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The Relationships between *Prorocentrum micans*-Growth and its Ecological Environment*

TAKUJI UCHIDA

Introduction

Numerous studies have been made to clarify the mechanisms of red tide occurrence (KETCHUM and KEEN 1945, COLLIER 1953, SLOBODKIN 1953, WILSON and COLLIER 1955, HICKEL *et al.* 1971, ADACHI 1972, IIZUKA 1972, IRIE 1973, IWASAKI 1973). The causative organisms of red tides spread widely over various taxonomical groups. Among them, dinoflagellates occupy considerable percentage of all the organisms (IRIE 1970, ADACHI 1972). Many studies on red tide are made to correlate phytoplankton abundance to the changes of environmental factors, such as sun light intensity, salinity and nutrient levels and to find a factor regulating the bloom. Such studies surely give the explanation for the development of phytoplankton mass, however, can hardly explain the fact that only a few species grow and mature into red tide among various phytoplankton species.

IWASAKI and his colaborators (IWASAKI and SASADA 1969, IWASAKI 1969, IWASAKI *et al.* 1969, IWASAKI 1971 a, b, IWASAKI 1973) carried out nutritional studies on some causing species to analyze the red tide occurrence from the nutritional characteristics of each species. As he indicated, such basal approach is indispensable to identify important factors for red tide maturing, which is variable among each species, and also to provide improved culture techniques for further studies.

As pointed by IIZUKA and IRIE (1969), biological interactions, as well as physical and chemical factors, can not be negligible in phytoplankton ecology. Especially in a eutrophic area, which has potential to support explosive phytoplankton growth, the dense populations of phytoplankton species may vigorously affect each other. Conversely, red tide is a phenomenon suitable for analyzing the relationship among phytoplankton species. Nevertheless, few attentions have been payed to this point of view (IIZUKA and IRIE 1969, HONJO *et al.* 1978).

In Muroran harbor, Hokkaido, there have been observed *Prorocentrum micans* red tide for several years. It appeared monospecific following the diatom blooms. This pattern has led to an interest in analyzing algal succession from diatoms to the red tide flagellate.

In the present study, for this purpose, the following procedures were performed.

* Based on a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Science, Institute of Algological Research, Faculty of Science, Hokkaido University in 1978.

1) Seasonal succession of phytoplanktons was studied to analyze algal community associated with *P. micans*. 2) The variations of some environmental factors were recorded to find a correlation to this species. 3) Nutritional study was conducted to identify important factors for *P. micans* growth, and also to develop defined medium for further experiments. 4) Bioassay was conducted to know a limiting nutrient for *P. micans*. 5) The two major diatom species, which had been dominant in advance of *Prorocentrum*, were isolated and their requirements for macro-nutrients were studied to compare their nutritional characters with those of *Prorocentrum*. 6) Some culture experiments were conducted whether there are interactions between diatoms and the flagellate.

From the results obtained, the cause and mechanism of *Prorocentrum micans* bloom was discussed.

Acknowledgements

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The writer was grateful to Dr. S. SAITOH of Osaka University and Dr. Y. NISHIHAMA of Hokkaido Institute of Mari-culture for their kind advice and suggestions and to the members of the Institute of Algological Research for their discussion and help in collection.

I. The relationship of phytoplankton seasonality to some environmental factors in Muroran harbor, Hokkaido, Japan

Prorocentrum micans is a cosmopolitan dinoflagellate species and sometimes forms red tide (ADACHI 1972, IIZUKA and KOMAKI 1974, FUJITA *et al.* 1976). Although the physical and chemical conditions of seawater are reported at the occurrence of this species (IIZUKA and KOMAKI 1974, FUJITA *et al.* 1976), there are few detailed informations on environmental aspects including biological phenomena linked with the bloom.

In Muroran harbor, Hokkaido, which is located in the eastern end of Funka Bay, *P. micans* red tide had been observed every autumn during several years. In the present study, periodical seawater analyses were carried out in the harbor to know the changes of phytoplankton community associated with *P. micans* and physical and chemical factors affecting phytoplankton growth with particular reference to this species.

I-1 Seasonal changes of phytoplanktons in Muroran harbor

In the coastal area of Funka Bay, NISHIHAMA *et al.* (1976) carried out a detailed

investigation on the seasonality of phytoplankton species. Since Muroran harbor is isolated by duplicate breakwaters, it may be expected that the harbor has different phytoplankton community from that in other parts of Funka Bay. Actually, red tide occurrence of the species had been limited to Muroran harbor and its vicinity (IIZUKA and KOMAKI 1974). It is of quite interest to compare the phytoplankton behavior in the harbor with that obtained by them.

In order to make clear a seasonal pattern of phytoplankton succession in the harbor, identification and counting were made periodically on dominant phytoplankton species.

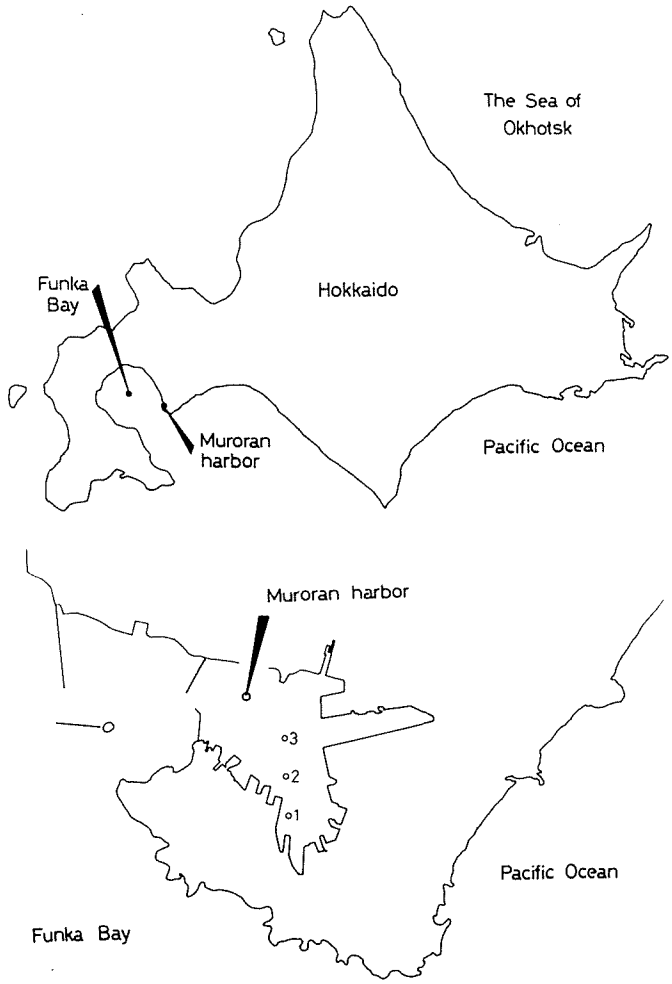

Fig. 1. The location of Muroran harbor where sampling had been made at three stations. Station 1; 7–8 m depth, station 2; 10 m depth, station 3; 14 m depth.

From the results obtained, characteristics of phytoplankton community in the harbor were described, and the relationships between *P. micans* and other members of phytoplanktons are considered.

Methods

Sampling was made at three stations (Fig. 1) and at each point both bottom and surface seawater were collected in one liter polyethylene bottle with a VAN DORN plastic sampler. This field work had been conducted monthly through two years from June, 1974 to May, 1976. One liter of each sample was fixed with 20 mℓ of neutralized formalin and concentrated by the settling method (The Meteorological Agency of Japan 1970). These treated samples were subjected to identification and counting of phytoplankton cells under a light microscope.

Results

The abundant period of phytoplankton was spring to autumn and the poor period was late autumn to winter through two years (Fig. 2). There were no remarkable

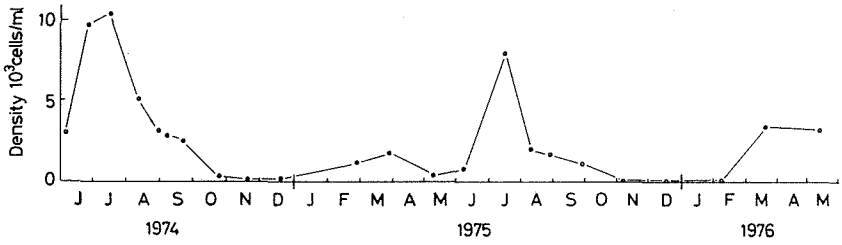

Fig. 2. Seasonal changes of phytoplankton density in surface seawater from June, 1974 to May, 1976.

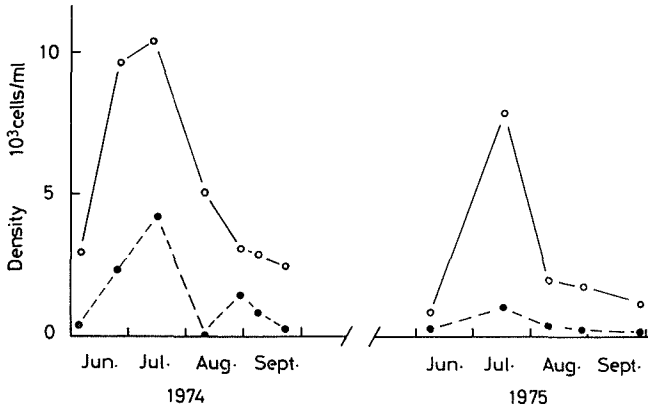

Fig. 3. Comparison of phytoplankton density between surface (——○——) and bottom (- - -●- - -) seawater during summer.

Table 1. Seasonality of 10 species of phytoplankton in Muroran harbor.

P; present in a certain amount, c; considerable amount, b; bloom by the species.

	Jun.	Jul.	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May.
Prorocentrum micans			p	b b	c	p	p					
P. triestinum			c									
Skeletonema costatum	p	p	b	c	p			p		c	c	p
Chaetoceros didymus	c	b	p	b								
Ch. curvisetus										p	p	
Ch. pseudocrinitus prox.										p	c	
Thalassiosira nordenskiördi										p	c	p
Th. hyalina										p	p	
Leptoclyindrus danicus			p							p		
Asterionella japonica		p	p	p	p					p	c	
month	Jun.	Jul.	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May.
				1974						1975		

	Jun.	Jul.	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May.
Prorocentrum micans			p	p	b	p						
P. triestinum			c									
Skeletonema costatum		b	c	c	p	p				p		
Chaetoceros didymus	c	p										
Ch. curvisetus						p				p		
Ch. pseudocrinitus prox.										p		c
Thalassiosira nordenskiördi		p	p	p					p	c		
Th. hyalina									p	p		
Leptocylindrus danicus			p						p	c		
Asterionella japonica					p	p				p		
month	Jun.	Jul.	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May.
				1975						1976		

differences in main constituents and their density between each sampling point except *Prorocentrum micans*, however, phytoplankton density of bottom seawater was less than that of surface seawater, especially in the summer period (Fig. 3). Although *P. micans* has a little motility, it drifted around the harbor in some masses, therefore, the density of this species was different between each station.

The major species which appeared comparatively at high density were choosen and their seasonal changes in surface seawater were shown in Table 1. Obviously, the main constituents were diatoms and dinoflagellates. Diatoms were dense in early spring and in summer. Summer diatom bloom was large scale as compared to that in early spring, and it was followed by *P. micans*. This species grew remarkably and formed a red tide. *P. triestinum* was also observed in the harbor but did not grow to a dominant species. Of 8 diatom species examined (Table 1), *Skeletonema costatum* and *Asterionella japonica* were observed through four seasons. Although the latter species never appeared at high density, the former species was observed to be a main constituent of summer diatom bloom. *Chaetoceros didymus* was also found to be dense in summer, 1974, but it occupied a less extent in 1975. *S. costatum* and *Ch. didymus* were main components of summer diatom bloom.

Early spring peak of phytoplankton density was composed of various species as compared to those in summer-autumn. They were mainly composed of *S. costatum*, two species of *Thalassiosira*, *Chaetoceros pseudocrinitus* prox., *Leptocylindrus danicus*, *Ch. curvisetus* and *Asterionella japonica*.

Ch. didymus, *S. costatum* and *P. micans* were prominently dominant species among

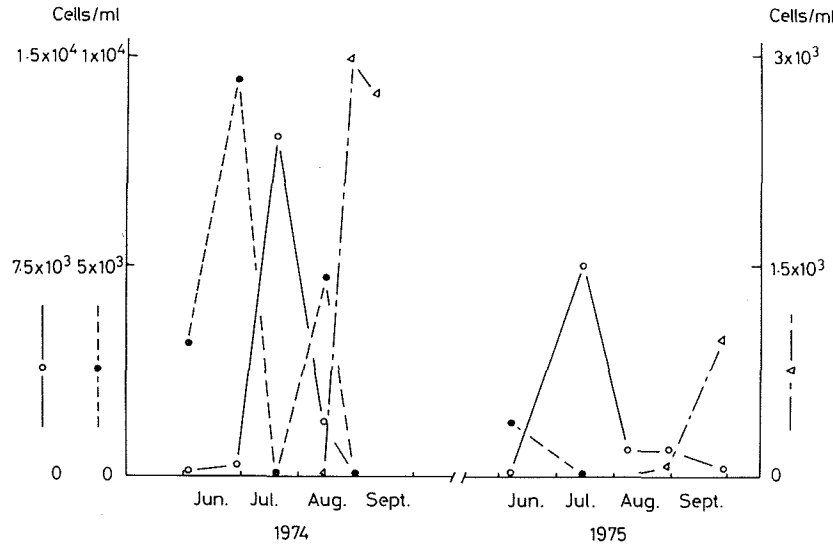

Fig. 4. Seasonal changes of three phytoplankton species.

phytoplankters appeared in Muroran harbor. They alternatively formed monospecific blooms (Fig. 4). Although the density of *P. micans* was far less than those of diatoms in a cell number, its cell size is much larger than diatoms (Fig. 5). The average cell volume of each species in abundant period was calculated $88 \times 10^2 \mu m^3$ for *P. micans*, $12.9 \times 10^2 \mu m^3$ for *Ch. didymus* and $1.67 \times 10^2 \mu m^3$ for *S. costatum*, respectively. Since a *Skeletonema* cell has a cylindrical form, it is easy to estimate its volume using a formula for geometric solid. On the other hand, another two species have more complicated forms, accordingly, it is difficult to measure their volume directly. Method

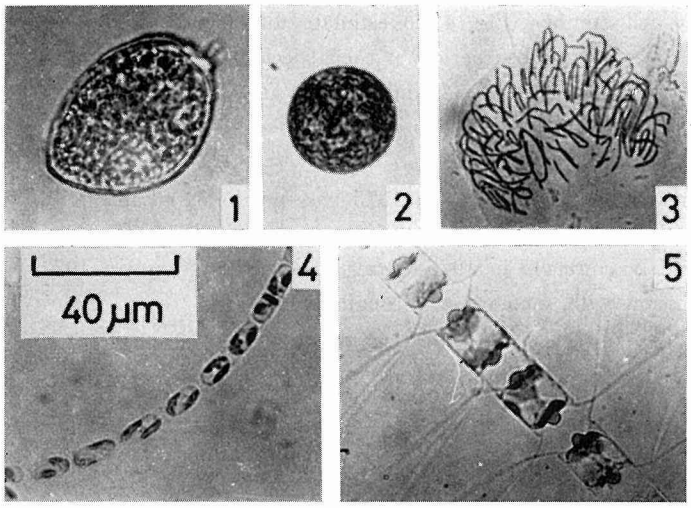

Fig. 5. Photographs of three phytoplankton species. 1–3; *Prorocentrum micans*, 1; a vegetative cell, 2; a cyst like cell, 3; chromosomes stained by aceto iron haematoxylin-chloral hydrate, 4; *Skeletonema costatum*, 5; *Chaetoceros didymus*.

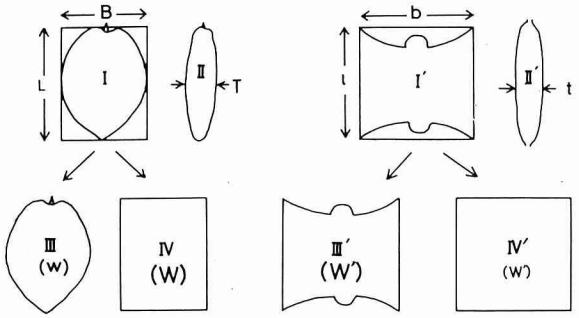

Fig. 6. Diagrams showing the method for the estimation of phytoplankton cell volume. I–IV; *P. micans*, I'–IV'; *Ch. didymus*, B and b; cell breadth, L and l;cell length, T and t; cell thickness.

used in the study was as follows (Fig. 6). At first, cell dimensions (length (L, l), breadth (B, b) and thickness (T, t)) were measured under microscope using micrometer. From the obtained values cell volume was calculated regarding that cell form was a rectangular prism (K•L•M and k•l•m). Then, such calculated volume was revised by a coefficient (w/W and w′/W′) which was derived by comparing weights of two printing papers cut off in different ways. One (W, W′) was a rectangular printing paper which was circumscribed to the cell form and the other was a printing paper of cell form itself. Finally, the cell volume was estimated by the expression of L•B•T•w/W for *Prorocentrum* and l•b•t•w′/W′ for *Chaetoceros*. Such calculated cell volume of each species was multiplied by cell number (Fig. 4) to estimate total cell volume contained in 1 mℓ of seawater. In the most dense period of each species, *P. micans* occupied 26.4×10^6 μm^3/mℓ, *Ch. didymus*, 12×10^6 μm^3/mℓ, and *S. costatum* 2.0×10^6 μm^3/mℓ, respectively. Obviously, *P. micans*-bloom occurred in the largest scale, which could be easily recognized as a red tide.

The density of *Ch. didymus* in 1975 was much less than that in 1974, and the succession with other two species was not so clear as in 1974. The cell densities of *P. micans* and *S. costatum* in 1975 were also less than those in 1974, however, made a clear succession with each other through two years.

Discussion

It is known that there is a spring phytoplankton increase in temperate waters (FOGG 1966, ARUGA 1973). Also in Funka Bay only spring outburst was observed and few phytoplanktons survived into summer (NISHIHAMA *et al.* 1976). On the other hand, three peaks of phytoplankton increase were observed in Muroran harbor. They appeared in early spring, summer and autumn. This characteristic pattern of phytoplankton growth surely comes from the peculiarity of this harbor, though the reason is unclear. However, it can be considered that this is, at least, partly due to the eutrophic seawater of the harbor. Red tide occurrence by *Prorocentrum* implies the possibility of this assumption. *P. micans* is known to form red tide frequently (ADACHI 1972, FUJITA *et al.* 1976), and in some cases it appeared with other phytoplankters (ADACHI 1972). On the other hand, in Muroran harbor and its vicinity *Prorocentrum micans* red tide was observed. The interest is in the reason why only *P. micans* grew such remarkably and matured into a red tide. Before the red tide occurence of this species, summer diatom bloom was always observed through two years. Advanced growth of diatom is a common phenomenon in many cases of flagellate blooms (IWASAKI 1971 c, HONJO *et al.* 1978). HONJO *et al.* (1978) observed a competitive phenomena between *Heterosigma* sp. and *Skeletonema costatum* which appeared alternatively at Hakozaki harbor in Hakozaki Bay. As indicated by them, the interactions between diatoms and *Prorocentrum micans* are predictable from these observations.

In accompany with the diatom growth, the differences of its density between surface and bottom seawater had become larger. This tendency was remarkable in summer. The dense phytoplankton populations in surface seawater reduce sunlight intensity to pass through the water column, and it may be possible that, in bottom layer, light limits the phytoplankton growth. In summer, thermocline developes by the rise of surface temperature. It prevents the vertical mixing of seawater. This makes it difficult that surface phytoplankton cells migrate into bottom layer. These two reasons can be considered to contribute the formation of such vertical microdistribution of phytoplanktons.

I-2 Seasonal changes of environmental factors in Muroran harbor

In Funka Bay, phytoplanktons were found only a few in the summer period while they were abundant in early spring (NISHIHAMA *et al.* 1976). This is considered to come from the poorness in concentrations of nutrients (NISHIHAMA *et al.* 1976). On the other hand, most remarkable increase was observed during summer-early autumn in Muroran harbor. Since the climate is considered not to be different between two areas, phytoplankton bloom in summer-early autumn may depend on higher concentrations of nutrients in the harbor.

In the present study seasonal variations of environmental factors, especially for nutrients, have been examined to discuss the relationships between these factors and *Prorocentrum micans* growth and other phytoplankton species.

Methods

The seawater samples were collected in the same manner as mentioned before (I-1). Seawater temperatures were measured immediately after poured seawater to polyethylene bottle from the sampler. Oxygen saturation was estimated with oxygen meter (EIL 1520). Hydrogen ion concentration (pH) was determined with pH meter (Toa HA 15) in two hours after sampling had finished. Salinity was measured with silver nitrate titration (The Meteorological Agency of Japan 1970). Transparency of seawater was determined with Secchi disc.

For the following chemical analysis, samples were previously passed through glass fiber filter (Whatman, pore size 1 μm) to remove granular contaminants such as plankton cells and detritus. The concentrations of nitrate were estimated as nitrite after reduction by passing through copper-coated cadmium column (The Meteorological Agency of Japan 1970). Nitrate was determined with a diazocoupling method (The Meteorological Agency of Japan 1970). Amonium was measured with CONWAY's microdiffusion units (ISHISAKA 1969). Inorganic phosphate and silicate were measured colorimetrically as molybdenum complexes (The Meteorological Agency of Japan 1970, Jap. Soc. Anal. Chem. Hokkaido 1971). Chemical oxygen demand was estimated using potassium permanganate as a oxydizing agent in alkaline condition. The concentrations of iron was

determined by ortho-phenanthroline method (Jap. Soc. Anal. Chem. Hokkaido 1971). For this analysis, 100 mℓ of seawater was added with 0.5 mℓ of concentrated nitric acid and then, it was concentrated in water bath to increase iron concentration. Such procedure did not affect the accuracy of this method.

Results

Seasonal changes of selected environmental factors from June, 1974 to May, 1976

Temperature

Temperature shift was observed from 1 to 23.6°C at the surface and 1.6-20.4°C at the bottom (Fig. 7). The highest temperature was measured in late August and the lowest one in February, through two years. There had been observed remarkable differences of temperatures between surface and bottom seawater during the summer period. Especially in August, 1974 (Fig. 7) surface seawater temperature was recorded as high as 23.6°C, though only 15°C was observed in the bottom. On the contrary, there were little differences from September to May between them.

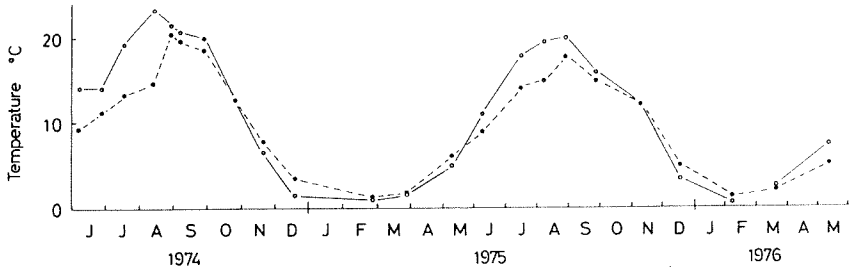

Fig. 7. Seasonal changes of seawater temperature from June, 1974 to May, 1976. ——○—— surface seawater, - - -●- - - bottom seawater.

Light energy

The monthly average of solar radiation energy (cal/cm^2/day) was illustrated in Fig. 8 which was indebted to the Muroran Regional Meteorological Observatory. The highest value (390 cal/cm^2/day in 1975 and 433 cal/cm^2/day in 1976) was obtained in May and the lowest value (87 cal/cm^2/day in 1974 and 93 cal/cm^2/day in 1975) in December. Annual cycle of solar radiation energy had been repeated in the similar pattern through two years.

pH

The pH values of surface seawater were higher than those of bottom seawater almost throughout the year. The higher pH was observed in summer-autumn and lower pH in winter (Fig. 9). From June to August, the difference of pH between surface and bottom seawater became larger. Especially in early August, 1974, pH of surface

seawater was 8.7, while that of bottom seawater was only 8.0 (Fig. 9). The similar tendency was observed in August, 1975, but the difference was less than that of the year before (Fig. 9).

Chlorinity

Chlorinity varied irregularly ranging from 15.5 to 18.5‰ (Fig. 10). Bottom seawater always kept higher chlorinity levels as compared to surface seawater except late August, 1974. From June to September, chlorinity of surface seawater showed considerable variations (15.5-17.7‰) in 1974, however, it was kept at 16.2-16.8‰ in 1975.

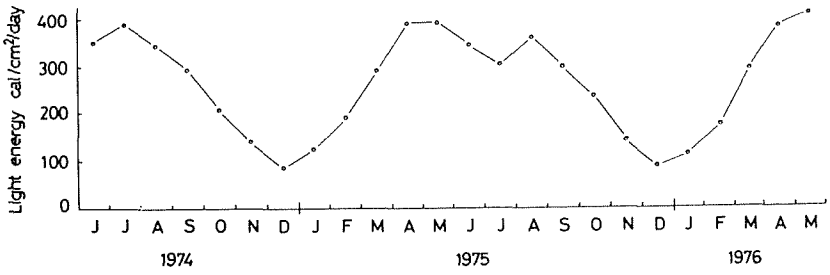

Fig. 8. Seasonal changes of solar radiation energy from June, 1974 to May, 1976.

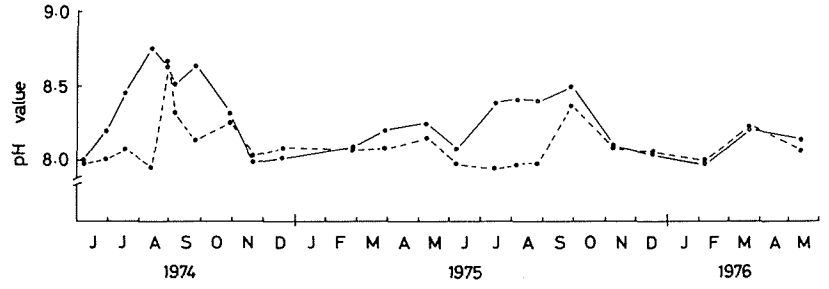

Fig. 9. Seasonal changes of pH from June, 1974 to May, 1976.
——○—— surface seawater. - - -●- - - bottom seawater.

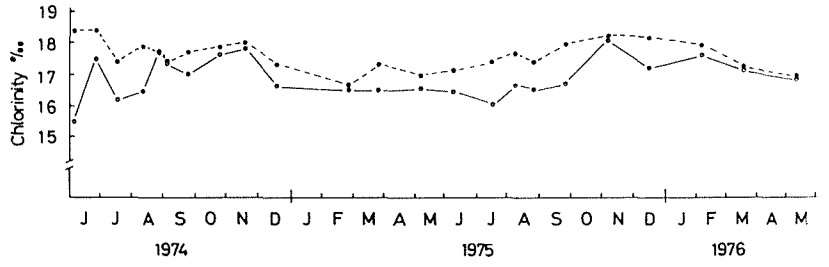

Fig. 10. Seasonal changes of chlorinity from June, 1974 to May, 1976.
——○—— surface seawater, - - -●- - - bottom seawater.

Nitrate

Higher nitrate concentrations were observed from November, 1974 to March, 1975 (6.6-13.7 μg at./liter) and from December, 1975 to February, 1976 (8.7-15.6 μg at./liter) (Fig. 11). On the contrary, nitrate concentrations were kept at lower levels from summer to autumn. During this period, there were considerable different variation patterns of nitrate concentration between bottom and surface seawater. From June to early August, 1974, surface nitrate concentration showed abrupt decreasing while in the bottom layer, the increase of nitrate concentration could be observed especially in 1974. However, such a difference in nitrate concentration between surface and bottom seawater had been disappeared in late August, 1974, when short term increase of nitrate concentration (7.8 μg at./liter in the surface and 7.6 μg at./liter in the bottom) was observed. During summer-autumn, 1975 (Fig. 11), there were two low peaks of surface nitrate concentration in July and in late September, on the other hand, bottom nitrate concentration showed no remarkable variation and was kept at lower levels.

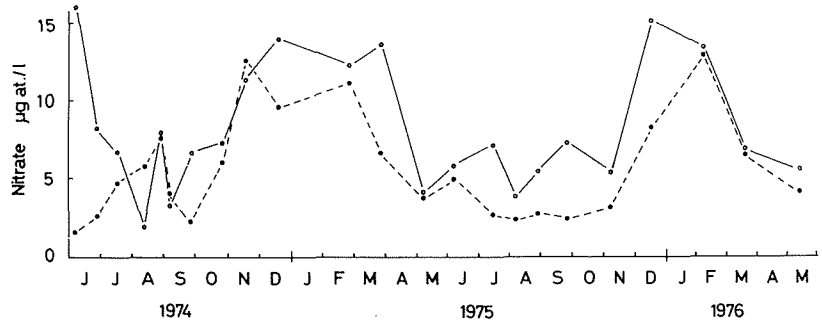

Fig. 11. Seasonal changes of nitrate from June, 1974 to May 1976.
——O—— surface seawater, - - -●- - - bottom seawater.

Nitrite

Nitrite never appeared in such high concentrations as nitrate throughout two years (Fig. 12). The maximum concentration was 2.85 μg at./liter in the surface and 1.4 μg at./liter in the bottom, which were observed in December, 1974 (Fig. 12). The variation pattern of nitrite was almost consistent with that of nitrate, that is, it was kept at high concentration in winter and found to be trace in summer.

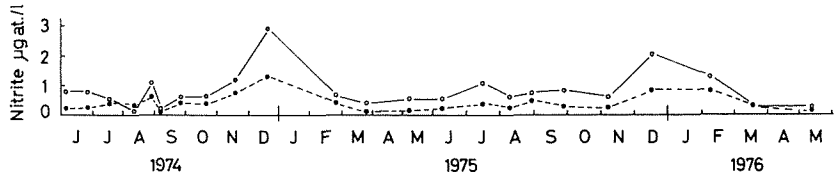

Fig. 12. Seasonal changes of nitrite from June, 1974 to May, 1976.
——O—— surface seawater, - - -●- - - bottom seawater.

Phosphate

Surface phosphate concentration was kept at lower levels from late June to early August, 1974 (0.057-0.15 μg at./liter) and from late March to late August, 1975 (0.13-0.27 μg at./liter) (Fig. 13). During these periods, bottom seawater contained phosphate in higher concentrations than the surface one. The poorness of surface phosphate concentration was recovered to 1.83 μg at./liter in late August, 1974 and 0.87 μg at./liter in late September, 1975. In winter phosphate was kept at higher concentrations in both surface and bottom seawater.

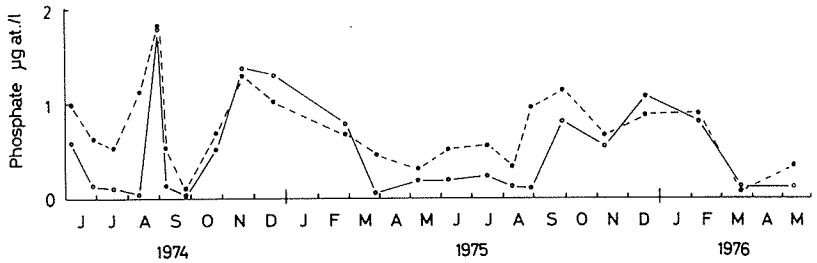

Fig. 13. Seasonal changes of phosphate from June, 1974 to May, 1976.
——○—— surface seawater, - - -●- - - bottom seawater.

Silicate

As other nutrients there were considerable variations in silicate concentration. It was kept at higher level in winter and lower in summer (Fig. 14). From June to August surface silicate concentration was decreased gradually, and reached extremely low value. Namely, it was measured for 0.54 μg at./liter in early August, 1974 (Fig. 14), and 0.72 μg at./liter in late August, 1975 (Fig. 14). On the contrary, bottom silicate concentration was kept at certain levels during the summer.

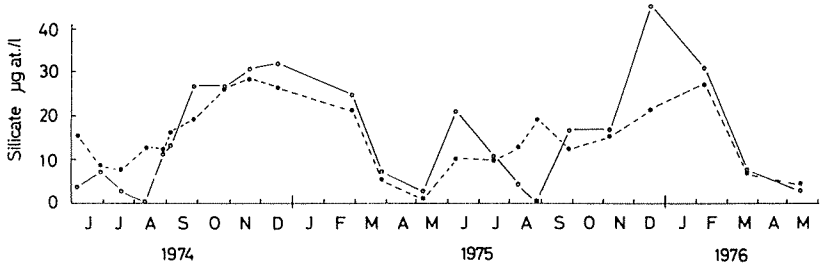

Fig. 14. Seasonal changes of silicate from June, 1974 to May, 1976.
——○—— surface seawater, - - -●- - - bottom seawater.

Seasonal variations of other environmental factors

In the course of laboratory experiments, it was found that *P. micans* preferred

ammonium to nitrate (Fig. 15) and also that it has a mixotrophic ability (II-5). In addition, iron requirement by this species was detected (II-2).

As indicated before (I-1), phytoplanktons of bottom seawater were less than those of surface seawater, particularly during the summer. On the contrary, the concentrations of nutrients were higher in bottom than those in surface seawater. These results suggest that sufficient light could not be provided to the bottom owing to high density of algal cells in the surface, and that active bacterial growth resulted in reproduction

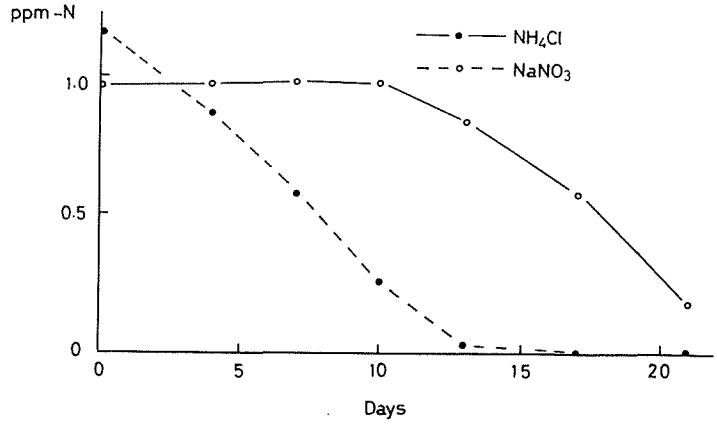

Fig. 15. Nitrogen uptake by *P. micans* when both ammonium and nitrate were provided together (UCHIDA 1978). Ammonium was preferentially utilized to nitrate. Nitrate uptake began when ammonium concentration decreased to a certain level.

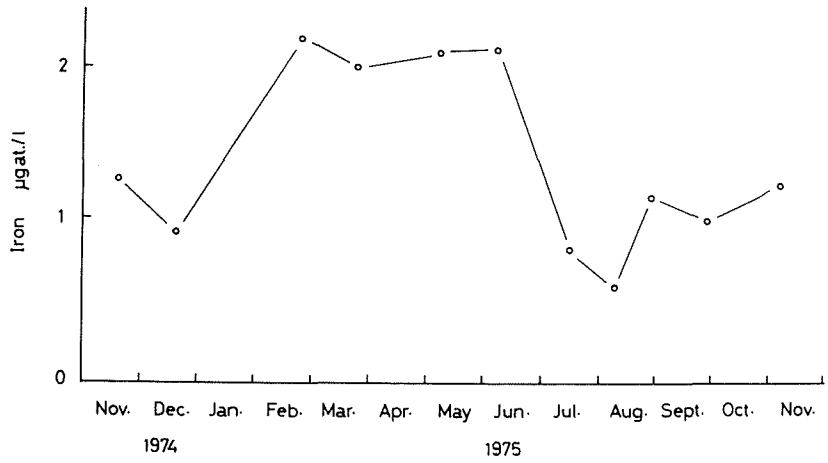

Fig. 16. Seasonal changes of iron in surface seawater from November, 1974 to November, 1975.

of nutrients in bottom seawater. From these reasons, the variations of iron, COD, ammonium, dissolved oxygen, and transparency of seawater have been also investigated.

Iron

Considerable amounts of iron had been recorded throughout four seasons (Fig. 16). From late February to June, it was kept in concentrations higher than 1.5 μg at./liter. In August the lowest value was recorded as much as 0.61 μg at./liter.

COD

Chemical oxygen demand (COD) was determined for the surface seawater collected from July to September, 1974 and from July to September, 1975 to examine the correlation to *P. micans* growth (Fig. 17). The peak of COD (19.7 mg O_2/liter) could be clearly observed in late August, 1974. On the other hand, there were no remarkable variations in 1975. From late August to late September, it was kept at higher levels (3.8–4.4 mg O_2/liter) as compared to other seasons in 1975.

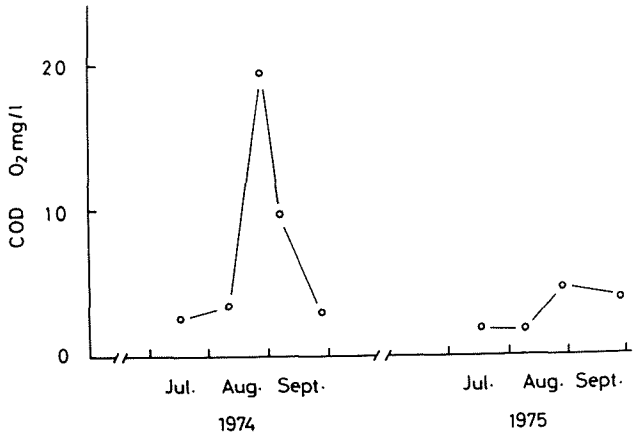

Fig. 17. Variations of COD in surface seawater from July to September in 1974 and 1975.

Ammonium

The extremely high concentrations of surface ammonium were observed in July, December and February, 1976, which were recorded 31, 40, and 28 μg at./liter, respectively (Fig. 18). In addition, there was a lower peak in late September. The seasonal changes of ammonium concentration showed similar pattern with that of nitrate in the surface seawater. On the other hand, bottom ammonium was always kept in lower concentrations than the surface one except August, 1975 and May, 1976. The peak of ammonium concentration in the bottom seawater (14.9 μg at./liter) was observed in February, 1976.

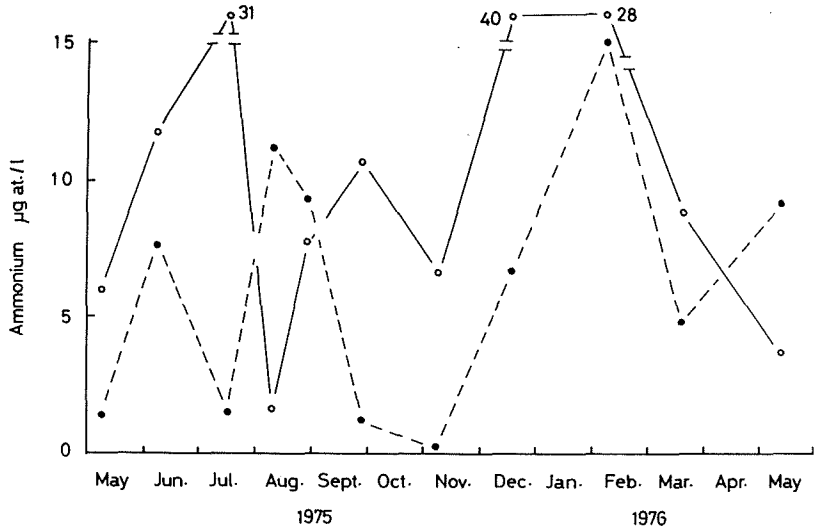

Fig. 18. Seasonal changes of ammonium from May, 1975 to May, 1976.
——○—— surface seawater, - - -●- - - bottom seawater.

Transparency of seawater

The changes of seawater treansparency were shown in Fig. 19. It varied from 1.45 to 4.7 m with two peaks in May-June and in November. The lowest value of seawater transparency (1.45 m) was observed in August-September.

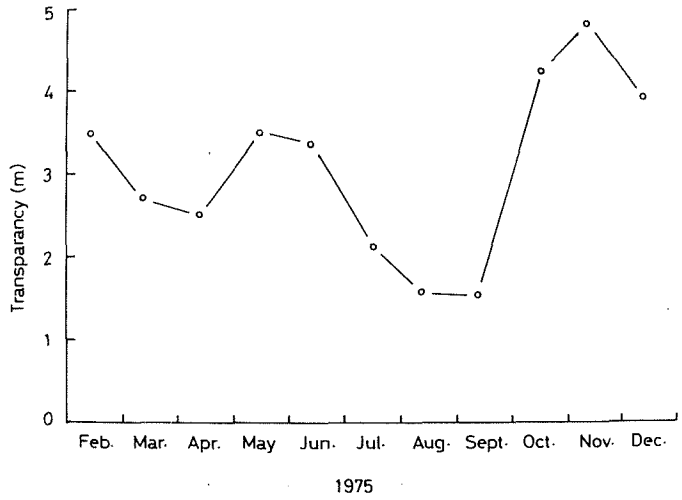

Fig. 19. Seasonal changes of seawater transparency from February to December, 1975.

Dissolved oxygen

Dissolved oxygen was supersaturated in surface seawater throughout the four seasons (Fig. 20). It was higher from July to August as compared to other seasons. On the contrary, saturation value in bottom seawater was higher in winter and lower in summer-autumn. Particularly, during late August-late September it decreased to lower than 80%.

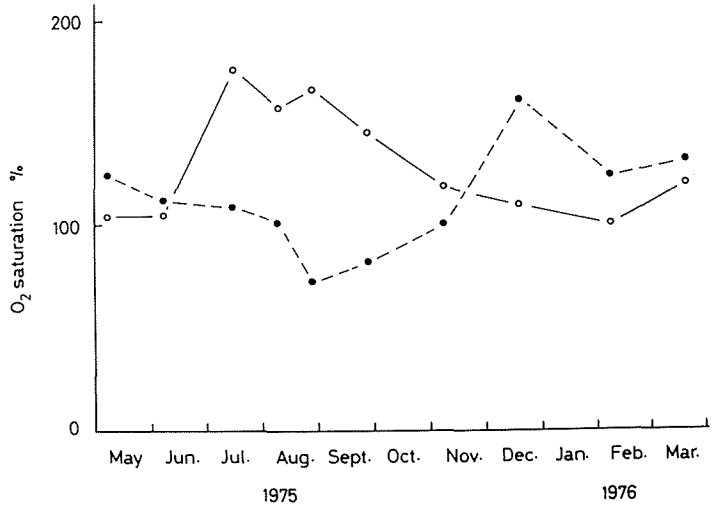

Fig. 20. Seasonal changes of O_2 saturation from May, 1975 to March, 1976. ——○—— surface seawater, - - -●- - - bottom seawater.

Discussion

General features

There were considerable differences of temperatures between surface and bottom seawater during the summer period. This indicates that thermocline had developed toward the summer with a rise of surface water temperature. Thermocline developed in summer 1975 had disappeared gradually. This indicates that small scale mixing of the upper and lower seawater repeatedly occurred toward early November with a drop of surface temperature. This pattern is typical in temperate waters (SVERDRUP *et al.* 1954 a, FOGG 1966, ARUGA 1973). On the other hand, thermocline in 1974 disappeared suddenly in late August when *P. micans* red tide was observed. This may be resulted from vertical mixing of seawater caused by rainfall and wind blows, namely, stormy weather had continued before the disappearance of thermocline. In some cases of red tide, advanced seawater mixing has been observed (IWASAKI 1971 c, MURAKAMI 1972, YANAGIDA 1976).

Silicate and phosphate in surface seawater were kept at lower concentrations during summer as compared to those in winter. This might come from the active consumption of these nutrients by diatom cells. As indicated previously (I-1), active diatom growth was observed only in surface seawater but not in the bottom. This resulted in low levels of these nutrients and also in raising pH values by the consumption of dissolved carbonate in surface seawater. On the other hand, nutrients levels in bottom seawater had become higher during summer, which is considered to come from decomposition of diatom cells by bacterial activity. Thermocline might prevent the supply of nutrients from the bottom layer to the surface. The poorness of nutrients in surface layer had recovered with the disappearance of thermocline.

Different from phosphate and silicate, nitrate and ammonium were kept at considerable concentrations during summer, though they were decreased with other nutrients by phytoplankton growth. The seasonal variations of nitrate and ammonium were almost consistent with each other. This may be due to the rapid oxidation from ammonium to nitrate caused by nitrifying bacteria (SVERDRUP *et al.* 1954 c).

High concentrations of iron have been observed in Muroran harbor as compared to those in the open sea (SVERDRUP *et al.* 1954 b, LEWIN and CHEN 1971). By the method used in the present study, soluble iron could not be distinguished from colloidal form because a portion of the latter which was small enough to pass through the glass fiber filter gradually changes to soluble form during the analysis procedure. Therefore, the value obtained is not exact to the soluble part. According to LEWIN and MACKAS (1972), *Chaetoceros armatum* in culture can utilize chelated iron but not ferric chloride as an iron source. Further they mentioned that clay particles attached to *Ch. armatum* serves as an iron source after chelated by the mucilage excreted by the organism. This suggests the diversity of iron utilization capability of marine phytoplanktons. It is generally known that trace metal elements in chelated form are well utilized by many planktonic algae (PROVASOLI *et al.* 1957, IWASAKI 1967, LEWIN and CHEN 1971). These facts were obtained from laboratory experiments, but it is very difficult to distinguish various forms of iron dissolved in natural seawater. For Muroran harbor seawater, it can be said that it has potential to supply high amount of iron for phytoplankton growth.

Dissolved oxygen (DO) of surface seawater was supersaturated during the summer, 1975. The active release of oxygen resulted from photosynthesis by dense phytoplankton cells is considered to contribute to this phenomenon. On the other hand, DO in bottom seawater has decreased during this period and was only 76% saturation in late August, 1975. Such decrease may come from the consumption of DO by bacterial activity in bottom seawater.

On the whole, the variations of seawater transparency were consistent with phytoplankton abundance. However, non-living granules such as detritus is not a negligible

factor. It decreased during summer and was lowest in September which was recorded as 1.45 m. In this condition, even if light intensity in surface is 100,000 lx (maximum light intensity in August), it decreased to only about 300 lx in 5 m depth. Accordingly, this light intensity is considered to limit phytoplankton growth in bottom seawater during summer.

Correlation to the succession from diatoms to *Prorocentrum*

The variations of phosphate concentration correlated with those of diatom cell density in summer, 1974, however, there were no clear correlationships between them in summer, 1975. On the contrary, silicate depletion was parallel to diatom decrease, which was observed during August-September every two years. The restoration rate of silicate is known to be lower than those of phosphorus and nitrogen (DUGDALE 1972), accordingly, silicate concentration is considered to affect diatom growth directly as compared to other two nutrients. Diatom can grow in considerable low concentrations of silicate, however, growth ceases in concentrations lower than 0.03-0.1 mg/liter-Si (LEWIN 1963, ARUGA 1973). In Muroran harbor the amount of silicate in the surface seawater decreased to 0.54-0.72 μg at./liter (= 0.015-0.020 mg/liter-Si). In such conditions diatom cannot survive. In spite of high concentrations of nutrients, diatom growth was inactive in bottom seawater during summer, which may be due to the deficiency of available sunlight in this layer.

After the decline of diatom blooms, *Prorocentrum micans* appeared and matured to the red tide when environmental conditions had become preferable. Namely, on that occasion, the transparency of seawater increased and surface nutrients were recorded. Especially for phosphate, its concentrations in the surface showed a close correlation with *P. micans* abundance, that is, the abundance of this species in 1974 was much more than that in 1975 which had lower phosphate concentration than that in 1974. FUJITA *et al.* (1976) also observed high concentration of phosphate at the occurrence of this species red tide in Kesennuma. As in the case of diatoms, active growth of *Prorocentrum* was mainly observed in the surface, accordingly, surface nutrients were considered to be closely related to the growth of this species than those of bottom. Chemical oxygen demand (COD) values also increased at the blooming of *Prorocentrum* especially in 1974. The possible origins of organic substances expressed in COD can be considered in two ways. One is the diatom cell decomposed matters and the other is the excrements by *P. micans*. IIZUKA and KOMAKI (1974) also observed high COD values in *P. micans* red tide and suggested that this may originated from active excretion of organic matter by this species. However, in early September, 1974, low COD values could only be measured, though high cell density of *Prorocentrum* was observed. Further, COD values in 1974 were higher than those in 1975, which were correspond to the fact that summer diatom bloom in 1974 occurred in large scale than that in 1975. Therefore, high concentration of organic matter could be considered to come,

in large part, from decomposed cells of diatoms.

II Culture experiments on three phytoplankton species

In Muroran harbor, as shown previously, *Chaetoceros didymus*, *Skeletonema costatum* and *Prorocentrum micans* were summer-early autumn phytoplankton population and appeared alternatively. To investigate growth phenomena of these species, nutritional studies and other culture experiments were conducted.

II-1 Culture maintenance of three phytoplankton species and their response to different light intensities and temperatures

There are many studies on culture experiments of marine phytoplanktons (PROVASOLI 1958, IWASAKI 1967, LOEBLICH 1967). However, it is not always easy to maintain successive cultures of some phytoplankton species in the laboratory. Particularly, it is sometimes unsuccessful to preserve diatom strains probably because diatom reduces its cell size by repeated cell divisions and the consequent sexual reproduction, which is necessary for cell size recovery, requires specific conditions (DREBES 1966, TAKANO 1967, SATOH and KANNO 1967, MIGITA 1967). In the present study, axenic cultures of these species were obtained and the responses of their growth to different light intensities and temperatures were examined.

Materials and Methods

The strains of *P. micans*, *S. costatum* and *Ch. didymus* were isolated in October, 1973, June, 1974, and July, 1974, respectively. Stock cultures of *Prorocentrum* were maintained in 100 mℓ Erlenmyer flasks containing 50 mℓ of BSW 4 medium (Table 3), or in screw cap tubes (18×130 mm) with 10 mℓ of ST 3 medium (PROVASOLI *et al.* 1957) at 20±1°C, and those of two diatom species were preserved in 1 liter Erlenmyer flasks with 500 mℓ of BSW 2 m medium (Table 2) at 14±1°C. Culture experiments were conducted in 100 mℓ Erlenmyer flasks with 50 mℓ of BSW 4 for *Prorocentrum micans*, and in screw cap tubes (18×130 mm) with 10 mℓ of BSW 2 m (Table 3) for diatoms. Each species was exposed to 14 : 10 light and dark cycle. Growth was measured with hemacytometer or counting all cells in 0.1 mℓ sample. From the data obtained, growth constant was calculated as the following formula (FOGG 1966);

$$K = \frac{2.3 \log Ne/No}{0.69\ t}$$

where

K; Growth constant

No; Initial cell number

Ne; Cell number after t days from inoculation

Table 2, 3; Culture media for diatoms (Table 2, left) and *Prorocentrum micans* (Table 3, right).

	BSW 2m	BSW 2Am
	Amount/liter	
NaCl	—	24 g
KCl	—	0.6 g
$CaCl_2$	—	0.8 g
$MgSO_47H_2O$	—	6 g
$MgCl_26H_2O$	—	3 g
$NaHCO_3$	—	0.1 g
Seawater	900 ml	—
$NaNO_3$	50 mg	50 mg
KH_2PO_4	5 mg	5 mg
$Na_2SiO_39H_2O$	0.1 g	0.1 g
P2 metals[1]	10 ml	10 ml
Vitamin mix T[2]	1 ml	1 ml
Tris	1 g	1 g
pH	8.0	8.0

	BSW 4	BSW 4A
	Amount/liter	
NaCl	—	18 g
KCl	—	0.4 g
$MgSO_47H_2O$	—	4.5 g
$MgCl_26H_2O$	—	3 g
$CaCl_2$	—	0.8 g
$NaHCO_3$	—	70 mg
H_3BO_3	—	25 mg
NaBr	—	50 mg
$SrCl_26H_2O$	—	15 mg
Seawater	900 ml	—
KH_2PO_4	10 mg	10 mg
$NaNO_3$	50 mg	50 mg
P2 metals[1]	2 ml	2 ml
Vitamin[2] mix T	1 ml	1 ml
Tris	1 g	1 g
pH	8.0	8.0

1) PROVASOLI *et al.* 1957. One ml of P2 metals contains Na-EDTA, 1 mg; Fe (as Cl), 0.01 mg; Mn (as Cl), 0.04 mg; Zn (as Cl), 0.005 mg; Co (as Cl), 0.001 mg; B (as H_3BO_3), 0.2 mg.

2) In an early study, vitamin mix 8 A (PROVASOLI *et al.* 1957) was used, but later it was replaced with vitamin mix T containing only B_{12}, thiamine and biotin in the same concentrations as vitamin mix 8 A. One ml of vitamin mix T contains B_{12}: 0.05 μg, thiamine: 0.2 mg, biotin: 0.5 μg.

Results

Isolation of three phytoplankton species

Photographs of the organisms used in the present study are shown in Fig. 5. These materials were isolated by repeated washings with sterilized seawater. Axenic cultures of *Prorocentrum* could be obtained by this method, however, it was unsuccessful to get those of another two species. Accordingly, two diatom species were first grown in media containing antibiotics mixture (penicillin G pottasium salt 10 mg/100 mℓ and streptomycin sulfate 5 mg/100 ml in final concentration), then they were washed and isolated in the same way as mentioned above. By this method axenic cultures of these species could be obtained. The sterility test of these cultures was carried out on ST 3 (PROVASOLI *et al.* 1957) and bouillon medium. The organism obtained by these methods grew well in media containing no organic substances except vitamins.

Maintenance of cultures

In small volume cultures the long-term maintenance of diatom species was impossible, namely, when the cell size was reduced to a certain extent after several times of cell division, the cells could survive no more. On the other hand, when they were preserved in large volume cultures (500 mℓ), successive maintenance of diatom species could be obtained.

Unlike these diatoms, the preservation of *P. micans* was easily obtained. In the course of culturing this species, it was observed that a small number of cells became round, non-motile and cyst like form (Fig. 5-2) in stationary phase. This type of cells gave rise to normal cells when they were transferred to fresh medium.

The culture of this species was left into an ice cabinet (1-3°C) to study its viability in darkness and low temperatures. As a result, a few cells could survive for 2 months, most were in spherical and few in normal form.

Chromosomes of *P. micans* was observed by the method of WITTMANN (1965). They were clearly stained (Fig. 5-3) and their number could be counted as many as 60±8. This result was almost consistent with that of previous report (DODGE 1966).

Growth curve

Logarism of cell numbers of each species was graphed as a function of incubation time (Figs. 21-23). Culture conditions were regulated to 20±1°C, 2,000 lx. Linear curve was obtained up to 5-10 days for *Pororcentrum*, 3-5 days for *Skeletonema*, and 4-5 days for *Chaetoceros*. Among these species, *Skeletonema* and *Chaetoceros* showed high growth constant which was calculated to 1.34 for the former and 1.15 for the latter. On the other hand, *Prorocentrum* grew very slowly. Its growth constant K was recorded to only 0.56. These values of K were calculated for the organisms show ing exponential growth.

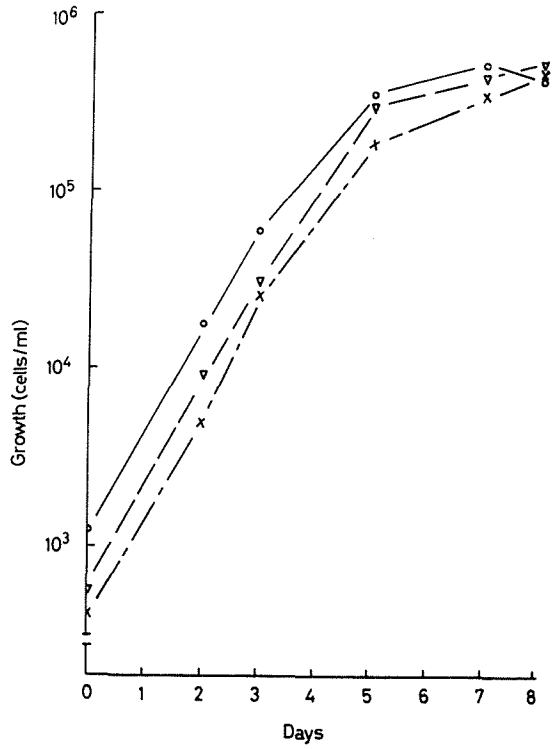

Fig. 21. Growth curve of *Skeletonema costatum*.

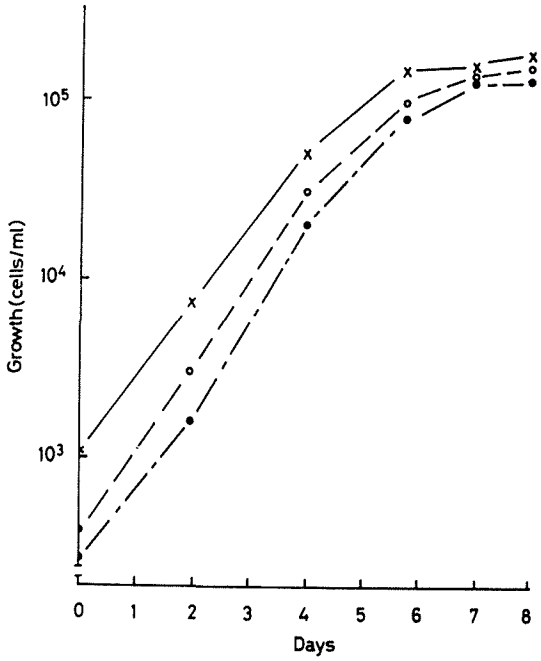

Fig. 22. Growth curve of *Chaetoceros didymus*.

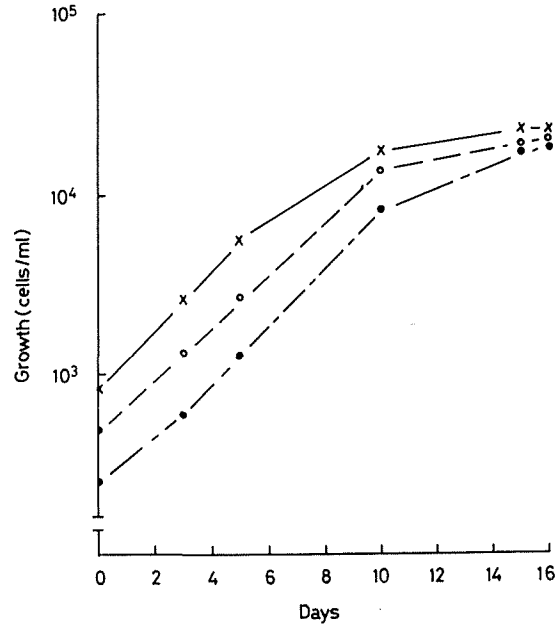

Fig. 23. Growth curve of *Prorocentrum micans*.

Effect of temperature

Growth rates of three phytoplankton species at different temperatures were examined at a light intensity of 2,000 lx. The results are shown in Figs. 24 and 25. Even at 5°C growth rates of diatoms were kept at certain levels, namely, the growth constant of *Skeletonema* was 0.25 and that of *Chaetoceros* was 0.12. At temperatures from 14 to 20°C, there were no remarkable differences in their growth rates. On the other hand, *P. micans* require higher temperature for its optimal growth. At 5°C growth constant of this species decreased to almost zero. With a rise of temperature, growth constant increased up to 0.56 which was recorded at 20°C.

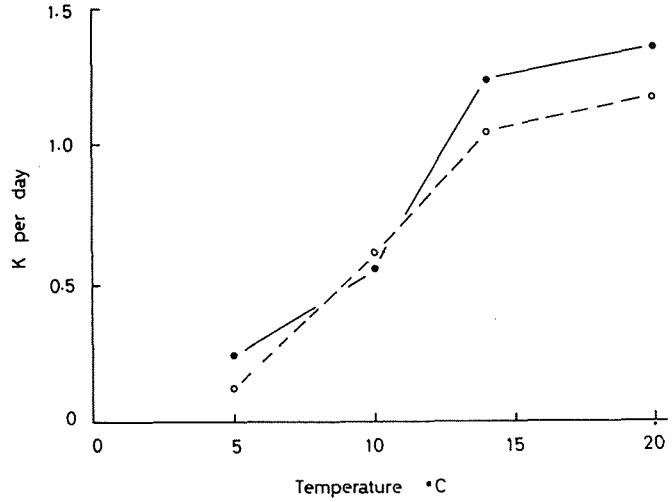

Fig. 24. Growth of two diatom species at different temperatures. —●— *S. costatum*, ---○--- *Ch. didymus*.

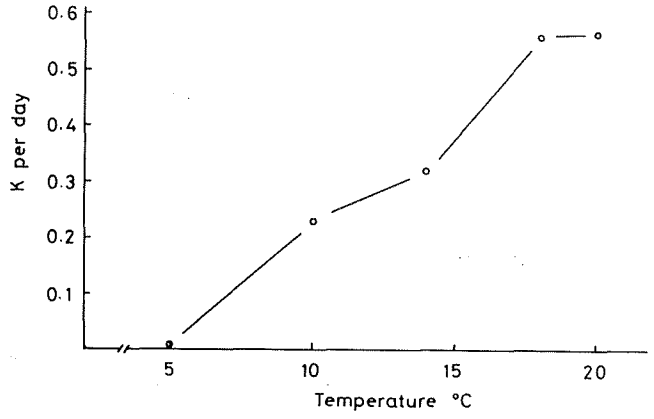

Fig. 25. Growth of *P. micans* at different temperatures.

Effect of light intensity

Effect of light intensity on the growth of three phytoplanktons were tested at 20± 1°C. As a result (Figs. 26, 27), their growths showed the same tendency at the intensity below 2,000 lx. However, there were not so much increment in *P. micans* growth between 2,000 and 7,000 lx, while the growth constants of two diatoms increased to 1.58 for *Chaetoceros* and 2.0 for *Skeletonema* by raising light intensity to 7,000 lx.

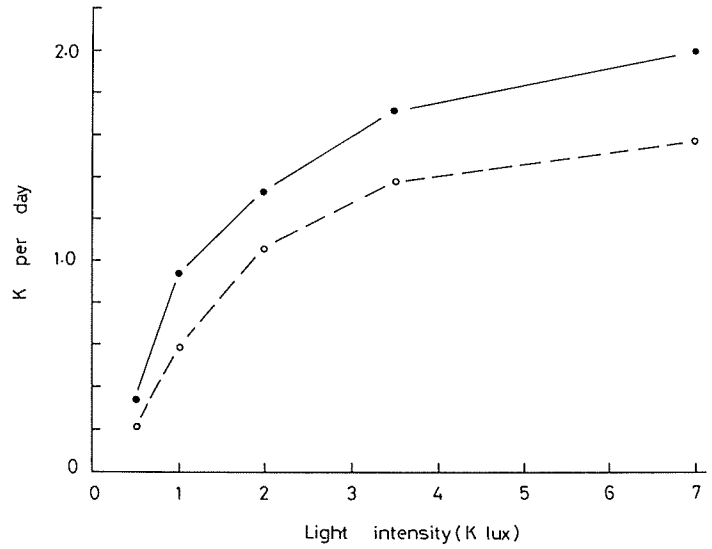

Fig. 26. Growth of two diatom species at different light intensities.
——●—— *S. costatum*, - - -○- - - *Ch. didymus*.

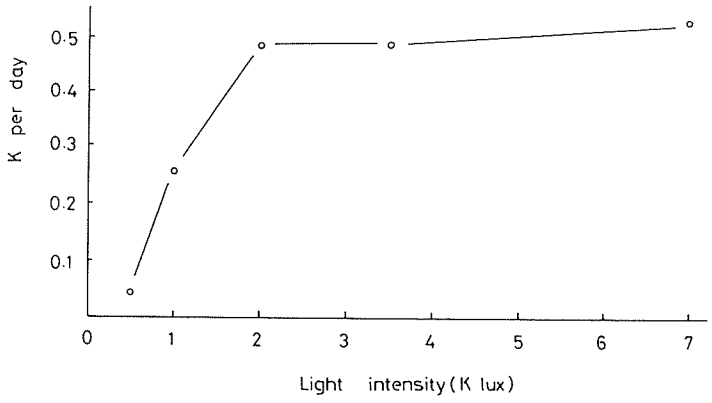

Fig. 27. Growth of *P. micans* at different light intensities.

Discussion

Culture maintenance

It is known that diatom gradually reduces its cell size through several cell divisions and that this is recovered by the formation of auxospores (VON STOSCH 1950, 1954, 1958, DREBES 1966, MIGITA 1967, TAKANO 1967). Accordingly, if this process is unsuccessful, culture maintenance is impossible. In the present study, large cells recovered through sexual process could not be obtained when they were grown in small volume medium. It is established in some species that gamete formation requires optimum temperatures and light intensities (DREBES 1966, TAKANO 1967, MIGITA 1967, SATOH and KANNO 1967). For the effect of light intensity, it is known that female gamete is formed in higher intensity (SATOH and KANNO 1967). If diatom is grown in large volume culture, there will be made various conditions of light in the medium as compared to smaller cultures, therefore, it may be deduced that recovery of cell size by the auxospore formation was performed more certainly in cultures with large volume.

In the last decade many studies have been reported on marine dinoflagellate cyst morphology and taxonomy using materials obtained from marine sediment (WALL *et al.* 1967, SASADA and FUJIYAMA 1975, DALE 1976, 1977 a, FUKUYO *et al.* 1977). However, studies on the encystment process of marine dinoflagellates are limited to few species belonging to *Peridinium* and its allied genera (VON STOSCH 1965, DALE 1977 b, TURPIN *et al.* 1978). *Prorocentrum micans* investigated in the present study formed cyst like cells without passing through sexual process. However, it is obscure whether this type of cell is a resting stage or a merely deformed cell since it has no specialized wall and red pigmented body as observed in other dinoflagellates (DALE 1976, 1977 a, b, FUKUYO *et al.* 1977).

Growth responses to different temperatures and light intensities

S. costatum and *Ch. didymus* showed a similar pattern of growth-light curve. Both species require high light intensity as compared to *P. micans.* The same tendency was observed in their response to different temperatures. Two diatom species showed wide tolerance at lower temperatures, namely, they could grow moderately even at 5°C and their optimal growth rate could be obtained from 14-20°C. The wide tolerance of *Skeletonema* to temperature can be approvable from the field observation that it appeared throughout the year. This speculation is supported by JITTS *et al.* (1964) in the strain originated from Long Island Sound. Different from these diatom, *Prorocentrum* could hardly grow at 5°C and its optimal temperature ranged from 18 to 20°C. The previous reports also showed the lower limit of temperature for the growth of this species to be about 5°C (BARKER 1935, KAIN and FOGG 1960). The preference of higher temperatures of this species seems to correspond with the fact that it appeared in late summer-autumn when seawater temperature was kept at high levels.

II-2 Nutrients requirements in *Prorocentrum micans* EHRENBERG

In addition to nitrogen and phosphorus, trace metal elements and vitamins are required for the phytoplankton growth both in the field and in culture (PROVASOLI 1958, MENTZEL and SPARTH 1962, IWASAKI 1967, LEWIN and CHEN 1971, O'KELLEY 1974). These nutrients are considered to regulate phytoplankton growth but there are much differences in nutritional characteristics among each phytoplankton species (PROVASOLI 1958, IWASAKI 1967, 1973) and sometimes among strains of the same species (PROVASOLI and CARLUCCI 1974).

In the present study the experiments were carried out to know nutritional characteristics of *Prorocentrum micans* and also to develop a defined medium for further studies. KAIN and FOGG (1960) studied on the nutrition of the Plymouth strain of this species, however, they used natural seawater as a basal medium and detailed informations are lacking.

Material and Methods

BSW 4 and BSW 4 A were used as basal media, which were sterilized by autoclaving (120°C, 20 min.). Culture experiments were conducted in 100 mℓ Erlenmyer flasks at 20±1°C under the continuous illumination of 2,000-3,000 lx which provided by cool-white fluorescent lamps. Growth was measured by counting all cells in 0.1 mℓ sample after about 15 days from inoculation. When necessary, the organism was once pre-cultured in medium lacking the compound to be tested. In the experiments of vitamins, culture vessels were treated at 200°C to decompose contaminants. The solution of each vitamin to be added to the basal medium was sterilized by passing through millipore filter (0.22 μm GS). The glass wares used in the experiments of metal requirement were previously left in 8 N HNO_3 for one day and then rinsed 10 times with distilled water.

Results

Initial pH

Optimal growth was obtained at pH of 7.8-8.6 (Fig. 28), and even at 7.0 slight growth was observed. At pH higher than 8.0, culture medium had become to make precipitation more easily with the rise of pH value, especially in the presence of high concentration of phosphate. The following experiments were performed at pH 8.0.

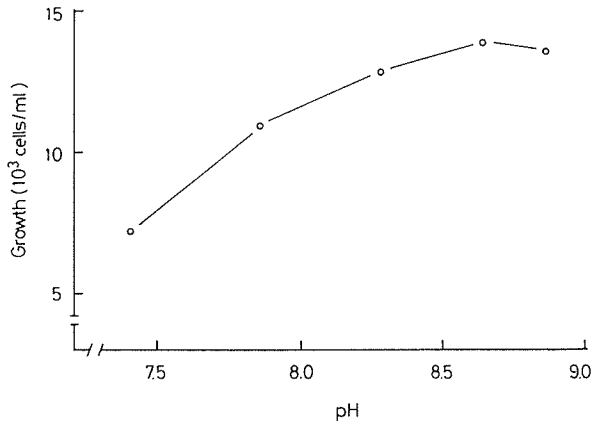

Fig. 28. Effect of initial pH on the growth of *P. micans*.

Chlorinity

Concentrated seawater was diluted to various chlorinity levels from 10-22‰ and it was enriched in the same way as BSW 4. There were no remarkable differences between the growths in each chlorinity level (Fig. 29).

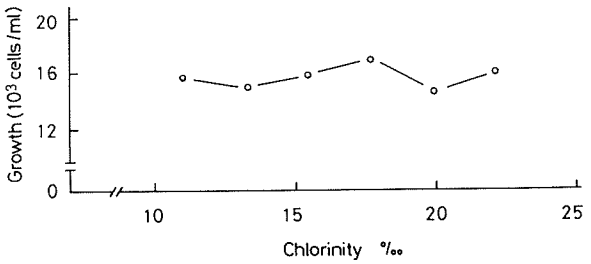

Fig. 29. Effect of chlorinity on the growth of *P. micans*.

Phosphorus sources

Sodium β-glycerophosphate and KH_2PO_4 were tested as phosphorus sources. Both could serve as phosphorus sources almost to the same extent (Fig. 30). Optimum growth was obtained from 13.1-327 μg at./liter for the former and 14.7-294 μg at./liter for the latter.

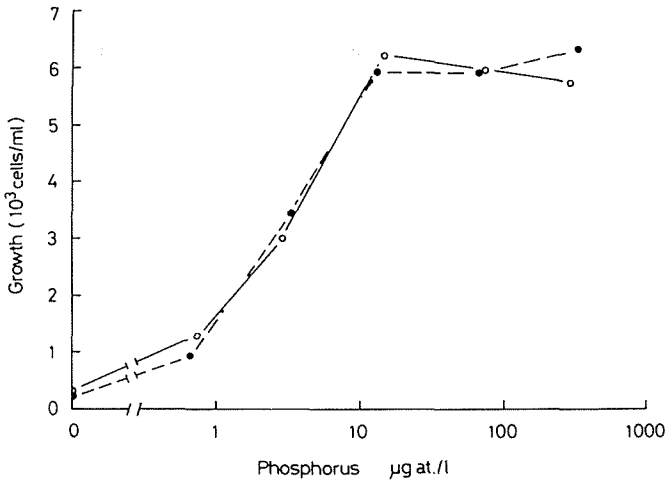

Fig. 30. Effect of phosphorus sources on the growth of *P. micans*.
——○—— KH_2PO_4, ----●---- β-glycerophosphate.

Nitrogen sources

As inorganic nitrogen sources, nitrate, ammonium and nitrite were examined. The former two compounds served as a good nitrogen sources (Fig. 31). The organism had capability to adapt high concentration of nitrate, at least, up to 1,765 μg at./liter of $NaNO_3$. On the other hand, it was sensitive to the change of ammonium concentration. Namely, the optimum growth was obtained at 187 μg at./liter of NH_4Cl while

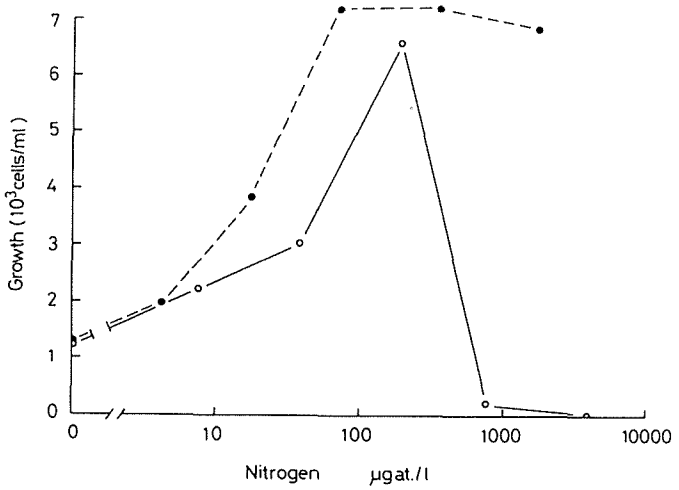

Fig. 31. Effect of nitrogen sources on the growth of *P. micans*.
——○—— NH_4Cl, - - -●- - - $NaNO_3$.

no growth was observed at 748 μg at./liter of NH_4Cl. Nitrite also permitted the growth (Table 4), however, cells became abnormal and bleached in the nitrite medium. Of 4 organic nitrogen compounds examined (Table 4), urea and ornithine could serve as nitrogen source for the species. Urea supported good growth as nitrate and ammonium but ornithine permitted only 1/3 growth as much as that in urea medium (Table 4, Fig. 32). On the contrary, no significant growth could be measured in media supplied with L-histidine or L-arginine as a sole nitrogen source.

Table 4. Available nitrogen compounds for the growth of *P. micans*.

Compounds	Concentrations mg/liter	Growth cells/ml
$NaNO_3$	30	7280
NH_4Cl	10	6570
$NaNO_2$	2	5080
Urea	2	7560
L-Ornithine	20	2350
L-Histidine	0.2~20	790
L-Arginine	0.2~20	800
Control	0	830

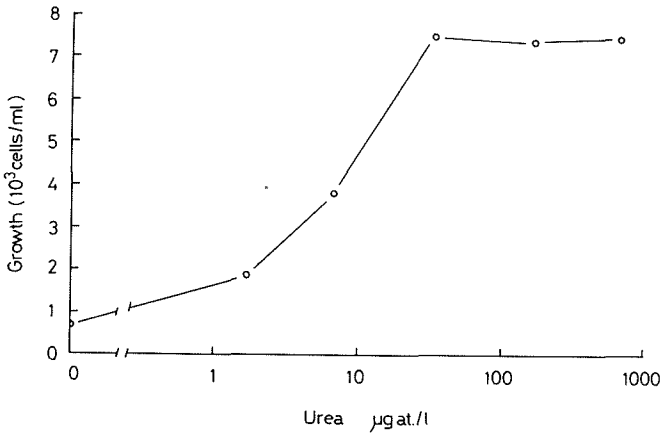

Fig. 32. Effect of urea on the growth of *P. micans*.

Silica

Silica requirement was studied using 50 mℓ polycarbonate bottles (LEWIN 1966). Even in media lacking silica, normal growth was obtained (Fig. 33), accordingly, this species has no requirement for silica.

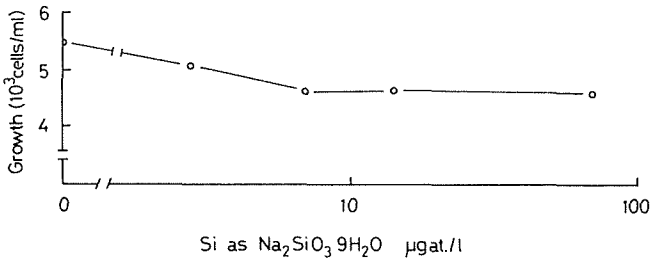

Fig. 33. Effect of silica on the growth of *P. micans*.

Vitamins

Vitamin requirements were examined for B_{12}, biotin, and thiamine. As a result (Figs. 34, 35), B_{12} and thiamine were required for the optimal growth. B_{12} starved cells were obtained by cultivating the organism for 10 days, and such prepared inocula could not survive in B_{12}-free medium. The optimal concentrations were $7.4\times10^{-3}-7.4\times10^{-1}$ ng at./liter (Fig. 34). On the other hand, thiamine had only stimulatory effect, since it could grow into 2.6×10^5 cells/mℓ even after three successive pre-cultures without thiamine. The optimum growth was obtained at the concentrations between 3×10^{-2} -30 ng at./liter (Fig. 35). Different from B_{12} and thiamine, growth was not supressed in biotin-free medium. The growth of the organism in the medium containing these three vitamins reached almost the same level as that in the medium with vitamin mix 8A, therefore, those compounds contained in this mixture except B_{12} and thiamine are considered to be inessential for the growth of this species.

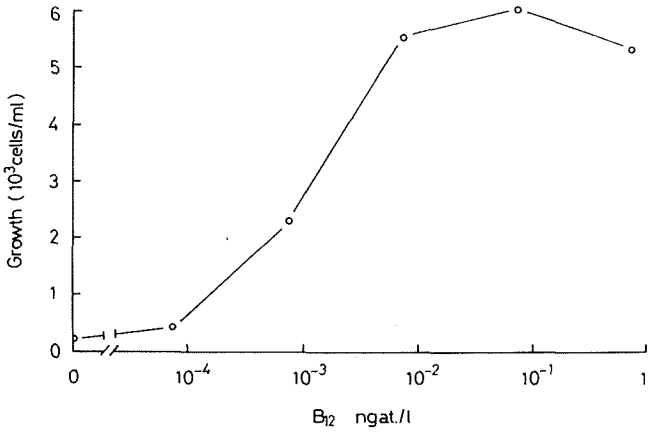

Fig. 34. Effect of B_{12} on the growth of *P. micans*.

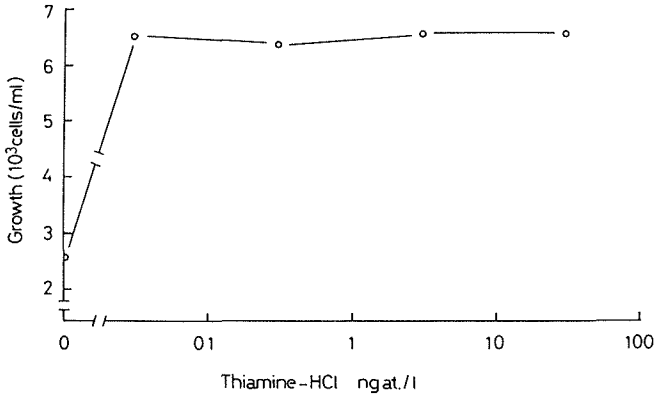

Fig. 35. Effect of thiamine on the growth of *P. micans*.

Trace metals

The requirements of trace metals were examined for iron, manganese, cobalt and zinc. Among them, only iron requirement was detected (Fig. 36). However, the addition of $1.8\times10^{-2}-1.8\,\mu$g at./liter of iron was adequate for the optimal growth.

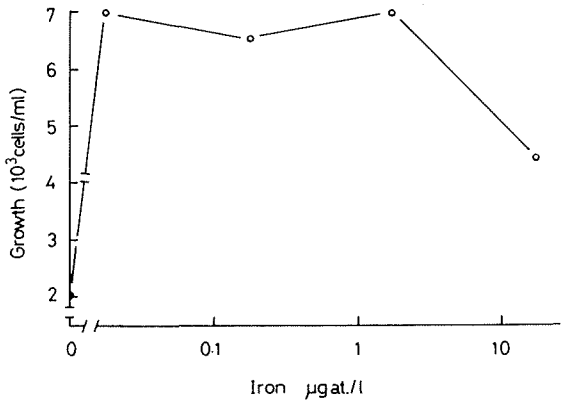

Fig. 36. Effect of iron on the growth of *P. micans*.

Discussion

IWASAKI (1973) reported that most red tide flagellate species which were examined by him were found to be euhaline. *P. micans* originated from Plymouth collection grew well in wide range of chlorinity of 11-20‰ (KAIN and FOGG 1960). This is almost consistent with the results obtained in the present study.

KAIN and FOGG (1960) showed that the Plymouth strain of *P. micans* could grow at various pH values of 7.5-8.75. The Muroran strain of this species also showed wide

tolerance to pH value. It could grow at pH of 7.0–9.0. Such adaptation to the wide range of pH and chlorinity seems to be correspond to the fact that the species is a cosmopolitan (WOOD 1968).

Other than ortho-phosphate, phosphomonoesters such as glycerophosphate and glucose-6-phosphate could serve as phosphorus sources (PROVASOLI 1958, KUENZLER and PERRAS 1965, IWASAKI 1967). In the present study, β-glycerophosphate was utilized by the species as well as ortho-phosphate.

Nitrate and ammonium serve as good nitrogen sources for many planktonic algae (PROVASOLI 1958, IWASAKI 1967, 1973). The organism examined in the present study well utilized these nitrogen compounds as the sole nitrogen source. In addition to these inorganic nitrogen compounds urea could serve as a nitrogen source and it permitted active growth as nitrate and ammonium. Ornithine was also found to support the growth but not so much as urea. Such ornithine cycle compounds could serve as nitrogen source for some phyto-flagellates (PROVASOLI 1958, IWASAKI 1967, 1973, ANTIA *et al.* 1975, 1977).

It is known that many algae require vitamins which are always limited to B_{12}, thiamine and biotin. According to the review by PROVASOLI (1958), of 90 microalgal species only 31 species required no vitamins and other 59 species showed requirements for three vitamins, alone or in combination. In *Prorocentrum micans* the Plymouth strain was reported to require B_{12} and biotin (KAIN and FOGG 1960). However, it is not clear whether these vitamins was absolute requirement or growth stimulator since seawater used as a basal medium may contain such organic substances. The strain used in the present experiments required B_{12} and thiamine, but not biotin. Since biotin requirement in Plymouth strain was shown in natural seawater medium, there may be some physiological differences between the two strains of *Prorocentrum micans*. Such differences between two strains of the same species were reported for some phytoplanktons (PROVASOLI and CARLUCCI 1974).

Iron requirement was demonstrated in some marine phytoplanktons (KAIN and FOGG 1960, IWASAKI 1971 b, LEWIN and MCKAS 1972, UCHIDA 1974). The organism used in the present study also showed iron requirement, however, trace amount was adequate for the optimal growth.

II–3 Effect of macronutrients and some environmental factors on the growth of two diatom species

Other than phosphorus and nitrogen, silicon is required in high concentrations for the growth of diatom species (LEWIN 1962). On the contrary, silicon requirement has not been found in dinophycean algae. *P. micans* investigated in the present study showed no requirement for silicon (II–2). The difference between diatom and dinoflagellate in nutritional source is expected to affect the formation of phytoplankton communities. In the present study, the requirements of silica by *Chaetoceros didymus* and

Skeletonema costatum were ascertained as well as phosphorus and nitrogen.

Methods

Screw cap test tubes and polycarbonate bottles were used as culture vessels (LEWIN 1966). As basal media, BSW 2 m and BSW 2 Am were used. Culture experiments were conducted at 20±1°C under the continuous illumination of 2,000 lx which was provided by cool white fluorescent lamps. For the inoculation the orgamisms were previously cultured in media lacking the compound to be studied. Growth was measured after 5-6 days from inoculation by counting a cell number with a hemacytometer.

Results and Discussion

Chlorinity

Effects of chlorinity levels on the growth of diatoms were investigated. There were no remarkable differences between 10 and 19‰ for *S. costatum* growth. At 22‰, growth was slightly supressed (Fig. 37). The preference of low chlorinity of this species was also ascertained by CURL and MCLEOD (1961). They measured photosynthetic ability of the organism at various salinity levels and found that optimum values of

Fig. 37. Effect of chlorinity on the growth of *S. costatum*.

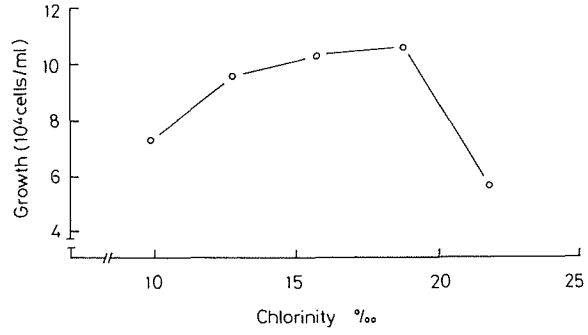

Fig. 38. Effect of chlorinity on the growth of *Ch. didymus*.

salinity were 16-22‰ (=8.6-12.2‰ chlorinity). The similar tendency was observed in *Ch. didymus* (Fig. 38). At 22‰ the growth of this species decreased to 1/2 as much as those in optimal conditions.

pH

Growth of each diatom was measured at various pH values. The results are shown in Figs. 39 and 40. *Skeletonema* had wide tolerance to pH as compared to *Chaetoceros*. The former species showed good growth even at pH of 7.5, on the other hand, growth of the latter species was supressed at this value.

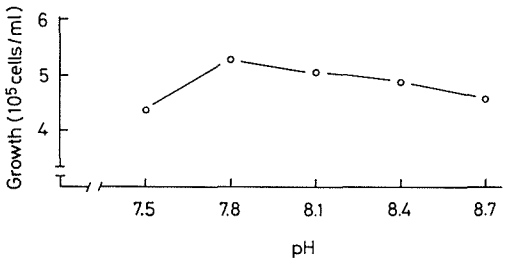

Fig. 39. Efftec of pH on the growth of *S. costatum*.

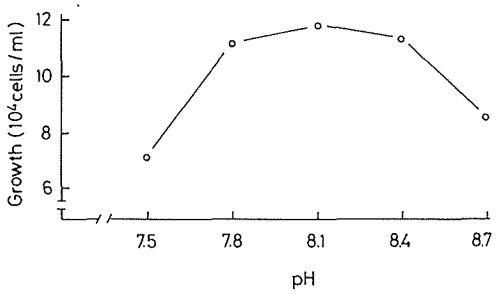

Fig. 40. Effect of pH on the growth of *Ch. didymus*.

Silicon

Both species required high concentrations of silicon. The optimal concentration of Na_2SiO_3 9 H_2O was 352-1,410 μg at./liter for *Skeletonema* and 352 μg at./liter for *Chaetoceros* (Figs. 41, 42). These results indicate that silicon concentration is an important factor regulating these diatom abundance in Muroran harbor where silicate concentration was recorded between 0.54-48 μg at./liter. According to SCHELSKE and STOERMER (1971), silica depletion by vigorous diatom growth is sometimes observed in eutrophic area owing to high concentrations of phosphorus and nitrogen.

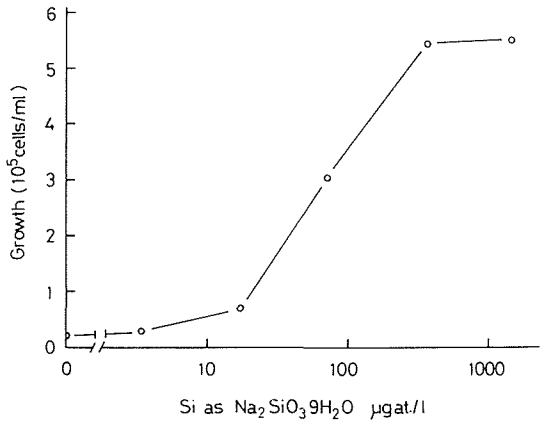

Fig. 41. Effect of silicate on the growth of *S. costatum*.

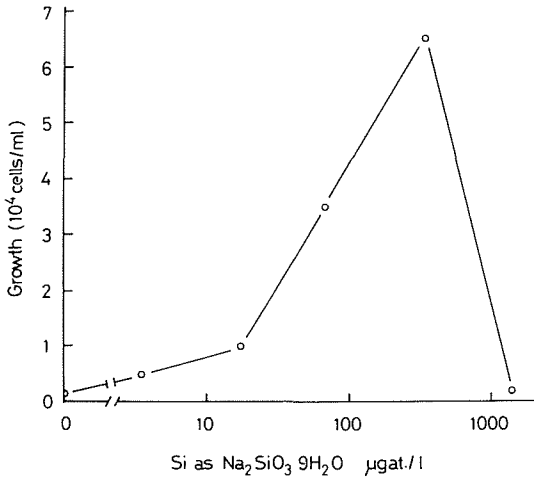

Fig. 42. Effect of silicate on the growth of *Ch. didymus*.

Nitrogen

Ammonium and nitrate were investigated in their ability to support the growth. The growth of both species was inhibited at high concentrations of ammonium (Figs. 43, 44) as in the case of other species of phytoplanktons (IWASAKI 1967). Active growth was obtained at 46.3–962 μg at./liter of NH_4Cl for *S. costatum* and 37–185 μg at./liter for *Ch. didymus*. In nitrate medium, on the other hand, optimal growth could be measured up to 3,530 μg at./liter of $NaNO_3$ for both species (Figs. 43, 44).

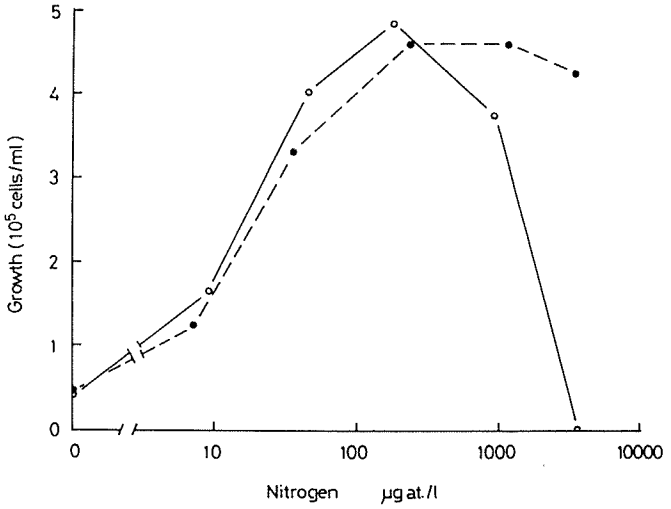

Fig. 43. Effect of nitrogen sources on the growth of *S. costatum*.
——○—— NH_4Cl, - - -●- - - $NaNO_3$.

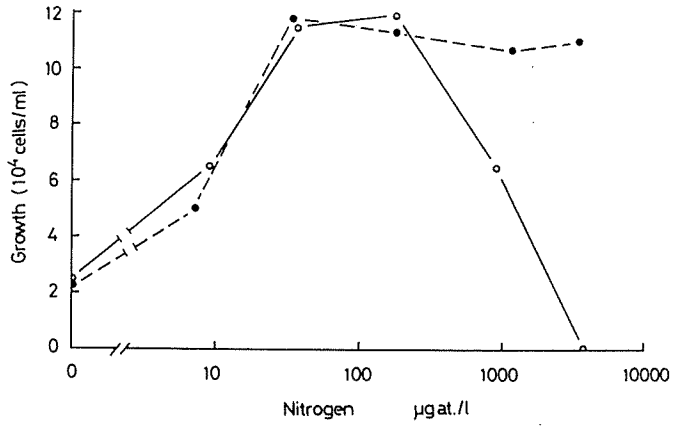

Fig. 44. Effect of nitrogen sources on the growth of *Ch. didymus*.
——○—— NH_4Cl, - - -●- - - $NaNO_3$.

Phosphorus

KH_2PO_4 and Na_2-β-glycerophosphate were used as phosphorus sources. The former compound permitted optimum growth at concentrations of 1.5－147.1 μg at./liter for *Chaetoceros* and 36.8-147.1 μg at./liter for *Skeletonema* (Figs. 45, 46). On the other side, the latter compound can serve as phosphorus source to a less extent. The active growth was observed at concentrations of 6.5-163.4 μg at./liter for *Skeletonema* and

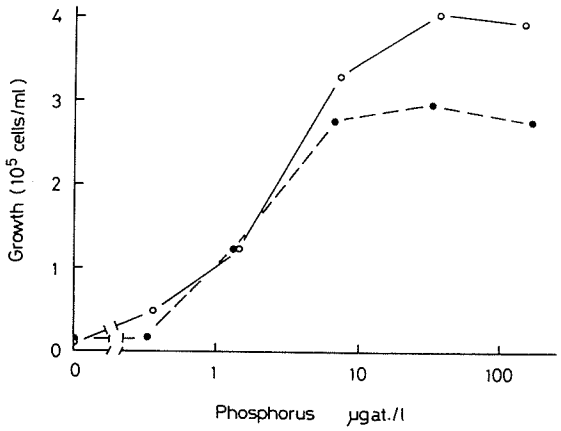

Fig. 45. Effect of phosphorus sources on the growth of *S. costatum*.
——○—— KH_2PO_4, - - -●- - - β-glycerophosphate.

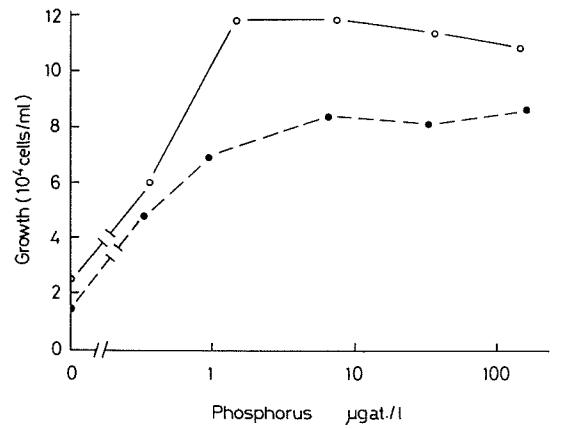

Fig. 46. Effect of phosphorus sources on the growth of *Ch. didymus*.
——○—— KH_2PO_4, - - -●- - - β-glycerophosphate.

0.98–163.4 μg at./liter for *Chaetoceros* (Figs. 45, 46). It is different from these species that glycerophosphate could be more effective than inorganic phosphate as phosphorus source for some phytoplankters (IWASAKI 1967).

In these ways, nutrient requirements by diatoms have definite difference from that of *P. micans* investigated in the present study in requiring silica in high concentrations.

II-4 Growth supporting potential of Muroran harbor seawater for *Prorocentrum micans*

The results obtained by the field observations in Muroran harbor indicate that nutrient increase was consistent with *Prorocentrum micans* growth. Among them,

phosphate concentration showed close correlation with *Prorocentrum* cell density. This result suggests that the amount of phosphorus limits the *Prorocentrum* growth in Muroran harbor. To ascertain this presumption the growth response of this species to Muroran harbor seawater, which had been collected periodically, was tested to know the relationships between the variations of phosphate concentrations and those of seawater potential supporting the growth of this species.

In spite of the existence of vitamin B_{12} and thiamine requirements by the organism, there is none of informations for these vitamins concentrations in seawater. The present bioassay experiments will answer whether the compounds regulate the growth of *P. micans* in the harbor or not.

Material and Methods

The growth rate of *P. micans* in exponential phase was measured in surface and bottom seawater which were enriched in various ways. Method used for the calculation of growth constant was the same as that described before (II-1). Seawater sampling had been carried in the same manner as that in I-1. Seawater samples collected at each station were mixed in equivalent amount, which were passed through glass fiber filter (pore size, 1 μm) and were deep frozen (below -20°C) untill the experiments were conducted. In the experiments, frozen samples were melted at room temperature and adjusted pH to 8.0 with tris-HCl buffer (tris lg/liter in final concentration), which was then millipore filtered (0.22 μm GS) to obtain axenic seawater. Such prepared seawater samples were enriched in the following ways using BSW 4 (Table 3) as a basal medium: 1) all nutrients 2) phosphate 3) vitamins (B_{12}, thiamine, biotin) 4) no nutrients. As culture vessels, 100 mℓ Erlenmyer flasks with 50 mℓ of the test medium were used. For the inoculum, the organism was grown in unenriched seawater for 5 days through two successive cultures to minimize carryover of nutrients and to cause nutrient limitation. Culture experiments were conducted at 20 ± 1°C under continuous illumination by cool white fluorescent lamps regulated to 2,000–3,000 lx.

Results

Growth supporting potential of seawater

The growth rate in unenriched seawater was measured to determine potentiality of each seawater sample collected at different times. The results are shown in Fig. 47. The potential of surface seawater was kept at lower level from June to August. During this period, growth constant K was measured below 0.2. On the other hand, bottom seawater showed high growth potentiality except early August, namely, growth in unenriched seawater was almost the same as in enriched seawater. In late September, significant difference could not be found between enriched and unenriched seawater

for both bottom and surface. Fig. 48 shows the changing pattern of K in surface seawater together with phosphate and nitrate concentrations. The changing pattern of growth supporting potential is consistent with that of phosphate concentration.

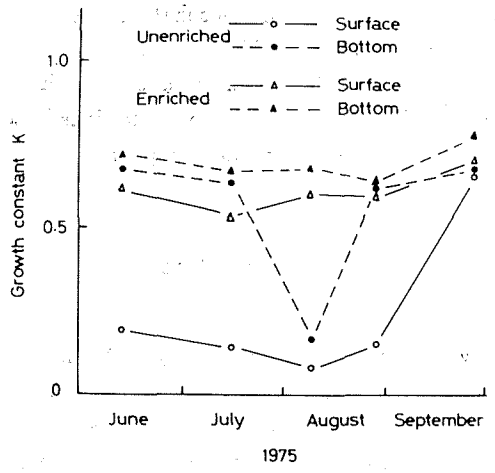

Fig. 47. Growth supporting potential of Muroran harbor seawater for *P. micans* from June to September, 1975.

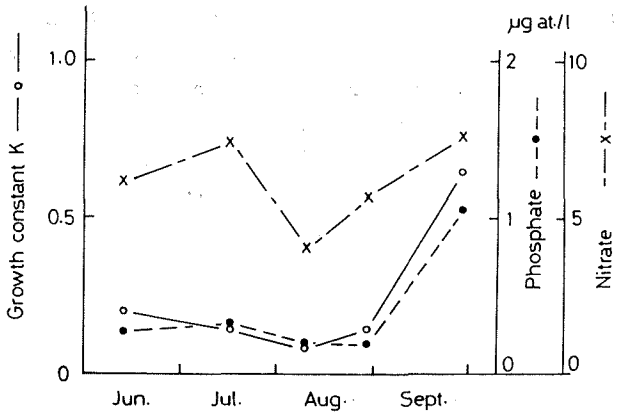

Fig. 48. Correlation of growth supporting potential to phosphate and nitrate concentrations in surface seawater from July to September, 1975.

Effect of phosphate addition

Phosphate was added to ascertain whether nutrient limited cells could recover the growth or not. As a result (Figs. 49, 50), the growth was increased almost to the

same level by addition of phosphate as in the seawater with complete nutrients. This is true for all seawaters tested.

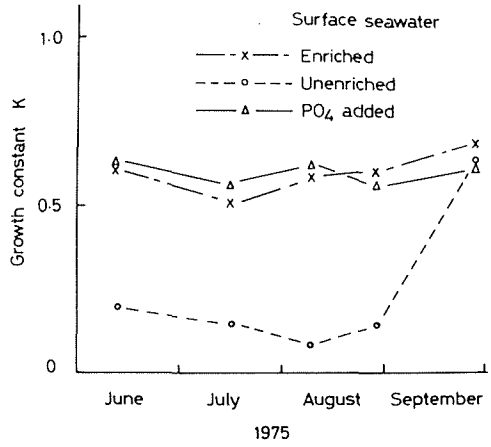

Fig. 49. Effect of phosphate enrichment on the growth of *P. micans* in surface seawater.

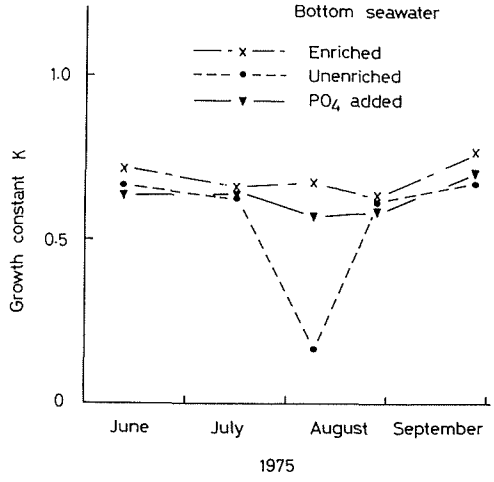

Fig. 50. Effect of phosphate enrichment on the growth of *P. micans* in bottom seawater.

Growth in seawater enriched with vitamins

The growth rate of this species was measured in seawater enriched with vitamins. As shown in Fig. 51, the additions of three vitamins could not promote the growth of this species. Growth constant measured in vitamin enriched seawater was almost the same as in unenriched seawater.

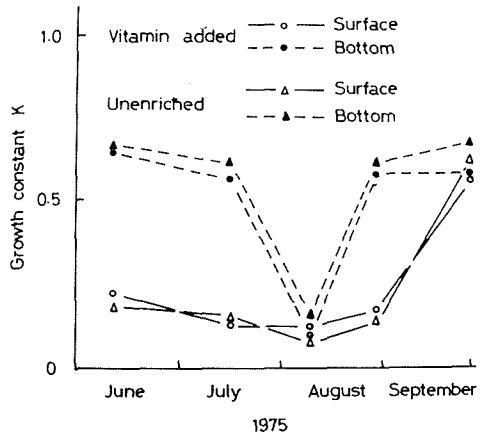

Fig. 51. Effect of vitamin enrichment on the growth of *P. micans*.

Discussion

The variations of growth supporting potential of seawater were almost consistent with those of phosphate concentrations. On the contrary, none of other nutrient factors showed the correlation to the growth supporting potential of seawater. However, growth supporting potential of bottom seawater collected in June and July was almost at the same level as that of September, though phosphate concentrations of summer seawater were less than those of autumn. This is considered to be due to the fact that phosphate amount of seawater in June and July was adequate to permit maximum growth rate in an early stage of the growth.

From the results obtained, phosphate is considered to limit the growth of *P. micans* in Muroran harbor. This is confirmed by further experiments. The addition of phosphate to natural seawater increased the growth of this species almost to the same level as that in medium with complete nutrients. It has been known that phosphorus and nitrogen often limit phytoplankton growth in the sea. HARVEY (1933) found that natural population of *Nitzchia closterium* was promoted in their growth with the addition of phosphate. On the contrary, RYTHER and DUNSTAN (1971) reported that nitrogen is considered to limit phytoplankton growth in the coastal area off Long Island from the results of enrichment experiments using *Nannochloris atomus* and *Skeletonema costatum* as assay organisms. For the red tide organisms phosphorus sometimes becomes a limiting factor (IRIE 1973). However, IWASAKI (1973) suggested that other growth factors such as trace metals, vitamins, and other organic compounds are more important than phosphorus and nitrogen for the red tide occurrence from the results of culture studies on red tide flagellates. The growth of *Gymnodinium breve*, which is a causative organism of red tide, was partially promoted with the addition of Fe-EDTA to natural

seawater (COLLIER *et al.* 1969). The importance of metals for phytoplankton growth in natural habitat was also emphasized in some other enrichment experiments (IGNATIADES and SMAYDA 1970, SMAYDA 1974). The close relationships between phytoplankton growth and vitamin concentration were clearly demonstrated in some areas (MENTZEL and SPAETH 1962, OHWADA 1972, OHWADA and TAGA 1972). However, Muroran harbor seawater is considered to have sufficient metals and vitamins necessary for the growth of this species because only phosphate addition recovered the growth in natural seawater.

II-5 Effect of organic substances on the growth of *Prorocentrum micans*

It has been considered that seawater contains biologically active substances other than vitamins, which play a part in regulating phytoplankton ecology (CRAIGIE and MCLACHLAN 1964, MCLACHLAN and CRAIGIE 1964, PRATT 1966, PRAKASH and RASHID 1970).

In some cases of red tide, it is believed that unknown substances give explosive growth of causative organisms (IWASAKI 1969, OKAICHI and YAGYU 1969, UENO and NAGAI 1973).

As shown in many cases of red tide caused by flagellate, diatom growth is advanced before its occurrence (IWASAKI 1971 c). It can be considered that the growth stimulating substances for red tide flagellates are supplied with the decomposition of these diatom cells. Also in Muroran harbor, diatom blooms always preceded that of *P. micans*, therefore, it is very amusing to examine the interaction between them. In the present study, mixotrophism of *P. micans* was investigated on various organic substances. In addition, the decomposed matter of diatom cells was tested on its ability to stimulate the growth of this species.

Methods

Culture experiments were conducted in the same conditions as used before (II-2). As a basal medium, BSW 4 A was used (Table 3). Growth was measured by counting all cells in 0.1 mℓ sample. The decomposed matter of diatom cells were prepared as follows. *Skeletonema costatum* and *Chaetoceros didymus* were grown in BSW 2 Am. When each culture reached about 5×10^5 cells/mℓ for *Skeletonema* and 2×10^5 cells/mℓ for *Chaetoceros*, 500 mℓ of each suspended medium was centrifuged at 10,000 rpm. Each concentrated material was homogenized with teflon-glass homogenizer, which was suspended in 100 mℓ of BSW 4 A medium without nutrients and decomposed by bacteria both in anaerobic and aerobic conditions for 8 days at 20°C. The anaerobic decomposition was conducted in oxygen bottle containing the medium which had been once boiled and removed dissolved oxygen. The aerobic decomposition was made in a 200 mℓ Erlenmyer flask. Muroran harbor seawater was used for the inoculation of bacteria. Such prepared solution was millipore filtered (0.22 μm GS) and used for assays.

Table 5. Effect of organic substances on the growth of *P. micans* (Growth after 15 days).

Compounds	Optimum Concentration (mg/liter)	Growth on minimal medium=100% (0% 100 200 300)
Acetate	2~200	
Pyruvate	2	
Glycerol	2~200	
Lactate	20	
Glycolate	200	
Fumarate	2	
Succinate	2	
Malate	200	
Citrate	2	
Malonate	200	
α-Ketoglutarate	20	
Oxalacetate	2~200	
Dextrose	2	
Sucrose	2	
Butyrate	2	
Maleate	2	
L-Aspartate	2	
DL-Glutamate	2	
Glycine	20	
L-Lysine	2	
L-Valine	2	
L-Proline	2	
L-Leucine	20	
L-Alanine	2	
L-Serine	2~200	
DL-Tyrosin	20	
L-Tryptophan	2	
Urea	2~20	
IAA	0.1	
Gibberellic acid	10	
Kinetin	0.1~1	
Hypoxanthin	0.1~1	

Results

Carbon sources

The mixotrophic ability of this species was examined on 28 organic compounds as carbon sources at concentrations of 2, 20, and 200 mg/liter. The results are shown in Table 5. Of these compounds examined, L-leucine stimulated the growth at 2 and 20 mg/liter, however, it inhibited the growth at 200 mg/liter. In an optimal concentration (20 mg/liter), this substance permitted 2.3 times growth as much as that in control. Malate also promoted the growth of this species. At concentration of 200 mg/liter, it permitted 1.9 times growth as compared to that in control.

Growth regulators

Four growth regulatory substances were tested at 0.1, 1 and 10 mg/liter for the effect on the growth of the species. Among them, gibberellic acid was found to be stimulatory (Table 5). It promoted the growth to 1.6 times at 10 mg/liter as much as that in control.

Diatom decomposed matter

The decomposed matter of diatom cells was also tested on their ability to promote the growth. As a result (Figs. 52, 53), the decomposed matters of *Skeletonema* and *Chaetoceros* had a stimulatory effect and promoted the growth 2.6–2.8 times as much as that in controls. There were no remarkable differences between anaerobically and aerobically decomposed matters. Non-treated samples with bacteria was required at

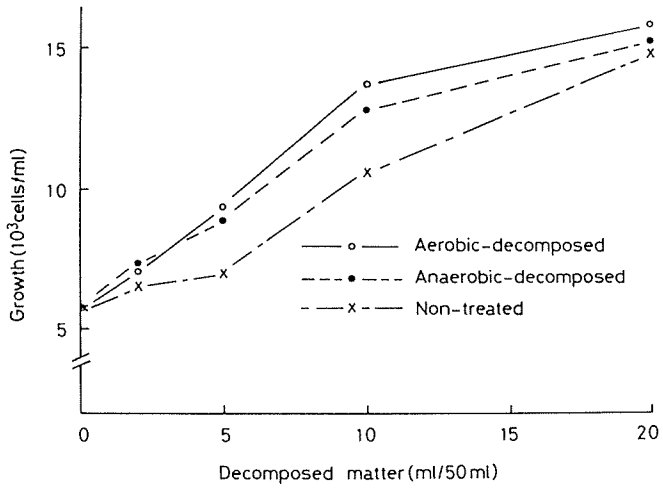

Fig. 52. Effect of *S. costatum* decomposed matter on the growth of *P. micans* (Growth after 15 days).

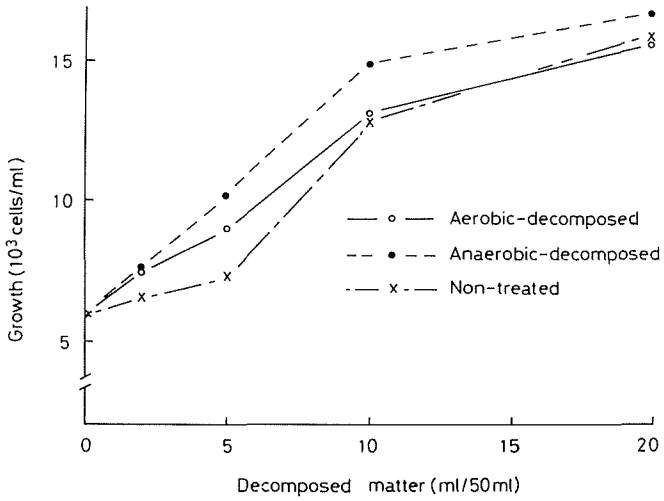

Fig. 53. Effect of *Ch. didymus* decomposed matter on the growth of *P. micans* (Growth after 15 days).

higher concentrations to support active growth, however, maximum growth reached the same level as those with decomposed materials by bacteria.

Discussion

Many species of red tide dinoflagellates are shown to be stimulated by some organic substances in their growth other than vitamins. Gibberellic acid promoted the growth of *Gymnodinium breve* (PASTER and ABBOTT 1970). In *Exuviaella* sp. (IWASAKI 1971 b) growth regulators including IAA, kinetin and gibberellic acid have growth promoting effect. The growth of *P. micans* studied in the present experiments was also stimulated by gibberellic acid. Other than growth regulatory substances, glutamate promoted the growth of *Peridinium hangoei* (IWASAKI 1969) and *Gymnodinium nelsoni* required organic nitrogenous compounds as a nitrogen source (IWASAKI 1973). *P. micans* investigated in the present experiments was promoted in their growth by malate and L-leucine in addition to gibberellic acid. Other than these substances, yeastolate, yeast extract and trypticase have stimulatory effect on red tide flagellates (IWASAKI 1969, 1971 a, b). In the present study, it is shown that diatom decomposed matter has a stimulatory effect on *P. micans* growth. Such mixotrophism is considered to concern with the red tide occurrence of some species (IWASAKI 1973), namely, effective organic substances in seawater promote the growth of red tide flagellates. Various ways can be considered to provide growth promoting substances to seawater. OKAICHI and YAGYU (1969) proved the stimulating effect of factory drainage on the growth of *Eutreptilla* sp. On the other side, IWASAKI (1969) reported that excrement of shell fish had growth-

stimulating effect on *Peridinium hangoei*. In the present study, the growth of *P. micans* was promoted in the presence of decomposed matter of diatoms which appeared in advance of this organism. Since a non treated sample with bacteria had a stimulatory effect, it seems to the writer that growth promoting substance was originally contained in diatom cells. Further, this substance may be produced by bacterial activity because lower concentration was adequate to support active growth when used decomposed materials by bacteria. These animal-phytoplankton and phytoplankton-phytoplankton relationships mediated by growth promoting substances are probably an important factor for flagellate red tide.

II-6 The interaction between *Prorocentrum micans* and two diatom species

The interaction among algal species has been shown by some workers (McLACHLAN and CRAIGIE 1964, PRATT 1966, HONJO *et al.* 1978). In an eutrophic area which has ability to support abnormal growth of phytoplanktons, biological interaction is considered to play an important role in phytoplankton ecology since dense populations affect each other.

In Muroran harbor it is shown that *Chaetoceros didymus*, *Skeletonema costatum* and *Prorocentrum micans* made a clear succession with each other. The decrease of these diatoms was found to be correlated to the disappearance of silicate in seawater. After the occurrence of *Prorocentrum* red tide, silicate concentration increased and other environmental conditions were prefereable to these species, however, few diatom growth was observed during this period. The present study was carried out to determine if diatom and *P. micans* interfere with each other by excreting growth inhibiting substances.

Methods

All experiments were conducted at 20±1°C in 14-10 : light-dark cycle and illumination by cool-white fluorescent lamps was regulated to 2,000-3,000 lx. Growth was measured with a hemacytometer or counting all cells in a 0.1 mℓ sample. As a culture medium BSW 4 was used with some modifications. As a sole nitrogen source NH_4Cl (8 mg/liter) or $NaNO_3$ was used. These two media with different source of nitrogen were supplied with 100 mg/liter of Na_2SiO_3 9 H_2O.

Results

Growth of two diatoms in bialgal cultures with *Prorocentrum*

The growth of *Skeletonema* and *Chaetoceros* was measured in media containing 1,000-2,000 cells/mℓ of *P. micans*. The results are shown in Figs. 54-57. In an early stage, there were no differences between growths in bialgal cultures and in controls for both species. However, after 3 days from inoculation the growth of both species

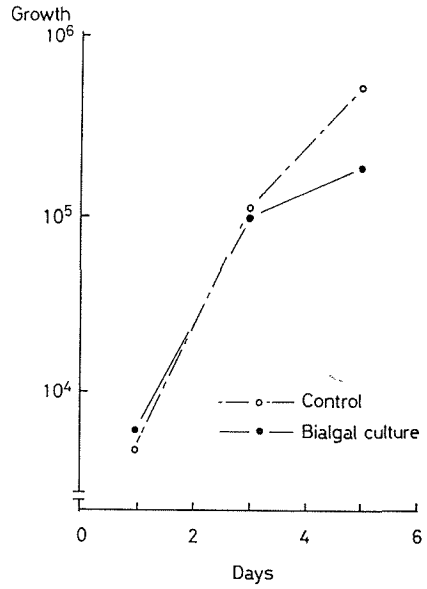

Fig. 54. Growth of *S. costatum* in bialgal culture with *P. micans* in nitrate medium.

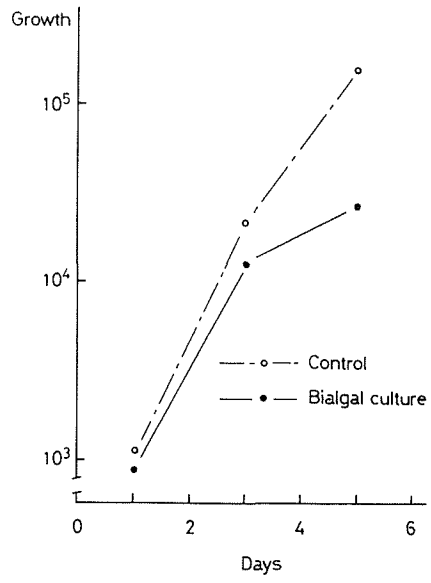

Fig. 55. Growth of *Ch. didymus* in bialgal culture with *P. micans* in nitrate medium.

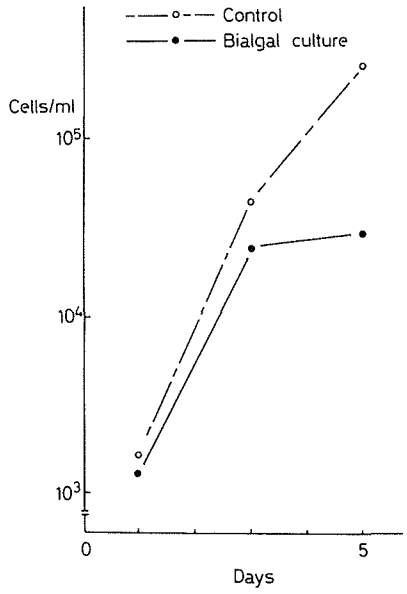

Fig. 56. Growth of *S. costatum* in bialgal culture with *P. micans* in ammonium medium.

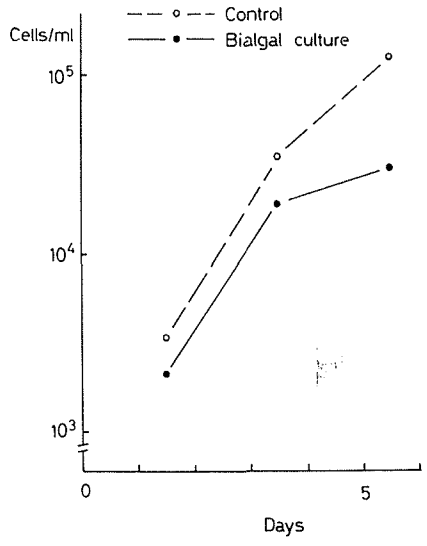

Fig. 57. Growth of *Ch. didymus* in bialgal culture with *P. micans* in ammonium medium.

was suppressed as compared to control. These tendency was observed both in NH_4Cl and $NaNO_3$ media.

Growth of two diatoms in *Prorocentrum*-conditioned medium

The results obtained in bialgal experiments show that the growth of diatom species is inhibited by the presence of *Prorocentrum*. However, it is still unknown how their growth is suppressed when cultured with this flagellate. Experiments were conducted to find out if a toxic substance mediated this phenomenon. For this purpose each diatom was grown in *Prorocentrum*-conditioned medium. *Prorocentrum* cells were grown in media supplied with NH_4Cl or $NaNO_3$ for 10 days, then they were filtered with millipore filter (0.22 μm GS) and the filtrates were enriched in the same way as the parent cultures. The growth of each diatom was suppressed remarkably in this conditioned medium when supplied with ammonium as a nitrogen source (Table 6). On the other hand, no inhibitory effect was observed in *Prorocentrum*-conditioned medium when supplied with nitrate (Table 7).

Table 6. Growth of two diatoms in *P. micans* conditioned medium supplied with ammonium as a nitrogen source (Growth after 5 days).

Species		Growth after 5 days (cells/ml)
Skeletonema costatum	Control	4.8×10^5
	Conditioned	8×10^3
Chaetoceros didymus	Control	1.2×10^5
	Conditioned	3×10^3

Table 7. Growth of two diatoms in *P. micans* conditioned medium supplied with nitrate as a nitrogen source (Growth after 5 days).

Species		Growth after 5 days (cells/ml)
Skeletonema costatum	Control	4.6×10^5
	Conditioned	4.5×10^5
Chaetoceros didymus	Control	1.3×10^5
	Conditioned	1.3×10^5

Growth of *Prorocentrum* in diatom-conditioned medium

In a general rule, diatoms showed fairly high growth rate, on the other hand, *Prorocentrum* grew very slowly. Accordingly, it is very difficult to assess diatom effect

on *Prorocentrum* in bialgal culture. The effect of diatom excrements on *Prorocentrum* growth was investigated only using diatom-conditioned medium. *Skeletonema* and *Chaetoceros* cells were grown respectively for 5 days, which was treated in the same manner as mentioned above. The results are shown in Table 8-9. The growth of this species was not inhibited in each medium supplied with nitrate and ammonium.

Table 8. Growth of *P. micans* in *S. costatum* conditioned medium (Growth after 15 days).

Nitrogen Source		Growth (cells/ml)
NH_4Cl	Control	6740
	Conditioned	6523
$NaNO_3$	Control	6560
	Conditioned	6538

Table 9. Growth of *P. micans* in *Ch. didymus* conditioned medium (Growth after 15 days).

Nitrogen Source		Growth (cells/ml)
NH_4Cl	Control	6390
	Conditioned	6128
$NaNO_3$	Control	6178
	Conditioned	6110

As mentioned above, *P. micans*-conditioned medium has an inhibitory effect on the growth of diatoms when supplied with ammonium as a sole nitrogen source. This implied that *P. micans* excretes an inhibitory substance to these diatoms. However, since conditioned medium was prepared by re-enrichment of *Prorocentrum*-cultured medium, amounts of nutrients in conditioned medium is considered to exceed those in the standard medium. Accordingly, it is possible that excessive nutrients inhibit the diatom growth in conditioned media. According to the following fact, however, this assumption could not be supported. Although these diatom species were inoculated in

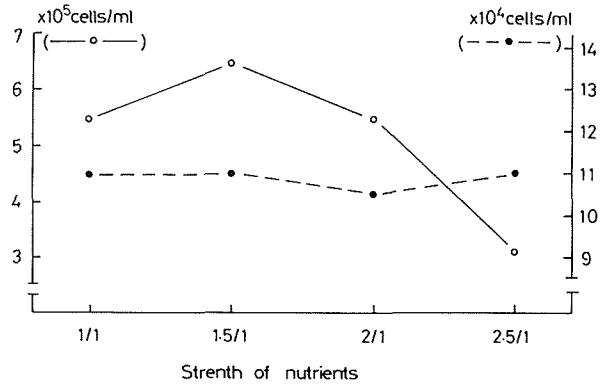

Fig. 58. Growth of *S. costatum* (——o——) and *Ch. didymus* (---●---) in different concentrations of nutrients (Growth after 5 days).

media containing different concentrations of nutrients from 1/1-2.5/1 times as much as in the standard medium, no unusual inhibition was observed for both species up to 2/1 times the concentration of the standard medium (Fig. 58).

Nature of the inhibitory substance

By means of the experiment on "Growth of two diatoms in *Prorocentrum*-conditioned medium", it is confirmed that *P. micans* secretes an inhibitory substance for both diatom species. This substance was subjected to dialysis and autoclaving (120°C, 15 min.). The conditioned medium was dialyzed using cellulose tubes in seawater at 14°C for 20 hrs. Both the outer and inner waters from such treated tubes were assayed with *Skeletonema*. The results are shown in Fig. 59 together with the results of growth in autoclaved conditioned medium. The inhibitory substance produced by *Prorocentrum* could not pass through the cellulose membrane and its inhibitory effect disappeared by autoclaving.

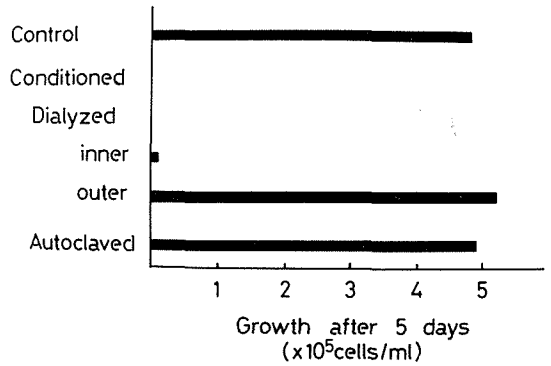

Fig. 59. Growth of *S. costatum* in dialyzed and autoclaved *Prorocentrum*-conditioned medium (Growth after 5days).

Discussion

In *Prorocentrum*-conditioned medium with nitrate the growths of two diatoms were not depressed, though in bialgal experiments both diatom species were inhibited by the presence of *Prorocentrum*. It must be remembered that nutrients in the medium are limited in volume. Since the available nutrients for diatoms in bialgal culture were less than those in conditioned media, it can be considered that nutrients become to limit diatom growth with the culturing age when grow with *Prorocentrum*. On the contrary, in the conditioned media supplied with ammonium the activity of inhibitory substance could be found. ELBRÄCHTER (1977) studied the interaction between *P. micans* and *Coscinodiscus concinnus* using a two-membered culture and concluded that none of inhibitory substance was excreted by both species. The medium used in his experiments contains nitrate as a nitrogen source. Also in the present study, the inhibitory

effect was not detected in the nitrate medium. It was observed when ammonium was used as a nitrogen source. It is expected that physiological conditions of the organism varied with the changes of its environment. AUBERT (1971) reported that *P. micans* excretes a kind of protein which inhibits the synthesis of antibiotics in *Asterionella japonica*, though he did not report its inhibitory effect on diatom growth. Other than *Prorocentrum*, some flagellates are known to excrete inhibitory substance for *Skeletonema costatum* (PRATT 1966). HONJO *et al* (1978) reported that the growth of *Skeletonema costatum* is inhibited by the excrements of *Heterosingma* sp. It is of interest that in any cases described above flagellates secrete an inhibitory substance to a diatom. It is worth examining as to whether these inhibitors are effective or not to other species of phytoplanktons.

Recently, PINTNER and ALTMEYER (1979) reported that many phytoplankton species excrete B_{12}-binding compound which inhibit B_{12} utilization by them, and that this substance is heat labile and non-dialyzable glycoprotein. The inhibitory substance detected in the present study is also considered to have a high molecular weight because it was non-dialyzable and heat labile. It can be considered that the inhibitor detected in the present experiment is the B_{12}-binding factor. However, the inhibitory effect could not be shown in the medium with nitrate as a nitrogen source. Since B_{12}-binding factor was detected in their medium with nitrate and amino acids as nitrogen sources, it seems different from the inhibitor observed in the present study. A possible consideration is that the inhibitory substance is more actively released by *Prorocentrum* in ammonium medium than in the medium with nitrate as a nitrogen source. If the conditioning period is fully prolonged, it is not unlikely that inhibitory effect can be observed in nitrate medium. In the present step, it is unclear whether it has the same function as a B_{12}-binding factor or not. Other than a B_{12}-binding substance, PINTNER and ALTMEYER (1979) showed species-specific low molecular inhibitors in some phytoplanktons. As for the nature of *Skeletonema*-inhibiting substance excreted by *O. luteus*, PRATT (1966) concluded it has a tannoid nature judging by the previous results reported by MCLACHLAN and CRAIGIE (1964).

Such inhibitory substances are considered regulating phytoplankton succession (PRATT 1966, UCHIDA 1977, HONJO *et al.* 1978, KAYSER 1979, SHARP *et al.* 1979, PINTNER and ALTMEYER 1979). The competition between *O. luteus* and *S. costatum* which demonstrated in culture has been observed in nature (PRATT 1966). HONJO *et al.* (1978) studied the red tide of *Heterosigma* sp. both in the field and in culture, and they found that the inhibitor excreted by this flagellate contributes to the monospecific bloom of this species. Also in Muroran harbor where high concentrations of ammonium were observed, the inhibitory substance examined in the present study is considered to affect the succession from diatoms to *Prorocentrum micans*.

Summary

Prorocentrum micans is a widely distributed dinoflagellate species and sometimes causes red tide. In Muroran harbor, Hokkaido, this species appeared late summer-erarly autumn and matured into red tide every year. Monthly observation through two years showed that diatom bloom always advanced the appearance of this flagellate, and that this diatom bloom was mainly composed of *Skeletonema costatum* and *Chaetoceros didymus*. The experiments were carried out both in the laboratory and the field to analyze the succession from diatoms to flagellate.

Three phytoplankton species including *P. micans*, *S. costatum* and *Ch. didymus* were isolated into axenic culture which have been maintained in the laboratory and used for experiments.

Nutritional characteristics of *P. micans* were, at first, studied to identify important factors for the growth of this species and also to develop defined medium. This species could utilize nitrate, ammonium and urea as nitrogen sources. As phosphorus sources, inorganic phosphate and β-glycerophosphate could be utilized. Of 3 vitamins tested, B_{12} and thiamine requirements were detected. Among trace metals, only iron requirement could be demonstrated. Optimum pH and chlorinity ranged from 7.8–8.6 for pH, and 10–22‰ for chlorinity. Nutritional study was also performed on *Ch. didymus* and *S. costatum* for nitrogen, phosphorus and silicate. From the results obtained, it is confirmed that the nutritional character of these diatoms was different from *P. micans* in requiring silicate.

Pararell to these laboratory experiments, the variations of environmental factors in Muroran harbor seawater were monitored at three stations in both surface and bottom seawater to know the relation between *P. micans* growth and these environmental factors. At the diatom blooming dense phytoplankton cells were observed only in the surface and few could survive in the bottom seawater. This is considered to be due to the poorness of available sunlight in this layer owing to the dense cells in the surface and also due to the presence of thermocline which blocks vertical mixing of seawater. Accompany with the decrease of silicate in the surface seawater, diatom cells gradually disappeared and bottom nutrients had been gradually increased, which resulted from the decomposition of diatom cells by bacteria. The decrease of dissolved oxygen in the bottom seawater implies the high activity of bacteria in this layer. In these ways, the difference of nutrient concentrations between surface and bottom seawater had become large, which continued to the breakdown of thermocline. After the thermocline had disappeared, nutrients prevailed to the surface seawater. At this time *P. micans* appeared and grew to form red tide. Among various ecological factors measured, phosphate concentration and COD showed close correlation to the density of *P. micans* cells. This result suggests that the amount of phosphate concentration in seawater

limits the growth of this species. This supposition was ascertained by bioassay experiments. Namely, the variations of seawater potential to support *P. micans* growth was almost consistent with the variations of phosphate concentrations in seawater. Further, phosphate supplied to natural seawater promoted the growth to the same level as that in seawater with complete nutrients. COD increase in the occurrence of *P. micans* blooming is considered to result from the decomposition of diatom cells by bacteria.

The effects of various organic compounds were tested for their ability to stimulate the growth of *P. micans*. Of 28 organic compounds tested, malate, L-leucine and gibberellic acid were found to be effective. From this result, it is considered that some organic compounds play a part in maturing of *P. micans*-red tide by stimulating the growth of this species, and that effective compounds were produced by the decomposition of diatom cells. From this point of view, diatom-decomposed matter by bacteria in both aerobically and anaerobically conditions was tested for the ability to promote the growth of this species. As a result, *P. micans* growth was promoted in the presence of diatom decomposed matter produced in both conditions. After *Prorocentrum* red tide occurrence, few diatom cells could be observed though nutrients conditions, especially for silicate, had recovered. This fact implies the biological interaction between diatoms and *P. micans*. Culture experiments were conducted to confirm this assumption. As assay organisms, *S. costatum* and *Ch. didymus* were used since they were major components of diatom bloom. The growth of each diatom was suppressed in bialgal cultures with *P. micans* and in *P. micans*-conditioned medium supplied with ammonium as a nitrogen source. From these results it was concluded that *P. micans* secretes an inhibiting substance to diatom growth. This substance was found to be non-dialyzable and its effect disappeared by autoclaving. In nature, it is considered to be a regulating factor for a diatom-*Prorocentrum* succession.

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