Plankton Biol. Ecol. 45 (2): 117–128, 1998

plankton
biology & ecology

Relationships between biochemical composition of *Gymnodinium mikimotoi* and environmental conditions during a red tide

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Received 28 February 1997; accepted 1 December 1997

Abstract: A red tide of *Gymnodinium mikimotoi* occurred in Uchinomi Bay, the Seto Inland Sea, Japan, in late September 1993. Water samples were collected at depths of every other meter from the surface to the bottom on 21 and 27 September to determine the contents of carbon, nitrogen, phosphorus, chlorophyll *a* and nucleotides in *G. mikimotoi* cells. The nutrient concentrations, temperature, salinity and dissolved oxygen concentrations of seawater were also determined. On 21 September, the concentrations of biochemical components in *G. mikimotoi* in the bottom waters were generally higher than those in the surface waters. This phenomenon was probably due to the fact that cells in the surface and bottom waters were in different growth stages. Through the nutrient spike experiment conducted on 21 September, nitrogen was found to be the basic limiting nutrient for *G. mikimotoi* growth.

Key words: Red Tide, dinoflagellate, *Gymnodinium*, biochemical components, nutrients

Introduction

After the first major outbreak of a red tide in the Seto Inland Sea (Tokuyama Bay, Japan) in 1957, the frequency of red tide occurrence has increased in proportion to the degree of industrial development in this region (Okaichi 1989). Many soy sauce industries have been established along the coast of Uchinomi Bay on Shodo Island, located in the eastern part of the Seto Inland Sea, and red tides have frequently occurred in this bay. In September 1993, a red tide of *Gymnodinium mikimotoi* occurred in this area and some fish mortality occurred, probably because of low oxygen concentrations in the water. Red tides of this species have occurred frequently in the coastal waters of Japan, especially in the south-western part, and have caused severe damage to fish mariculture since 1965 (Takayama & Adachi 1984; Iizuka et al. 1989).

Attempts to explain the mechanism of red tide formation have frequently considered the importance of factors associated with terrestrial run off, such as nitrogenous and phosphorous nutrients, organic substances, trace metals and some physical oceanographic conditions (Okaichi 1975; Pakash 1975; Anderson & Wall 1978, Provasoli 1979; Anderson et al. 1983;

Frank & Anderson 1992). To explain the formation mechanism of *G. mikimotoi* red tides, studies on the ecological and physiological characteristics of *G. mikimotoi* have been carried out by a number of authors (e.g. Iizuka & Mine 1983; Iizuka et al. 1989; Honjo 1994; Yamaguchi 1994). Honjo et al. (1990) have reported that exponential growth occurred during both spring and autumn in Japanese coastal waters. Furthering this research, the enhancement of growth by anoxic bottom water or low oxygen concentrations in bottom water was discussed (Honjo 1994). In a study on phytoplankton growth under laboratory conditions, Yamaguchi (1994) showed that the highest growth rate of this organism is obtained at a salinity and temperature combination of 25 psu and 25°C, respectively. Since such low salinity regimes in the field are commonly induced by heavy rainfall, one factor contributing to blooms of this organism has been suggested to be the marked decrease in the salinity of the surface layer induced by rainfall (Yamaguchi 1994).

Although several studies have been conducted to explain the mechanism of red tide outbreaks, the mechanism is still not well understood. Studies on the biochemical composition of *G. mikimotoi* may be another way to clarify the outbreak mechanism of red tides. Since research on the biochemical composition of this organism has only rarely been done (e.g. Yamaguchi 1992, 1994), the present study has concentrated on relating the biochemical composition of this organism to changes in environmental conditions during a red tide outbreak. This study should provide additional information helpful to understanding the cause of outbreaks of *G. mikimotoi* red tides.

Materials and Methods

Field Study

Sampling and site description

Red tide samples were collected from Stns 1, 2 and 3 in Uchinomi Bay, the Seto Inland Sea, Japan. Stations were ca. 400 m apart, with bottom depths of 7 m (Stn 1), 14 m (Stn 2), and 14 m (Stn 3), respectively (Fig. 1). For chemical analyses, samples were collected on 21 September, 1993 at depths of 0, 2, 4 and 6 m at Stn 1 and at depths of 0, 2, 4, 6, 8, 10 and 12 m at Stns 2 and 3. The samples were also collected again at Stn 3 on 27 September. Water samples were collected from discrete depths with a Van-Dorn bottle sampler. The physicochemical characteristics of the water such as salinity, temperature and dissolved oxygen con-

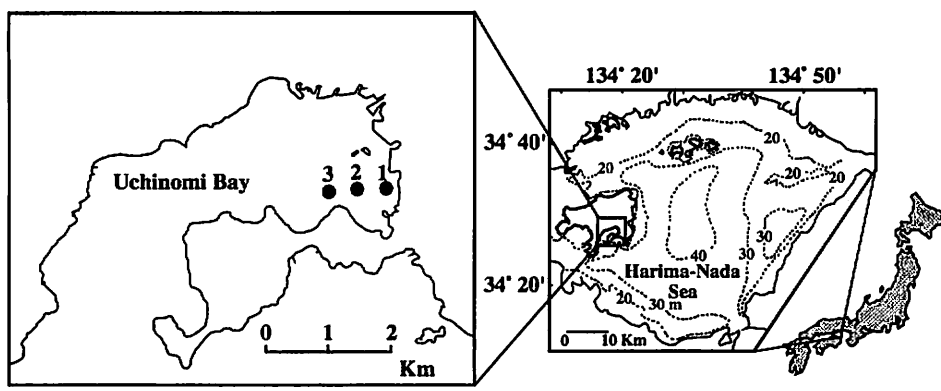

Fig. 1. Map of Shodo Island and Uchinomi Bay, with designated sampling stations.

centration were also determined. Cell numbers of *Gymnodinium mikimotoi* were counted directly under a light microscope.

Analytical procedures

Cellular carbon, nitrogen and phosphorus analysis.—One-hundred milliliter aliquots of water samples were filtered through Whatman GF/C filters (freed of organic matter by ignition at 450°C for 2 h) and the filters were stored at −80°C till analysis. Filters were freeze-dried below −20°C, and carbon and nitrogen content was measured by a Yanaco MT-3 CHN Analyzer. Total cellular phosphorus content was determined by the methods of Anderson et al. (1985).

Pigment (chlorophyll a*) analysis.*—Fifty milliliter aliquots of water samples were filtered through Whatman GF/C filters and pigments were extracted from the filters with 3-ml dimethylformamide (DMF). Pigments were analyzed by an HPLC system (Jusco 880-PU intelligent HPLC pumps, Jusco 870-UV intelligent UV/VIS detector, using a wavelength of 440 nm). The separation column was a Tosoh TSK-GEL ODS 80Tm analytical column (250×4.6 mm ID). The elution profile was as follows: a 10-min concave gradient from 50% buffer A to 100% buffer B; 30-min isocratic elution with 100% buffer B where buffer A was 70% methanol containing 25.5-mM tetrabutyl ammonium acetate and buffer B ethylacetate containing 80% methanol (v/v). The flow rate was 0.8 ml min^{-1}. The column temperature was kept at 35±1°C.

Nucleotide and related compounds analysis.—One-hundred milliliter aliquots of water samples were first filtered through Whatman GF/C filters. Immediately after filtration, the filters were immersed in boiling TRIS buffer (0.025 M, pH 7.75) at 100°C for 5 min to inactivate all enzymes. The samples so extracted were analyzed by an HPLC system (Meksumpun et al. 1993a).

The adenylate energy charge (EC) was calculated after Atkinson (1968) as follows: EC=(ATP+1/2ADP)/(ATP+ADP+AMP).

Nutrient analysis.—Samples for nutrient measurements were kept frozen at −80°C and analyzed by a Technicon autoanalyzer II (Parsons et al. 1984) within one week of sampling.

Laboratory Experiment

Red-tide water collected from the surface at Stn 3 on 21 September was inserted into two 10-liter glass carboys. To one of the two glass carboys K_2HPO_4 and $NaNO_3$, 5 and 30 mg, respectively, was added. The other carboy was kept as a control with no addition of nutrients. The carboys were incubated at 21±1°C. Illumination was provided by cool-white fluorescent lamps at an irradiance level of approximately 80 $\mu E\, m^{-2}\, s^{-1}$ with a 14 : 10 LD cycle. Subsamples were taken every day for 4 d. Cellular contents of chlorophyll *a* (Chl *a*), carbon (C), nitrogen (N), phosphorus (P), amino acids and nucleotides were analyzed. Three replicates were generally analyzed for each sample except for nucleotides, where a single analysis on each sample was conducted.

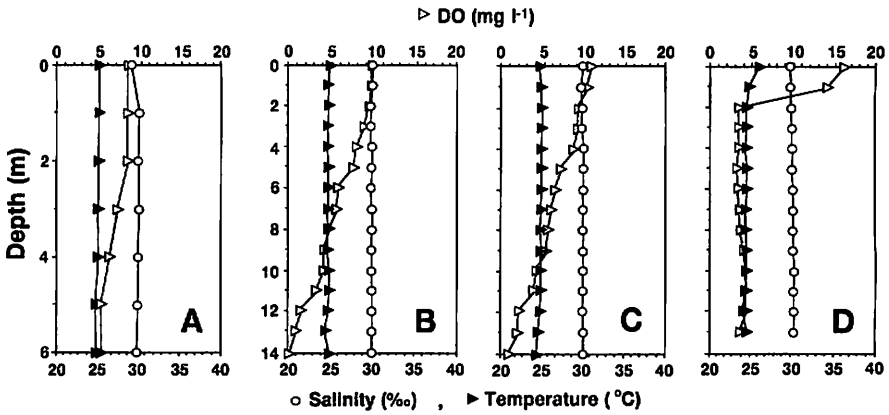

Fig. 2. Vertical profiles of dissolved oxygen, temperature and salinity at Stns 1 (A), 2 (B) and 3 (C) on 21 September 1993 compared to the profiles at Stn 3 on 27 September (D).

Results

Field Study

The vertical profiles of nutrient concentrations, temperature, salinity and dissolved oxygen concentrations are shown in Figs 2 and 3. Dissolved oxygen (DO) concentrations in the surface water (0 m) at Stn 3 on 21 and 27 September were higher than 10 and 16 mgO_2l^{-1}, respectively, while the DO concentrations in the bottom water (12 m) at this station were lower than 3 and 5 mgO_2l^{-1}, respectively. A thermocline and/or pycnocline was not observed at any station (Fig. 2). The concentrations of PO_4^{3-}-P, $NO_3^-+NO_2^-$-N and NH_4^+-N in the bottom water at Stns 2 and 3 on September 21 were considerably higher than that in the surface water (0 m) at the same station (Fig. 3). At depths of 0–2 m at all sampling stations on 21 September, $NO_3^-+NO_2^-$-N was undetectable.

At Stn 3 on 27 September, the concentration of PO_4^{3-}-P in the surface water (0 m) was higher than that in the lower water layers (2–12 m). However, the concentrations of $NO_3^-+NO_2^-$-N in the bottom water (8–12 m) (5.5 μM) was higher than that in the surface water (0.3 μM).

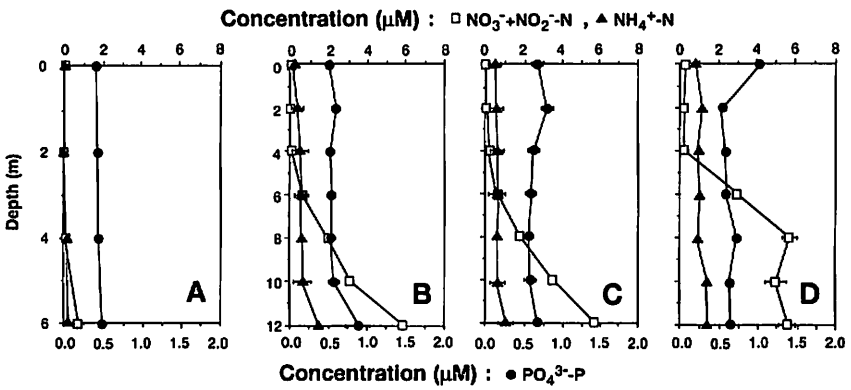

Fig. 3. Vertical profiles of nutrient concentrations at Stns 1 (A), 2 (B) and 3 (C) on 21 September 1993 compared to the profiles at Stn 3 on 27 September (D).

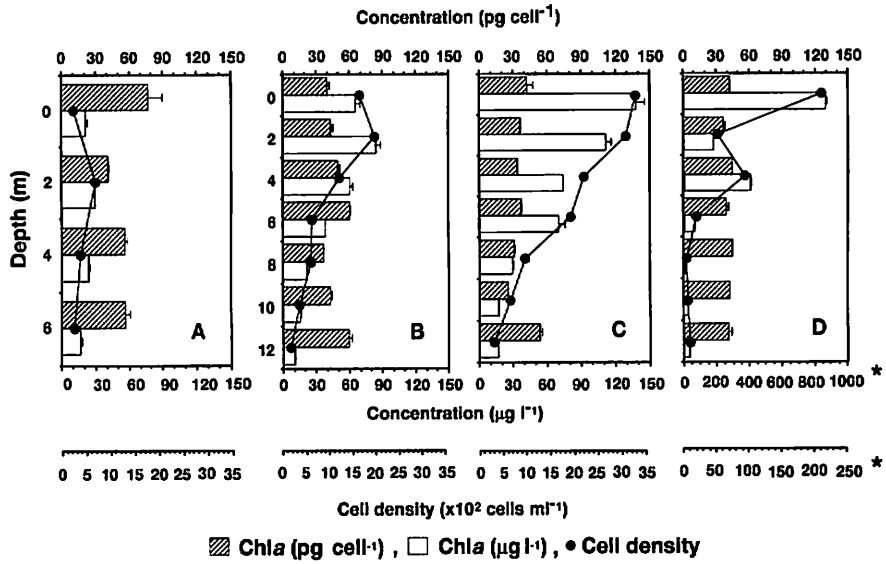

Fig. 4. Vertical distributions of cell densities, cellular contents of Chl *a* in *Gymnodinium mikimotoi*, and total Chl-*a* concentrations at Stns 1 (A), 2 (B) and 3 (C) on 21 September 1993 compared to the distributions at Stn 3 on 27 September (D) (* different scale).

Vertical profiles of cell densities and Chl-*a* concentrations at Stns 1, 2 and 3 are shown in Fig. 4. Contamination by other phytoplankton species was observed to be less than 5% in all water samples. During this study, the weather during the sampling period on 21 September was cloudy, whereas it was very clear on 27 September. On 21 September, the highest cell densities of *G. mikimotoi* at Stns 1 and 2 were observed at 2-m depth. Approximately 3280 cells ml^{-1} were observed in the surface waters at Stn 3 where the Chl-*a* concentration was 138 $\mu g l^{-1}$. The amounts of Chl *a* per cell of *G. mikimotoi* in the surface waters (0 m) at Stns 2 and 3 were lower than those in the bottom waters (12 m). At Stn 3, the cell density in-

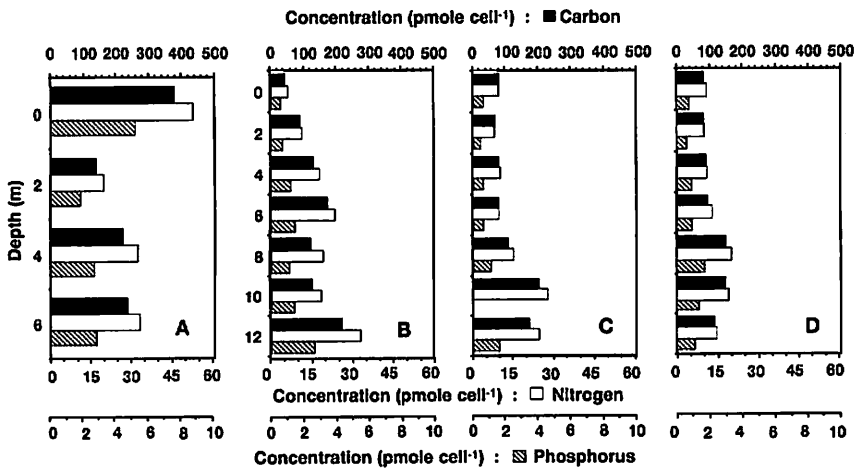

Fig. 5. Vertical profiles of cellular contents of carbon (C), nitrogen (N) and phosphorus (P) in *Gymnodinium mikimotoi* at Stns 1 (A), 2 (B) and 3 (C) on 21 September 1993 compared to those at Stn 3 on 27 September (D).

Table 1. The C : N and N : P ratios at Stns 1, 2 and 3 on 21 September 1993 compared to values at Stn 3 on 27 September.

Depth (m)	Stn 1		Stn 2		Stn 3		Stn 3 on 27.9.93	
	C:N	N:P	C:N	N:P	C:N	N:P	C:N	N:P
0	7.1	10.1	7.9	10.0	8.2	16.6	7.6	16.2
2	7.0	10.8	8.4	16.0	8.5	18.0	8.1	16.0
4	6.8	12.0	7.6	14.8	7.9	17.9	7.8	12.8
6	7.0	12.1	7.6	15.9	8.2	16.2	7.6	13.0
8	—	—	6.6	17.0	7.3	14.0	7.6	11.6
10	—	—	7.0	12.9	7.4	—	8.0	13.0
12	—	—	6.8	12.3	7.0	15.1	8.0	12.2

creased to 20,900 cells ml^{-1} with a Chl-*a* concentration of ca. 800 $\mu g l^{-1}$ on 27 September. The Chl-*a* concentration decreased gradually with depth from 4 to 10 m. However, the Chl-*a* content of cells in the bottom water (12 m) was almost the same as that of cells in the surface water (0 m) (Fig. 4D).

Figure 5 shows the cellular contents of carbon (C), nitrogen (N) and phosphorus (P) in *G. mikimotoi*. On 21 September at Stn 1, the carbon content in cells from the surface water was almost twice that in the bottom water (6 m). But at Stns 2 and 3, the value for cells in the surface waters was less than half of that in the bottom waters (12 m). The carbon content in cells ranged from 80 to 148 pmole over the whole water column at Stn 3 on 27 September (Fig. 5D). The carbon content of cells in the bottom water (12 m) was slightly higher than that of cells in the surface water (0 m).

The cellular nitrogen content of *G. mikimotoi* ranged from 5.8 to 31.9 pmole over the water column at Stns 2 and 3 on 21 September (Fig. 5). On the same day, it ranged between 19.8 and 52.0 pmole at Stn 1. The values for cells in the surface and bottom waters at Stn 3 on 27 September were 10.4 and 14.2 pmole, respectively. Vertical profiles of cellular N content at these three stations on both 21 and 27 September followed the same pattern as that of carbon. The ratios of C : N (by atom) of *G. mikimotoi* were nearly constant at about 7.0 throughout the water column at Stn 1 (Table 1). The ratios of C : N in the surface water at Stns 2 and 3 on 21 September were higher than those in the bottom water (12 m). However, the C : N ratio of cells in the surface water at Stn 3 on 27 September was lower than that in the bottom water (Table 1).

The cellular phosphorus (P) contents of *G. mikimotoi* in the surface water at Stns 1, 2 and 3 on 21 September were 5.2, 0.6 and 0.6 pmole, respectively, and those for cells in bottom waters on 21 September were 2.7, 2.6 and 1.6 pmole, respectively (Fig. 5). On 27 September, the value for cells in the surface water was half of that in the bottom water. The vertical profiles of cellular phosphorus content in this organism were similar to that for carbon. Nitrogen and phosphorus ratios of *G. mikimotoi* in various layers at Stns 1, 2 and 3 are shown in Table 1. The N : P ratios of cells in the surface layer (0 m) on 21 September were lower than those in directly underlying layers (2–4 m). The N : P ratios of cells between the depths of 2–6 m at Stns 2 and 3 on the same day were higher than those in cells from the bottom waters (12 m). At Stn 3 on 27 September, the N : P ratios of cells in the surface layers (0–2 m) were higher than those of cells in the bottom layers (10–12 m).

Vertical profiles of nucleotide content and energy charge (EC) values for *G. mikimotoi* at

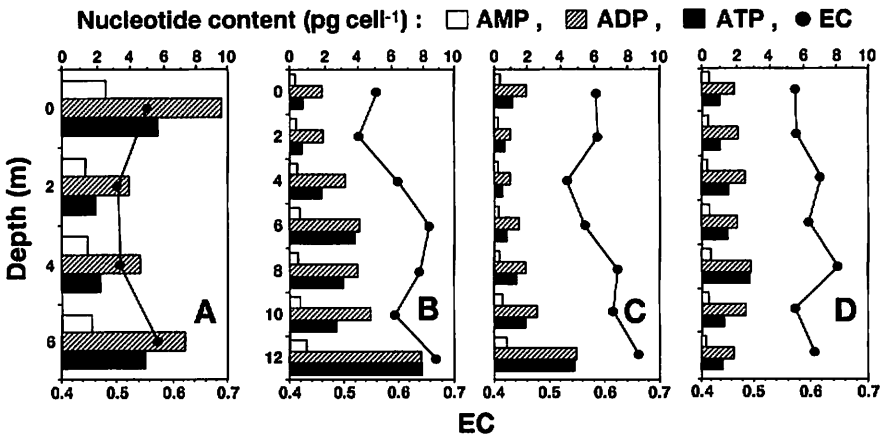

Fig. 6. Vertical profiles of cellular contents of nucleotides and energy charge (EC) in *Gymnodinium mikimotoi* at Stns 1 (A), 2 (B) and 3 (C) on 21 September 1993 compared to those at Stn 3 on 27 September (D).

Stns 1, 2 and 3 during the bloom are shown in Fig. 6. The cellular content of nucleotides at Stn 1 on 21 September ranged from 7.3 to 17.6 pg cell^{-1} over the water column, and the EC values were between 0.49 and 0.57. At Stns 2 and 3, cellular nucleotide content at depths between 10 and 12 m was definitely higher than that at depths between 0 and 2 m. The EC values were between 0.52 and 0.67 throughout the water column at these stations. At Stn 3 on 27 September, the cellular nucleotide content of *G. mikimotoi* ranged from 3.1 to 6.4 pg cell^{-1} over the water column, while EC values fluctuated between 0.57 and 0.64.

Laboratory Study

Figure 7 shows cell densities and nutrient concentrations during the incubation. After incubation for 3 d, the cell densities of *G. mikimotoi* had increased from 2633 to 3246 cells ml^{-1} in the control bottle and from 2633 to 7307 cells ml^{-1} in the nutrient-supplied bottle, respectively, whereas the concentrations of PO_4^{3-}-P decreased from 0.49 to 0.45 μM and from 3.79 to 0.45 μM, respectively. In the nutrient-supplied bottle, the concentrations of $NO_3^-+NO_2^-$-N decreased from 33.4 to 0.23 μM after incubation for 2 d.

Changes in the cellular Chl-*a* content of *G. mikimotoi* in the control and nutrient-supplied

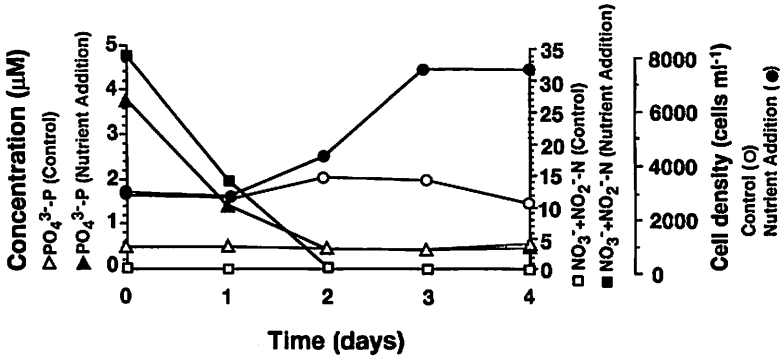

Fig. 7. Changes in nutrient concentrations and cell densities during incubation under laboratory conditions.

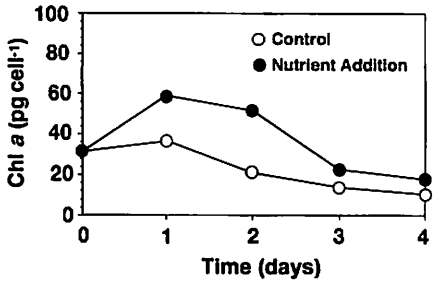

Fig. 8. Changes in cellular contents of Chl *a* in *Gymnodinium mikimotoi* during incubation under laboratory conditions.

bottles are shown in Fig. 8. The cellular content of Chl *a* in the nutrient-supplied bottle increased rapidly upon incubation and decreased gradually from the first day to the end of the experiment.

The changes in cellular contents of carbon, nitrogen and phosphorus in *G. mikimotoi* during the incubation are shown in Fig. 9. The cellular carbon content increased rapidly to double after the first day for both the control and the nutrient-supplied bottles. The cellular carbon contents in the nutrient-supplied bottle decreased gradually over the following days. The cellular nitrogen contents ranged from 8.5 to 11.7 pmole in the control bottle and 7.5 to 16.7 pmole in the nutrient-supplied bottle, respectively, during the experiment. The C : N ratio increased rapidly during the first day in the control bottle and thereafter only increased slightly (Table 2). The time profiles for cellular phosphorus content in both the control and nutrient-supplied bottles were similar to that for carbon. The N : P ratios of *G. mikimotoi* in the control bottle were effectively constant during the incubation. In the nutrient-supplied bottle, the ratios increased after the first day and then decreased gradually from 16.6 to 11.3.

The changes in nucleotide content and energy charge (EC) values in *G. mikimotoi* during the incubation are shown in Fig. 10. The total nucleotide content for this organism in the control bottle ranged from 4.8 to 5.8 pg cell^{-1} and the EC values fell between 0.55 and 0.61. The total nucleotide content of *G. mikimotoi* in the nutrient-supplied bottle increased rapidly and reached the maximum level of 8.8 pg cell^{-1} on the second day of incubation. Thereafter, it decreased. The EC values of cells in this bottle were approximately constant (0.6) for the first

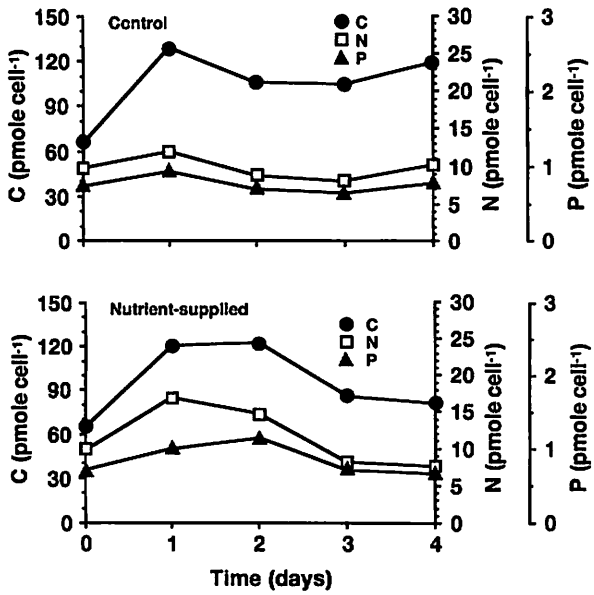

Fig. 9. Changes in cellular contents of carbon (C), nitrogen (N) and phosphorus (P) in *Gymnodinium mikimotoi* in the control bottle (upper) and nutrient-supplied bottle (lower) during incubation under laboratory conditions.

Table 2. Changes in C : N and N : P ratios of *Gymnodinium mikimotoi* during incubation under laboratory conditions.

Time (Days)	Control		Nutrient-supplied	
	C : N	N : P	C : N	N : P
0	6.7	13.4	6.7	13.4
1	10.9	13.0	7.1	16.6
2	12.2	12.7	8.2	12.7
3	13.2	12.3	10.5	11.4
4	12.0	13.0	10.6	11.3

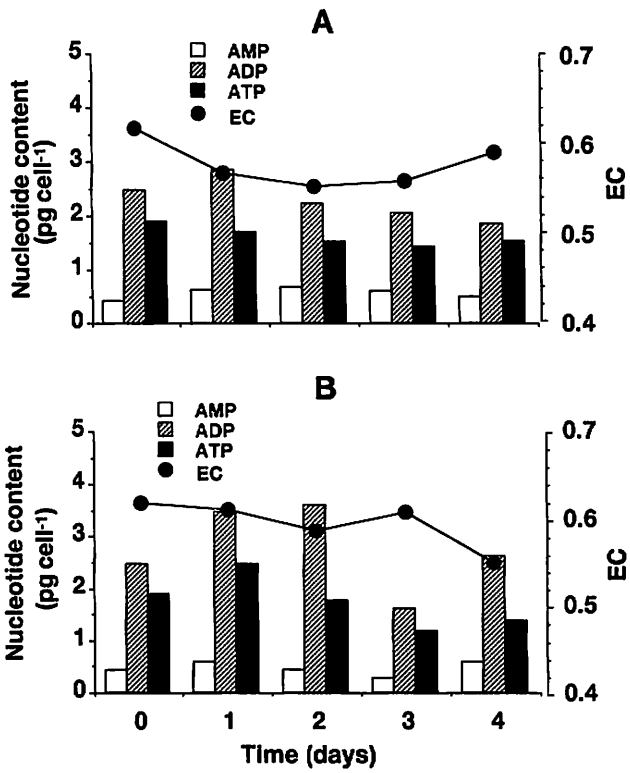

Fig. 10. Changes in cellular contents of nucleotides and energy charge (EC) in *Gymnodinium mikimotoi* in the control bottle (A) and nutrient-supplied bottle (B) during incubation under laboratory conditions.

3 d of incubation and then decreased rapidly.

Discussion

In this study, it was demonstrated that the biochemical composition of *Gymnodinium mikimotoi* at the beginning of the outbreak of a red tide (on 21 September) differed from that at the peak of the red tide. The results of the incubation experiment indicate that cell density can increase rapidly at the outbreak of a red tide if the seawater contains enough nutrients for growth. The rapid decrease in PO_4^{3-}-P and $NO_3^- + NO_2^-$-N (Fig. 7) during the first day of incubation while cell density remained stable indicates that *G. mikimotoi* can increase cell activity in nitrogen and phosphorus uptake for cell growth, and, accordingly, a rapid increase in

cell density became possible. Moreover, the results show that the amounts of Chl *a*, carbon, nitrogen and phosphorus per cell increased rapidly during the first day of incubation. This may be because the cells can increase the level of their photosynthetic activity and accumulate the biochemical components necessary for cell division. Cell density did not increase as much in the control bottle as that in the nutrient-supplied bottle and the N : P ratio was approximately constant during the course of the experiment. This may have been due to the depletion of seawater $NO_3^-+NO_2^-$-N in the control, as the PO_4^{3-}-P concentration was high enough for growth. The incubation experiment showed that the N : P ratio increased to a level approximating the Redfield ratio (Goldman et al. 1979) after the first day of incubation. Thereafter, this ratio decreased gradually to levels near that found in natural cells in the surface layers (0 m) of Stns 1 and 2. In this layer, the concentration of $NO_3^-+NO_2^-$-N in seawater was so low as to be undetectable. These results suggest that $NO_3^-+NO_2^-$-N is the basic limiting nutrient that controlled the occurrence of this red tide.

In the natural environment, since the temperature and salinity at the sampling stations on 21 and 27 September were vertically constant, good vertical mixing of sea water must be a characteristic of this area at this time. However, the concentrations of $NO_3^-+NO_2^-$-N and PO_4^{3-}-P in the bottom water (12 m) at all stations were higher than those in the surface waters (0 m). Moreover, $NO_3^-+NO_2^-$-N was undetectable in the surface water at Stns 2 and 3. These results indicate that most of the $NO_3^-+NO_2^-$-N in the surface waters was used up by *G. mikimotoi*. Based on the results from the incubation experiments, we can predict that cell density at Stn 3 would not increase above 4000 cells ml^{-1} because the concentrations of $NO_3^-+NO_2^-$-N and PO_4^{3-}-P were about 0.3 and 1.0 μM, respectively. However, cell densities in the surface waters at Stn 3 on 27 September were in fact higher than 20,000 cells ml^{-1}. This suggests that cells of *G. mikimotoi* have obtained nutrients from adjacent areas and from domestic waste drainage following an extended period of heavy rainfall in the days preceding the day of sampling. Low salinities in seawater, initiated by rain fall, may also have enhanced the growth of *G. mikimotoi*. This hypothesis is supported by the suggestion by Yamaguchi (1994) that the highest growth rate of *G. mikimotoi* is obtained at the low salinity of 25 psu. The apparently high level of vertical mixing of the seawater in this area should simultaneously supply these rain-derived nutrients to the bottom layer. Yamaguchi (1994) reported that *G. mikimotoi* can grow at such low irradiances as 10 $\mu E\, m^{-2} s^{-1}$. Cells in the bottom layer under low light conditions are therefore able to increase in cell number and vertically migrate up into the surface layer. The speed of vertical migration of this organism has been estimated to be about 1.3 m h^{-1} (Honjo 1994). Thus, the development of this red tide may have been significantly affected by vertical migration.

The concentration of organic components in *G. mikimotoi* in the bottom water (12 m) at Stns 2 and 3 on 21 September was, as a rule, higher than that in cells in the surface waters. This was probably due to the fact that cells in the surface and bottom waters were in different stages of growth. Much of the previously published data has suggested that the quantities of biochemical compounds in phytoflagellates vary with the stage of growth (Binder & Anderson 1990; Lirdwitayaprasit et al. 1990a, b; Kim et al. 1993; Meksumpun et al. 1993a, b). In particular, the amount of ATP per cell and the EC values of dinoflagellates during the exponential phase of growth have been found to be higher than those during the stationary phase (Lirdwitayaprasit et al. 1990a, b; Meksumpun et al. 1993b). In this study, the results show that the amount of ATP per cell and the EC values of *G. mikimotoi* in the bottom layers were higher than those of cells in the surface layers. These results thus suggest that cells earlier in the growth cycle occurred mostly in the bottom layers. The vertical profile of the biochemical

composition of *G. mikimotoi* at Stn 3 on 27 September was different from that on 21 September at the same station. The cellular Chl-*a* content of this organism was effectively constant throughout the water column, although the ambient nutrient concentrations varied. These results indicate that the majority of cells in the water column were probably in the same stage of the growth cycle. It was during this period that the peak in cell density occurred. These cells were most likely in the stationary phase of growth and the density of cells should gradually have decreased as nutrient levels were reduced.

Overall, this study has provided more detailed information, clarifying the cause of outbreaks of *G. mikimotoi* red tides. It demonstrated that the bloom might be heightened by the action of cells in earlier growth phases active in the bottom layers. These cells stayed in the bottom layers and produced the biochemical compounds which are necessary for cell division. When nutrient concentrations in seawater are high enough for cell growth, this organism can rapidly replicate and occur as a red tide. Further studies need to be carried out to understand the role vertical migration plays in the outbreak of *G. mikimotoi* red tides.

Acknowledgments

We gratefully acknowledge the assistance of Prof. Dr T. Ochi. We are also grateful to Dr A. Hoshika and Dr C. Chareonpanich for their very helpful comments. We especially thank Mr T. Hamagaki for ensuring successful field operations.

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