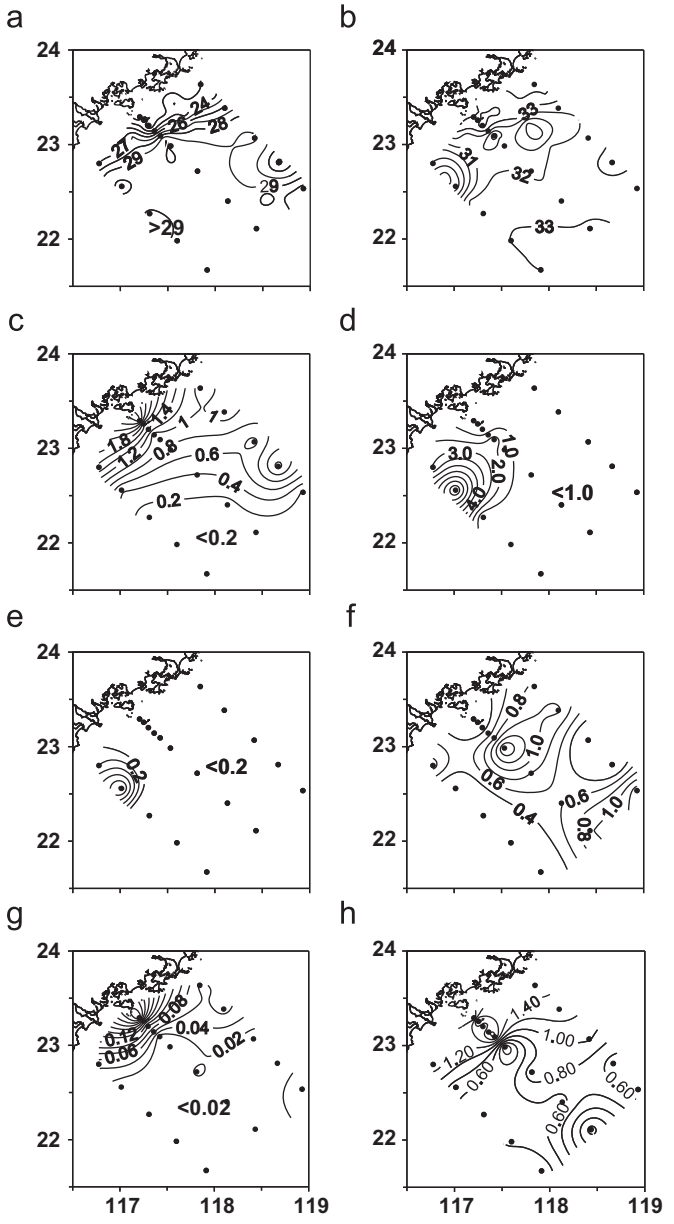

Fig. 3. Distribution of NRA, GSA, nutrient concentrations and other variables at the sampling stations. (a) Temperature (°C); (b) Salinity; (c) Chl-*a* ($\mu g\,l^{-1}$); (d) Nitrate ($\mu mol\,l^{-1}$); (e) Nitrite ($\mu mol\,l^{-1}$); (f) Ammonium ($\mu mol\,l^{-1}$); (g) NRA (U); (h) GSA (U).

up to 4.1 $\mu g\,l^{-1}$ at the surface on 10 July, before decreasing gradually on 11 and 12 July and reaching a low concentration of about 2.6 $\mu g\,l^{-1}$ on 12 July. On the other hand, the Chl-*a* concentration in the 10 and 15 m layers decreased remarkably to 0.8 $\mu g\,l^{-1}$ on 8 July, and maintained this concentration until 10 July (Fig. 4c).

The concentrations of nitrate were high at all layers in the first two days except for a relatively low nitrate concentration (0.6 $\mu mol\,l^{-1}$) in the 10 m layer on 7 July (Fig. 4d). Nitrate concentration decreased sharply in the surface layer from 7 July and reached its lowest on 8 July, and then a low nitrate concentration (less than 0.4 $\mu mol\,l^{-1}$) was maintained over the next several days. Meanwhile nitrate concentrations in the 5 m layer reached their highest on 8 July and declined to their lowest on 9 July. Concentrations of nitrate in other layers varied a little but maintained high levels. Ammonium concentration at the surface was low from 6 July to 11 July with the lowest on 9 and 10 July accompanied by the highest Chl-*a* concentration, then increased to about 1.0 $\mu mol\,l^{-1}$ on 12 July. A high ammonium concentration (2.18 $\mu mol\,l^{-1}$) was observed in the 5 m layer on 6 and 7 July, and then declined to a low level (Fig. 4e). Ammonium concentration in the 10 m layer was low during the tracing period except for a relatively high level (0.8 $\mu mol\,l^{-1}$) on 9 July. Ammonium concentrations at other layers remained relatively constant.

High NRA was first observed in the 10 m layer on 6 July, then at the surface and in the 5 m layer on 10 July with the highest value of 0.341U at the surface layer, and maintained a high value (0.277U) until 12 July (Fig. 4f). The NRAs in other layers varied a little but were maintained at low levels during the tracing period. The high NRA was always located in the low nitrate concentration area, indicating that a negative relationship existed between nitrate concentration and NRA. High GSA was observed at 5 m on 6 July with a high concentration of ammonium (2.18 $\mu mol\,l^{-1}$), and at the surface layer on 9 July (0.804U) with a high concentration of Chl-*a*, then at the upper-layer on 11 and 12 July accompanied by a high concentration of ammonium and Chl-*a*. No GSA was detected at the surface or in the 10 m layers on 6, 7 and 8 July, and low GSAs were observed at the surface and in the 5 m layers on 10 July (Fig. 4g).

3.3. The results of mesocosm experiments

The results of the mesocosm experiments are shown in Fig. 5. Nitrate was exhausted within two days in M. 2 with the addition of 12 $\mu mol\,l^{-1}$ nitrate and three days in M. 3 with the addition of 48 $\mu mol\,l^{-1}$ nitrate (Fig. 5a). There was a two-day time lag between the addition of nitrate and the highest concentration of Chl-*a*. In M. 2 and M. 3, Chl-*a* increased on the second day and reached its highest values on the third day (15.0 $\mu g\,l^{-1}$ in M. 2 and 49.6 $\mu g\,l^{-1}$ in M. 3) (Fig. 5b). The concentrations of nitrate and Chl-*a* in M. 1 varied little.

NRA in M. 2 and M. 3 reached a peak on the third and fourth day after nitrate was added, up to 1.55 and 14.54U, respectively (Fig. 5c). There was a two to three- day time-lag between nitrate addition and the highest value of NRA depending on the nitrate concentration. This time-lag was also found between nitrate addition and the NRA/Chl-*a* increase both in M. 2 and M. 3 (Fig. 5d). GSA was low during the first two days after nitrate was added, and then increased gradually as the nitrate was consumed by the phytoplankton (Fig. 5e). Moreover, GSA maintained a high level even when the Chl-*a* concentration decreased to a low level, which was consistent with the *in situ* upwelling tracing results. The GSA/Chl-*a* decreased to a low value on the fourth day after increasing slightly in the first two days, then increased remarkably during the following days (Fig. 5f).

4. Discussion

4.1. Influence of environmental factors on NRA and GSA

Many studies both on phytoplankton cultures and field samples have shown that NR synthesis is induced by ambient nitrate, elevated by light and repressed by ammonium. Availability of ambient nitrate and illumination directly influence *in vivo* NRA (Eppley et al., 1969; Packard et al., 1971; Hipkin andSyrett, 1977; Blasco et al., 1984; Smith et al., 1992). However, in our *in situ* investigation, no correlation was found during the mapping survey in the southern Taiwan Strait (Fig. 6a).On the contrary, a negative relationship between NRA and nitrate concentration was observed during the upwelling tracing at Stn. Bo (Fig. 7a: $n=16$, $R^2=0.41$, $P<0.01$) and the reason for this is not clear at present. NRA has been postulated to represent the nitrate reduction

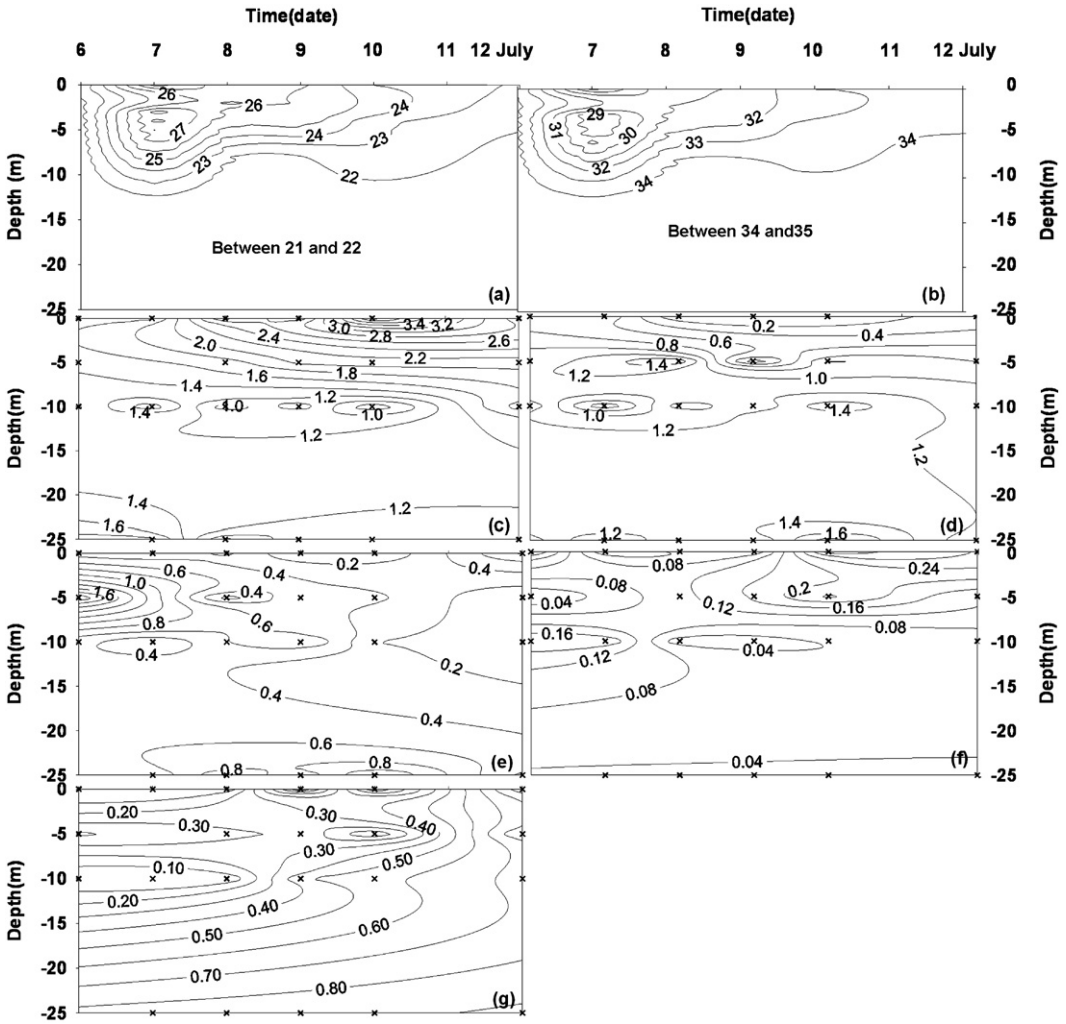

Fig. 4. Variations of NRA, GSA and nutrient concentrations at Stn. B0 from 6 to 12 July, 2005 (sampling stations are marked in the figure). (a) Temperature (°C); (b) Salinity; (c) Chl-*a* (μg l^{-1}); (d) Nitrate (μmol l^{-1}); (e) Ammonium (μmol l^{-1}); (f) NRA (U); (g) GSA (U).

capacity, and its level reflects the effects of the environment before the sampling time (Blasco et al. 1984). In a diatom species, *Skeletonema costatum*, NR biosynthesis involves a complex process at the molecular level, including gene transcription, RNA translation, and post translational modulation of enzyme activity, which results in a temporal lag between ambient nitrate concentration and NRA (Smith et al., 1992). Therefore, NRA may reflect the ambient nitrate status previously rather then the present sampling time nitrate concentration. On the other hand, some studies indicate that NR is controlled by intracellular nitrate concentration rather than ambient nitrate concentration (Collos and Slawyk, 1976; 1977; Dortch et al., 1979; Joseph et al., 1997), and that phytoplankton can use the internal nitrate pool to synthesize macromolecular components even after the depletion of external nitrate. Another study also demonstrates that NRA is related to the nitrate taken up inside the cells instead of the nitrate in the medium (Joseph and Villareal, 1998). This internal nitrate pool has been found in many phytoplankton species, especially in some diatom species (e.g., *S. costatum*) (Dortch et al., 1979), and is suggested to provide a buffer against fluctuation of external nutrient concentration (Joseph et al., 1997). The previous study on nitrate storage in phytoplankton cells during the early phases of upwelling in coastal stations off northern Spain between 1990 and 1994 indicates that nitrate storage by phytoplankton cells is characteristic of early phases of upwelling and is linked to patterns of carbon fixation (Bode et al., 1997). Diatoms are often the dominant species when external nitrogen inputs to the euphotic zone occur (Dugdale and Goering, 1967; Malone, 1980). Our results are in agreement with these findings. During the upwelling period in the southern Taiwan Strait, phytoplankton composition was dominated by the diatom species *S. costatum* and *Asterionellopsis glaciali* (data not shown). These species might have an internal nitrate pool, which resulted in the time-lag between nitrate addition and NRA in the phytoplankton. The internal nitrate pool could maintain the high NRA of cells even at low nitrate concentration in the medium.

Nevertheless, factors other than nitrate or ammonium might affect the uptake of nitrate and subsequent specific NRA. High concentrations of Chl-*a* also could result in high NRA (Berges, 1997; Touchette and Burkholder, 2001). In our study, we also found a good correlation between phytoplankton biomass (Chl-*a* concentration) and NRA (Fig. 6b: $n=18$, $R^2=0.69$, $P<0.01$; Fig. 7b: $n=22$, $R^2=0.31$, $P<0.01$). Moreover, we found an interesting phenomenon that higher NRA was detected in those phytoplankton samples collected in the daytime and lower in those collected at night, and hypothesizing that NRA here might also be regulated by light. Without a detection of radiation we couldn't give a quantitative statistical analysis. However, the other researchers have also mentioned that NRA is expressed most strongly in the morning

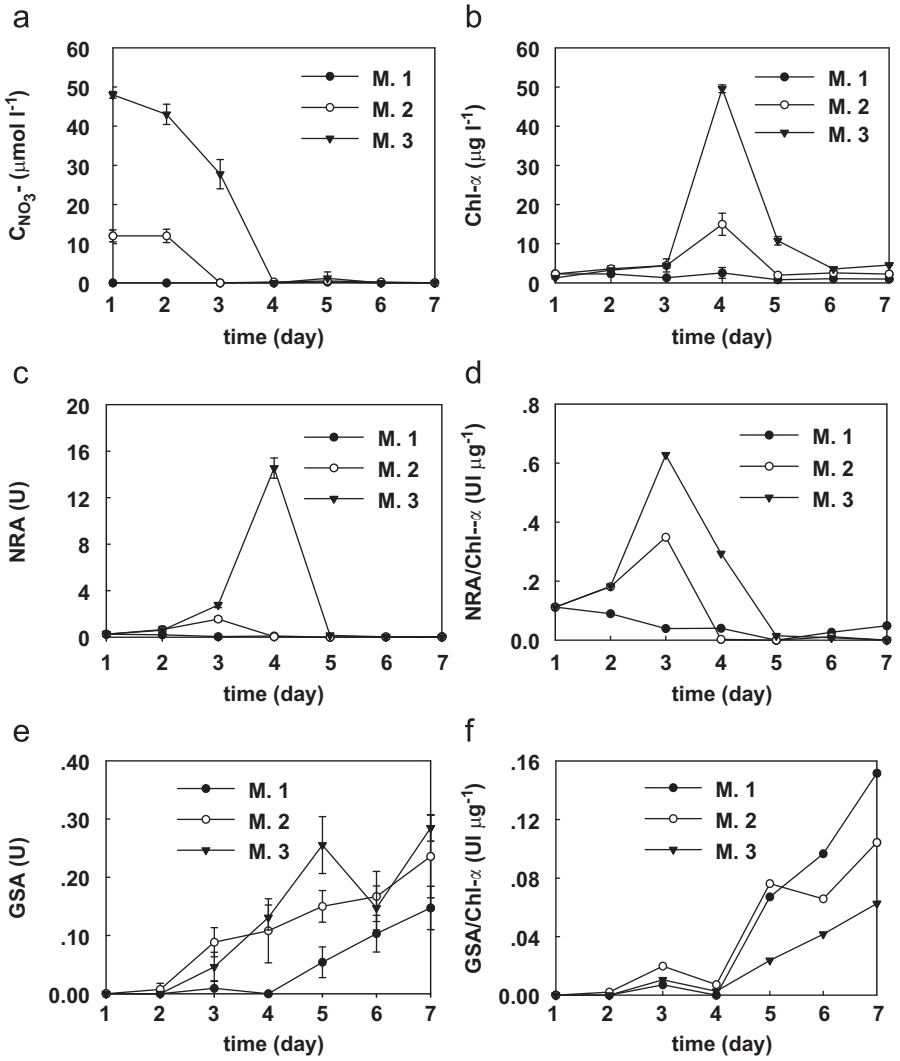

Fig. 5. Variations of nitrate concentration, NRA, GSA and other variables in mesocosm culture experiments. Variables are expressed as the mean value ($n=2$), and error bars ($\pm$SE) are given. M. 1=control, M. 2=low nutrient treatment, and M. 3=high nutrient treatment mesocosm. (a) Nitrate (μmol l^{-1}); (b) Chl-*a* (μg l^{-1}); (c) NRA (U); (d) NRA/Chl-*a* (U μg^{-1}); (e) GSA (U); (f) GSA/Chl-*a* (U μg^{-1}).

in their study (Berges, 1997; Touchette and Burkholder, 2001). Another study also demonstrates that both NRA and nitrate uptake rate exhibit a positive response to light irradiation in the Peru upwelling region (Blasco et al., 1984).

GS is a highly regulated enzyme whose synthesis and activity are regulated by environmental factors (Maurin and Gal, 1997). However, the effects caused by environmental factors vary significantly among phytoplankton species. For example, GSA is enhanced by nitrogen starvation and inactivated by darkness in many phytoplankton species, but this does not occur in *Prochlorococcus* (El Alaoui et al., 2001; 2003). The relationship between ammonium and GSA is complex: in *Emiliania luxleyi*, GSA is inhibited by the addition of ammonium, depending on the physiological status of the phytoplankton cells (Maurin and Gal, 1997). *In vivo* ammonium concentration is inversely related to the GSA in *Dunaliella primolecta* in the culture medium, but *in vitro* it causes no inhibitory effect on GSA (Seguineau et al., 1989). In our study, GSA was influenced by multiple environmental factors, such as nitrate concentration, ammonium, light and Chl-*a*. Our field results showed a positive relationship between GSA and the concentrations of ammonium and Chl-*a* in the mapping survey (Fig. 6d: $n=16$, $R^2=0.46$, $P<0.01$; Fig. 6e: $n=18$, $R^2=0.64$, $P<0.01$). The high GSA observed along the transect of Stn. A1, B1 and C1 might be caused by the high concentrations of Chl-*a* and ammonium, while the high GSA around Stn. B9 might be induced by the high concentration of ammonium. Around Stn. B0, the concentration of Chl-*a* was high and the GSA was low, while high NRA but low concentration of ammonium were observed. In this area, nitrate might be the main nitrogenous source for the phytoplankton, which depressed the expression of GSA. In our mesocosm experiment, we also observed that GSA was low when nitrate was added, indicating that GSA was depressed by nitrate.

Although high GSA was found in those stations with high ammonium concentration (Fig. 4e, g), no positive relationship was found between GSA and the concentrations of ammonium and Chl-*a* during the upwelling tracing period (Fig. 7d: $P>0.05$; Fig. 7e). The upwelling is a dynamic process, and GSA was not only affected by ammonium concentration but also by other environmental factors, which might result in an uncoupling between ammonium concentration and GSA in this upwelling system.

4.2. Physiological response of phytoplankton to upwelling

It is known that nutrients supplied by upwelling play important roles in regulating phytoplankton growth and population succession (Hong et al., 1991; Mann and Lazier, 1996; Pennington

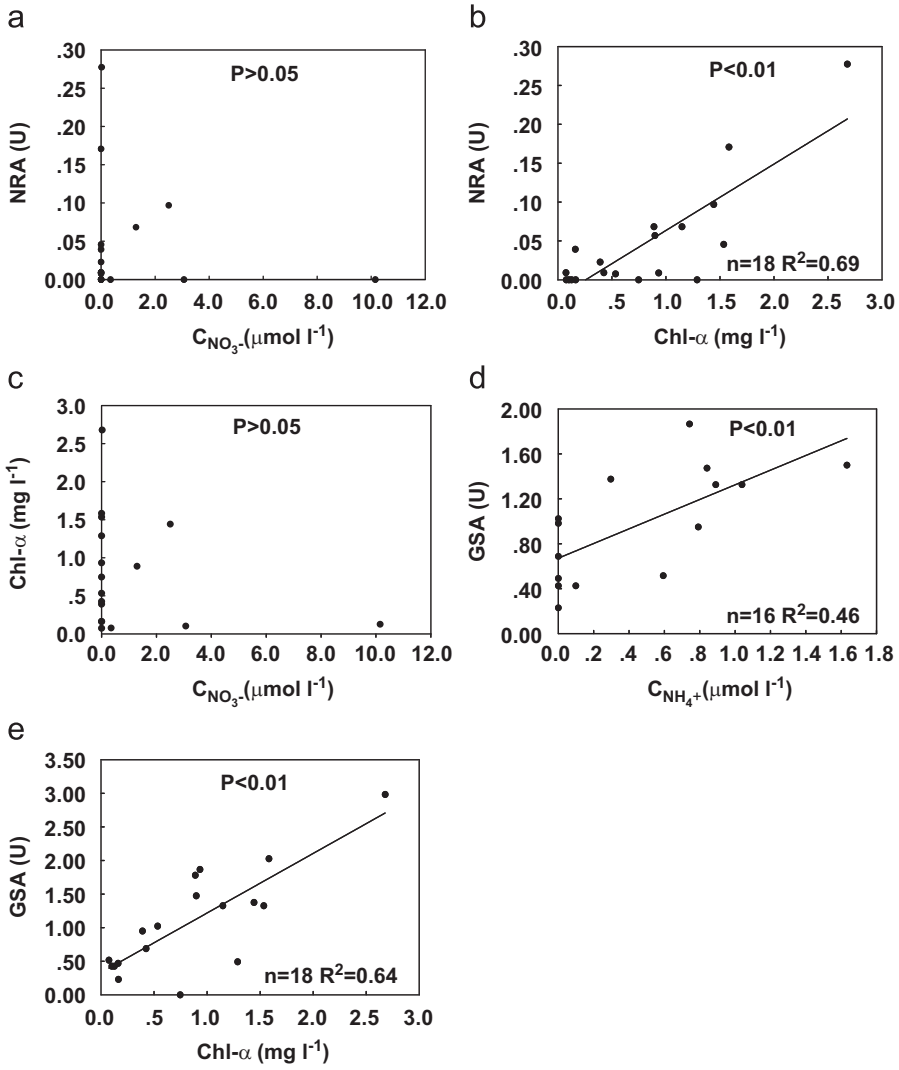

Fig. 6. Relationship between NRA and nitrate concentration (a), NRA and Chl-*a* concentration (b), Chl-*a* concentration and nitrate concentration (c), GSA and ammonium concentration (d), GSA and Chl-*a* concentration (e) in the southern Taiwan Strait.

and Chavez, 2000; Shang et al., 2005). Many studies are devoted to gaining an understanding of nutrient uptake by phytoplankton and the physiological responses of the phytoplankton to nutrient addition during the upwelling period (Li and Wang, 1991; Yang et al., 1991; Wang et al., 2002). NRA has been regarded as representing the velocity of nitrate assimilation and so is used to estimate the assimilation of nitrate *in vivo*, and a positive relationship between nitrate uptake and NRA is observed in previous studies (Blasco et al., 1984; Berges and Harrison, 1995). Although GSA does not give a quantitative estimation of the ammonium assimilated into the cells, it does reflect variations in the ammonium assimilating capacity of the enzyme (Maurin and Gal, 1997). However, little is known about the mechanism by which the phytoplankton responds to nutrient addition and variation during the upwelling period. Blasco et al. (1984) in their investigation of the relationship between NRA and nitrate uptake in phytoplankton in the Peru upwelling region found that nitrate uptake and nitrate reduction are significantly correlated, but they are two different processes, and the former system responds faster to ambient nitrate variation than the latter. A study on molecular and physiological responses of a diatom species (*S. costatum*) to variable levels of irradiance and nitrogen in simulated upwelling conditions shows that nitrate addition induces NR gene expression and both enhance the synthesis of enzyme and activity modulation of the existing NR protein and the cellular changes in laboratory cultures are qualitatively similar to the responses of natural phytoplankton assemblages to the improved growth conditions characteristic of coastal upwelling (Smith et al., 1992). In our study, high NRA was first detected at the 10 m layer on 6 July, then it moved up to the surface layer gradually with time, and reached its highest value on 10 July. High GSA was found at the 5 m layer on 6 July and the surface layer on 9 July. These results suggested that nutrients supplemented by upwelling induced NR and GS synthesis in phytoplankton cells and enhanced NRA and GSA during the upwelling of deep seawater to the upper layer. Thus, variations of NRA and GSA could be used as potential indicators of the nutrient assimilation process and the physiological response of phytoplankton to nutrient enrichment during the upwelling period.

Many previous studies report that the temporal and spatial extent of phytoplankton bloom formation depend on the temporal lag in physiological acclimation of the phytoplankton population to improved growth conditions (Collos, 1986; Zimmerman et al., 1987; Smith et al., 1992). A time-lag of phytoplankton response to the addition of nutrients is observed both in the field and laboratory culture (Smith et al. 1992). In this study, we also found this phenomenon during the upwelling tracing period: a time-lag was observed between nitrate

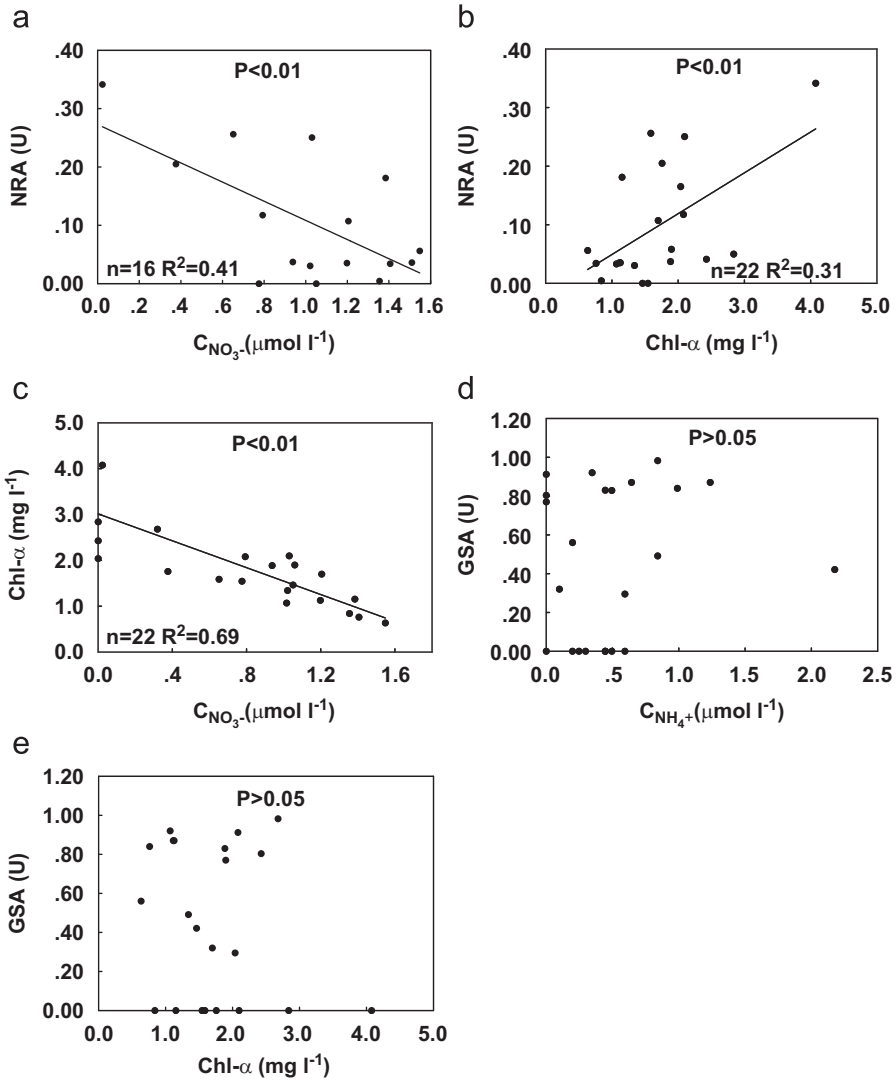

Fig. 7. Relationship between NRA and nitrate concentration (a), NRA and Chl-*a* concentration (b), Chl-*a* concentration and nitrate concentration (c), GSA and ammonium concentration (d), GSA and Chl-*a* concentration (e) during the upwelling tracing.

supplementation and phytoplankton biomass (Chl-*a*), and Chl-*a* concentration exhibited a negative relationship to nitrate concentration (Fig. 7c: $n=22$, $R^2=0.69$, $P<0.01$) and a positive relationship to NRA (Fig. 7b: $n=22$, $R^2=0.31$, $P<0.01$). The mesocosm results also showed that there was a two-day time-lag between nitrate addition and the expression of NR, and between nitrate addition and high Chl-*a* concentration. In phytoplankton cells nitrate assimilation involves a complex process: nitrate taken up into the cells is reduced to nitrite and ammonium sequentially by NR and nitrite reductase (NiR), ammonium is further transformed into organic nitrogen by the action of GS through GS/GOGAT, and finally macromolecular components (e.g. Chl-*a*) related to cell growth are synthesized, which results in the time-lag between nitrate addition and phytoplankton growth, and the negative relationship between high Chl-*a* concentration and nitrate concentration. Laboratory studies of unialgal cultures subjected to N starvation suggest that the temporal response to N resupply results in an uncoupling between N assimilation and cell division and leads to variable lags in the accumulation of cellular metabolites (Smith et al. 1992). Therefore, modulation of the expression of NR and GS and enzyme activity by environmental factors may influence the temporal lag between nitrate utilization and bloom formation in the upwelling.

In this study, high GSA was observed with high ammonium concentration in the 5 m layer on 6 July, indicating that ammonium might enhance the GSA of phytoplankton samples. However, high GSA was also found in the surface layer after nitrate was consumed on 11 and 12 July. The mesocosm results also showed that GSA increased alongside the decrease of nitrate concentration and reached its highest level when nitrate was exhausted (Fig. 5e, f). Previous studies also report that nitrogen starvation enhanced GSA in many phytoplankton species (Seguineau et al., 1989; Maurin and Gal, 1997). Maurin and Gal (1997) note that GSA increases by a factor of 2 after 24 h of nitrogen starvation when nitrogen becomes limiting. This contradiction might be caused by the differing assimilation pathways of different nitrogen sources. Ammonium absorbed to the phytoplankton cells could be transformed directly to amino acid by GS through GS/GOGAT, no time-lag was found between ammonium addition and GSA, and they exhibited a positively correlated relationship. However, nitrate absorbed by the phytoplankton cells must be sequentially reduced to nitrite and ammonium with the help of NR and NiR, then transformed to amino acid by GS, which results in the uncoupling between nitrate

concentration and GSA. Thus, analysis of *in situ* GSA as well as nitrate and ammonium concentrations will provide useful information concerning the nutritional status of phytoplankton and their response mechanism to different nitrogen supplements.

5. Conclusion

Our results indicate that NRA and GSA are influenced by ambient nitrogen concentrations and phytoplankton biomass (Chl-*a*). Temporal and spatial variations of NRA and GSA provided useful indicators of the nutritional status of the phytoplankton and the stage of the upwelling event. The assimilation process of nitrate inside phytoplankton cells resulted in the time-lag between nitrate uptaken and NRA, and between nitrate addition and phytoplankton growth (Chl-*a* concentrations). Clearly the simultaneous measurement of NRA and GSA reported in this paper is an important way to enhance the understanding of how phytoplankton responds to periodic nutrient supplementation within an upwelling system. By measuring the NRA and GSA in the phytoplankton of newly upwelled water, it should be possible to trace the sequence of events leading to enhanced productivity.

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