Jpn. J. Phycol. (Sôrui) 38: 155–161, June 20, 1990

Growth characteristics of a dinoflagellate *Gymnodinium nagasakiense* Takayama et Adachi

Hideo Iwasaki, Chang-Hoon Kim and Masataka Tsuchiya

Faculty of Bioresources, Mie University, Kamihama-cho 1515, Tsu, 514 Japan

Iwasaki, H., Kim, C.-H. and Tsuchiya, M. 1990. Growth characteristics of a dinoflagellate *Gymnodinium nagasakiense* Takayama et Adachi. Jpn. J. Phycol. 38: 155–161.

Gymnodinium nagasakiense, which is one of the most harmful red tide organisms appearing in the coastal waters of Japan, was obtained in axenic clonal culture by micropipette washings. The growth characteristics of the strain were examined. Optimal growth rate was obtained at temperatures of 20–25°C, salinities of 25–26‰, light intensities above 130 μE/m^2/sec, and pH 8.0. Both inorganic and organic nitrogen, and phosphorus served as good nitrogen and phosphorus sources. Several organic substances were utilized and encouraged growth, however, they did not support growth in the dark. Iron and manganese promoted growth remarkably at a concentration of 200 μg/*l*. The organism needed vitamin B_{12} for growth. Addition of thiamine increased growth in the presence of vitamin B_{12}. The critical concentration of vitamin B_{12} was 10 ng/*l*. The pattern of specificity is similar to that of *Escherichia coli* 113-3. Benzimidazole cobalamine and 5-methylbenzimidazole cobalamine supported as much growth as vitamin B_{12}.

Key Index Words: Dinophyceae—growth rate—Gymnodinium nagasakiense—*nutrients requirement—red tide.*

The dinoflagellate *Gymnodinium nagasakiense* Takayama et Adachi is one of the most harmful organisms to mariculture. The organism was first found in Ōmura Bay, the northwestern part of Kyūshū, and tentatively called *Gymnodinium* type-'65 by Iizuka and Irie (1969). Later the organism was called by various other tentative names, such as *Gymnodinium* sp., or *Gymnodinium nagasaki* until the proposal by Takayama and Adachi (1984). The blooms of this organism frequently occur in the coastal waters of Japan and Korea, killing a large number of farm fish and causing great economic damage to other fisheries.

Many ecological and physiological studies on the organism have been made by Iizuka, Hirayama and their collaborators (Iizuka 1972, 1976, 1979, Iizuka and Irie 1966, 1969, Iizuka and Nakajima 1975, Hirayama and Numaguchi 1972, Hirayama *et al.* 1972, Numaguchi and Hirayama 1972, Abe and Hirayama 1979, Hirayama and Kawabata 1982), and Nishimura (1982).

Iizuka (1982) found the *G. nagasakiense* tolerated anoxic or near anoxic conditions in Ōmura Bay and also that it utilized sulfide from the sediment. Hirayama and Numaguchi (1972), in assaying the organism, suggested that the dissolution of anaerobically decomposed products of the bottom mud into seawater might be one of the cause of red tide outbreak in Ōmura Bay. Nishimura (1982) reported that seawater samples collected from a fish farm supported good growth of *G. nagasakiense*; its growth was promoted by the addition of extracts from mackerel meat and yellowtail feces to seawater in low concentration. He suggested that dissolved organic matters in fish farms may play important role in the occurrence of red tide of *G. nagasakiense*. However, the basal nutrition and growth response to other environmental factors have not yet been studied in axenic culture. Therefore, the present paper deals with growth response to ecological factors, such as light, temperature, salinity, pH, and main nutrients requirement.

Materials and Methods

An axenic clone of *Gymnodinium nagasakiense* obtained from Gokasho Bay in 1984 by micropipette washings was used for the experiments. Seawater base medium SWII was used to study the effects of light, temperature, salinity, and pH, while PROVASOLI's (1957) artificial medium ASP_2NTA was used to ascertain the nutrients requirement (Table 1). The cells which had been precultured for 14 to 18 days in medium lacking the compound to be tested were inoculated to the test media so as to give an initial concentration of 200 to 400 cells/m*l*. The cells were grown in 20 × 125 mm screw cap tubes containing 10 ml of medium under illumination with "cool white" fluorescent lamps (about 4000 lux) for 12 hours daily.

Table 1. Composition (w/v) of culture media.

	(m)SWII	ASP_2NTA
Distilled water		1,000 m*l*
Filtered seawater	1,000 m*l*	
NaCl		28 g
$MgSO_4 \cdot 7H_2O$		7 g
$MgCl_2 \cdot 6H_2O$		4 g
KCl		700 mg
Ca (as Cl)		400 mg
$NaNO_3$	72 mg	100 mg
KH_2PO_4	4.5 mg	
K_3PO_4		10 mg
Na_2-glycerophosphate	10 mg	10 mg
$Na_2SiO_3 \cdot 9H_2O$	10 mg	10 mg
Fe (as Fe-EDTA)	500 μg	
Vitamin B_{12}	(20 ng)	20 ng *
Biotin	(1 μg)	1 μg *
Thiamine	(100 μg)	100 μg *
P II metals**		10 m*l*
S 2 metals***		10 m*l*
"Tris" buffer	500 mg	1 g
Nitrilotriacetic acid		100 mg
pH	7.9–8.1	7.7–8.0

* Vitamin mix I.

** One m*l* of P II metals contains: EDTA, 1 mg; Fe (as Cl), 10 μg; B (as H_3BO_3), 0.2 mg; Mn (as Cl), 40 μg; Zn (as Cl), 5 μg; Co (as Cl), 1 μg.

*** One m*l* of S 2 metals contains: Br (as Na), 1 mg; Sr (as Cl) 0.2 mg; Rb (as Cl), 20 μg; Li (as Cl), 20 μg; I (as K), 1 μg; Mo (as $NaMoO_4$) 50 μg.

Temperature was kept at 19 to 20°C.

The seawater collected from Kuroshio current waters in Kumano-nada was heated gently up to 70°C, and passed through a glass-fiber filter (Whatman GF/F) after cooling. Glass-distilled water was passed through charcoal and ion-exchange columns before distillation.

In order to avoid trace metals contamination, laboratory ware used for culture was, as far as possible, made of teflon. Culture tubes were cleaned with soap, soaked in 0.05 percent EDTA solution for a day, rinsed thoroughly with distilled water, and placed for one hour at 250°C to eliminate organic traces. Growth yield was measured by direct counting the cells number or by using a Coulter counter after two to three weeks of incubation. The data were compared on the basis of growth yield. Growth rate (cell division rate) in exponential phase was calculated by;

Table 2. Sterility test medium ST3(s).

Filtered seawater	700 m*l*
Distilled water	250 m*l*
Soil extract	50 m*l*
$NaNO_3$	50 mg
Na_2-glycerophosphate	10 mg
Hy-case (Sheffield Chemical)	20 mg
Yeast extract (Difco)	10 mg
Liver oxoid L-25 (Oxo, LTD)	20 mg
Vitamin B_{12}	100 ng
Vitamin mix 8Am*	1 m*l*
Carbon source mix II**	20 m*l*
Glycylglycine	400 mg
Agar	(12 g)
pH	7.9

* Putrescine and folinic acid were omitted from original Vitamin mix 8A. One m*l* of Vitamin mix 8Am contains: thiamine HCl, 0.2 mg; nicotinic acid, 0.1 mg; Ca-pantothenate, 0.1 mg; riboflavin, 5 μg; pyridoxine 2HCl 40 μg; pyridoxamine 2HCl, 20 μg; *p*-aminobenzoic acid, 10 μg; biotin, 0.5 μg; choline H citrate, 0.5 mg; inositol, 1 mg; thymine, 0.8 mg; orotic acid, 0.26 mg; B_{12}, 0.05 μg, folic acid, 2.5 μg.

** One m*l* of Carbon source mix II contains: glycine, 1 mg; DL-alanine, 1 mg; L-asparagine, 1 mg; Na-acetate $3H_2O$, 2 mg; glucose, 2 mg; L-glutamic acid 2 mg.

$$D = 1/\ln 2 \cdot 1/t \cdot \ln N/N_0,$$

where N_0 is the initial cell concentration, and N is the cell concentration after t days.

All experiments were done in triplicate and contamination by bacteria was checked using ST3 medium (Table 2) of IWASAKI (1965).

Results and Discussions

Effect of light intensity: Growth at different light intensities was examined at 20±1°C and 25±1°C. In this experiment, different intensities of light were obtained by neutral density screening. Light intensity inside the tubes was measured by a spherical quantum sensor (Bio-spherical Instrument, Inc., U.S.A., model QSL-100). The results were shown in Fig. 1.

Growth was recognized at light intensity above 40 μE/m^2/sec, and growth rate reached its maximal level at 130 to 150 μE/m^2/sec. Saturating light intensities were about 130 μE/m^2/sec at 25°C, and about 150 μE/m^2/sec at 20°C. Maximal growth rates of 0.98 div./day at 25°C and 0.95 div./day at 20°C in this strain, and in another strain isolated from Suō-nada 1.20 div./day at 25°C were obtained.

These results coincide with the maximal growth rate of 1.05 div./day obtained by IIZUKA (1983) in natural population at surface water. Saturating light intensities obtained show that the organism has the ability to form dense populations even in turbid coastal

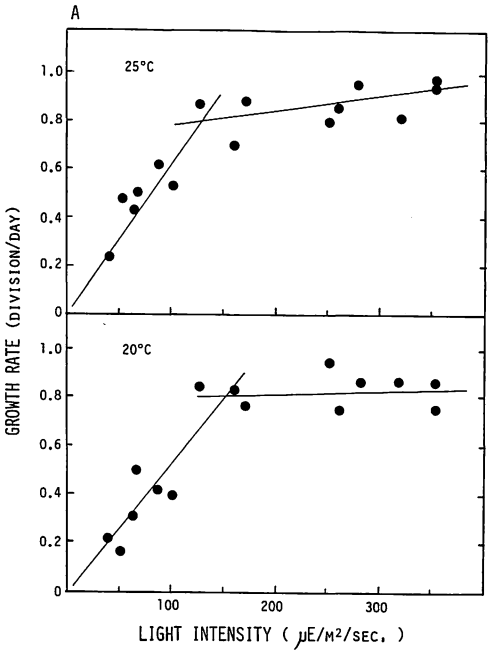

Fig. 1. Effect of light intensity on the growth rate of *Gymnodinium nagasakiense* at 25°C and 20°C.

waters of low light intensity, and may help to explain its occurrence at the subsurface (2-5 m in depth) water.

Effect of salinity: Several enrichments were added to seawater base and distilled water as in SWII formula, and further enriched with soil extract in 10 m*l*/*l*. The pHs were adjusted to 8.0. The latter medium was used to dilute the seawater base medium to the desired salinities varying from 7.9 to 34.5‰. Fig. 2 shows the growth of *G. nagasakiense* at

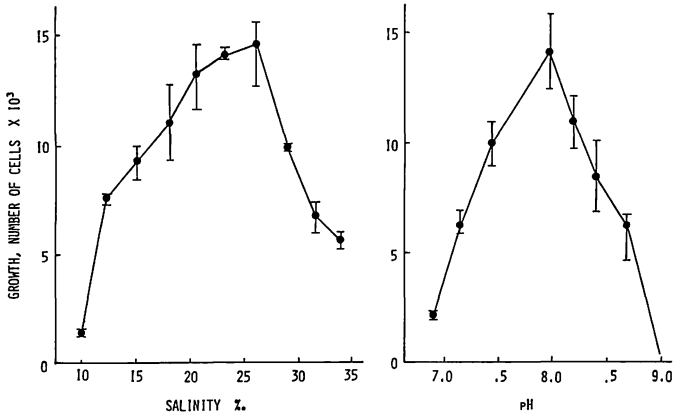

Fig. 2. Growth of *Gymnodinium nagasakiense* at different salinities (left) and pH (right).

various salinities. The organism showed preference for low salinities, grew well at the salinity of 12.0 to 31.0‰, and the maximal growth occurred at 26.0‰, which is lower than oceanic water. Growth was very much reduced at 10‰, and not recognized at 7.9‰.

Effect of pH: Growth at different pHs was examined using SWII based on seawater diluted to 26‰ S. Within the range of pH 7.0-8.8, the final pH of culture media changed very slightly from the initial pH (within 0.04 units). Maximal growth occurred at pH 8.0, as shown in Fig. 2, which is at slightly acid side of normal seawater. Upper side of the maximum growth dropped off fairly rapidly. However, in the range of pH 7.8-8.4, which includes almost the extreme variations for natural seawater, the organism grew well over half maximum. It is clear that the organism is not sensitive to small changes of pH as other neritic red tide flagellates, though it preferred slightly acidic than normal seawater.

Utilization of nitrogen and phosphorus sources: All the nitrogen compounds tested served as nitrogen sources; growth was better in low concentration (30 μg N/l) of ammonium chloride, and in high concentration ($>$300 μg N/l) of sodium nitrate and glutamic acid. Table 3 indicates that the increase in each nitrogen source produces no significant variation in the growth of the organism. Both ammonium chloride and urea inhibited growth when higher than 300 μg N/l.

The growth response to various phosphorus sources and concentrations are shown in Table 4. The organism utilized both inorganic and organic phosphate. The highest growth was produced at higher than 3 mg P/l of Na_2-glycerophosphate. However, no deviation in growth was observed at a concentration of 30 μg to 10 mg P/l with other phosphorus sources.

Table 3. Growth of *Gymnodinium nagasakiense* in ASP_2NTA with different nitrogen sources and concentrations (after 21 days).

Wt./l (as N)	Growth, number of cells/ml			
	$NaNO_3$	NH_4Cl	Urea	Glutamic acid
None added	3,490	3,490	3,490	3,490
30 μg	4,880	7,660	4,140	3,530
100 μg	5,050	5,830	6,160	3,630
300 μg	5,650	100	2,160	3,930
1,000 μg	8,020	0	0	8,120

The bloom of *G. nagasakiense* has been observed in high organic nutrients waters such as Gokasho Bay, and in poor inorganic nutrients waters as Kumano-nada coast and Suō-nada. The results make clear that one of the reasons why the organism appears in oceanic water such as Kumano-nada coast is its extremely low requirements of nitrogen and phosphorus, and also suggest that the organism can grow even in incomplete mineralization of nitrogen and phosphorus sources; this is particularly important for phosphorus which is often limiting, or close to a limiting condition.

Utilization of carbon sources: The experiments were conducted to find out whether the organism has heterotrophic abilities. As

Table 4. Growth of *Gymnodinium nagasakiense* in ASP_2NTA with different phosphorus sources and concentrations (after 19 days).

Wt./l (as P)	Growth, number of cells/ml				
	KH_2PO_4	Na_2-glycerophosphate	Adenosine 5′-monophosphate	Guanosine 5′-monophosphate	Citidine 5′-monophosphate
None added	1,700	1,700	1,700	1,700	1,700
30 μg	3,830	2,430	2,160	2,610	—
100 μg	3,160	2,290	—	—	—
300 μg	2,490	2,920	2,450	3,230	2,400
1 mg	2,470	3,260	2,760	3,140	2,170
10 mg	2,300	4,850	3,400	2,590	2,170

organic substances, 4 amino acids—glycine, DL-alanine, L-glutamic acid, and L-asparagine—, and 10 substances—acetate, sucrose, glucose, thiotone, trypticase, Hy-case, glutamate, yeast extract, yeastolate, and DNA—were examined in artificial medium ASP_2NTA containing nitrate. Sucrose, glutamate, Hy-case did not aid growth. All the other substances utilized encouraged growth (Fig. 3). Thiotone, yeastolate, and asparagine were effective at high concentration (100 mg/*l*). However, these organnic substances did not support any growth in dark condition.

Since the culture medium contains P II metal mix, which is in over chelation with EDTA, it is probable that these organic substances served only as carbon source. The result indicates that *G. nagasakiense* is limited heterotrophic.

Growth response to trace metals: To have preknowledge about trace metal requirement, growth in mSWII medium enriched with trace metal mix P II and S 2 of Provasoli was tested in advance. As clear in Table 5, an addition of P II metals to seawater medium stimulated remarkably growth, but S 2 metals showed no effect. Consequently, growth

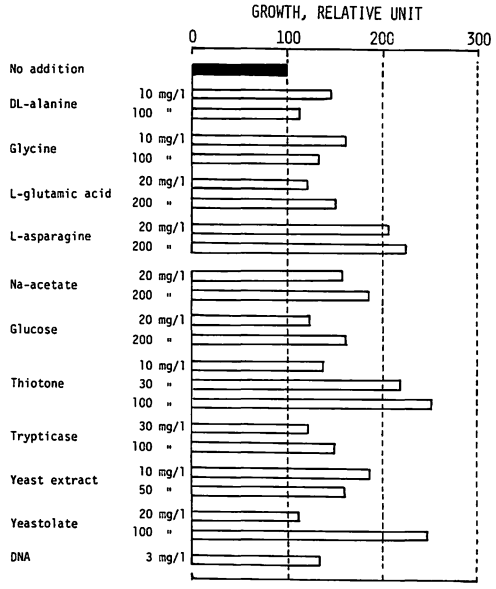

Fig. 3. Growth response of *Gymnodinium nagasakiense* to organic substances.

Table 5. Growth of *Gymnodinium nagasakiense* in diluted seawater medium mSWII (26‰ S) with addition of P II and S 2 metals (after 21 days).

Addition ml/100 ml		Growth, number of cells/ml
None added		1,500
P II metals	1 ml	10,930
S 2 metals*	1 ml	1,120
P II metals	1 ml	9,620
S 2 metals	1 ml	

* One ml of S 2 metals contains: Br (as Na) 1 mg, Sr (as Cl) 0.2 mg, Rb (as Cl) 20 μg, Li (as Cl) 20 μg, I (as K) 1 μg, Mo (as Na) 50 μg.

responses to the trace metals contained in P II were examined. Among these metals, only iron and manganese were very stimulative, and cobalt was slightly effective. As shown in Table 6, manganese and iron at 200 μg/*l* produced, respectively, a 16 to 18 fold higher growth when compared with natural seawater in 22 days incubation.

The growth response to iron and manganese is similar to the response of *Chattonella antiqua, Fibrocapsa japonica*, and *Alexandrium (=Protogonyaulax) tamarense* (Iwasaki 1979, Achiha and Iwasaki 1990). Ishimaru *et al.* (1989) found that selenium also stimulated remarkably growth of the organism. These results suggest that iron, manganese, and selenium play an important role in forming the blooms.

Vitamin requirements: Although the above

Table 6. Growth of *Gymnodinium nagasakiense* in diluted seawater medium mSWII with addition of different amounts of iron, manganese, and cobalt (after 22 days).

Addition, μg/*l*		Growth, number of cells/ml
None added		420
Fe (as EDTA)	30	980
	100	3,010
	200	7,780
Mn (as EDTA)	100	2,630
	200	7,000
	400	6,880
Co (as Cl)	10	900
	20	680

Table 7. Growth response to vitamins of *Gymnodinium nagasakiense* (after 17 days).

Vitamins		Growth number of cells
No addition		50
Vitamin mix I	10 ml/*l*	11,060
Vitamin mix 8Am	1 ml/*l*	8,210
Biotin	1 μg/*l*	0
Thiamine	100 μg/*l*	0
Cyanocobalamine (=B_{12})	200 ng/*l*	6,500
Vitamin B_{12} +biotin	200 ng/*l* 1 μg/*l*	6,800
Vitamin B_{12} +thiamine	200 ng/*l* 100 μg/*l*	8,350

experiments were carried out with media containing vitamin mix I which consists of vitamin B_{12}, biotin, and thiamine, the vitamin requirements were still unknown. Therefore, these requirements were examined. As shown in Table 7 and Fig. 4, *G. nagasakiense* needed vitamin B_{12} for growth. The highest growth was attained in presence of vitamin mix I. Biotin and thiamine alone did not support growth, however, thiamine promoted growth in the presence of vitamin B_{12}. The organism responded to all vitamin

Table 8. Growth response to vitamin B_{12} analogs of *Gymnodinium nagasakiense* (after 21 days).

B_{12} analogs at 100 ng/*l*	Growth number of cells × 10^3
Control	0.35
B_{12} (5,6-dimethylbenzimidazole)	5.35
5-methylbenzimidazole cobalamine	5.26
Benzimidazole cobalamine	5.32
Factor III (5-hydroxy-benzimidazole)	0.95
Factor A (2-methyladenine)	4.14
2-mercaptoadenine	3.28
Pseudovitamin B_{12}	0.2
Aetiocobalamine (=Factor 1B)	0.45
Factor B (no nucleotide)	0.78
Factor Z_1	1.25
Factor Z_2	1.28
Factor Z_3	1.97
B_{12}+penicillin (1000 I.U./*l*)	6.63

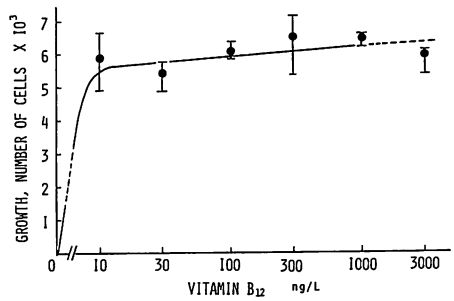

Fig. 4. Growth response of *Gymnodinium nagasakiense* to vitamin B_{12}.

B_{12} analogs except pseudovitamin B_{12} (Table 8). Vitamin B_{12}, 5-methylbenzimidazole cobalamine, benzimidazole cobalamine, and Factor A (2-methyladenine) analogs were nearly equal in their effeciency. A better yield was also obtained with 2-mercaptoadenine. The specificity was similar to that of *Escherichia coli* 113-3. An antibiotic penicillin also stimulated growth in the presence of vitamin B_{12}.

Red tide flagellates have been classified into three types by IWASAKI (1973) from the standpoint of nutritional characteristics. The experimental results showed that *G. nagasakiense* had the characters presented by both type II and type III. These growth responses to trace metals and organic substances, and the broad specificity to vitamin B_{12} analogs may constitute a significant ecological advantage.

We wish to express thanks to Dr. K. BERNHAUER who kindly supplied most of B_{12} analogs and to Dr. HALINA NEUJAHR who kindly made available the scarce factors Z_1, Z_2, and Z_3 that she isolated from sewage sludge. This work was supported in part by the sponsorship of Fisheries Agency, Japan.

References

ABE, T. and HIRAYAMA, K. 1979. Lethal effect of *Gymnodinium* sp. on the rotifer, *Branchionus plicatilis*. Bull. Fac. Fish. Nagasaki Univ. (46): 1–6. (in Japanese with English abstract)

ACHIHA, H. and IWASAKI, H. 1990. Growth characteristics of the toxic dinoflagellate *Alexandrium tamarensis*. Jpn. J. Phycol. **38**: 51–59. (in Japanese with English abstract)

HIRAYAMA, K., IIZUKA, S. and YONEJI, T. 1972. On culture of *Gymnodinium* type-'65 in the sea water sampled in Omura Bay during summer 1971. Bull. Fac. Fish. Nagasaki Univ. (31): 11–20. (in Japanese with English abstract)

HIRAYAMA, K. and KAWABATA, T. 1982. Growth of *Gymnodinium* sp. (type-'65) cultured in the seawater sampled at the southern part of Omura Bay-I. Bull. Fac. Fish. Nagasaki Univ. (52): 29–40. (in Japanese with English abstract)

HIRAYAMA, K. and NUMAGUCHI, K. 1972. Growth of *Gymnodinium* type-'65, causative organism of red tide in Omura Bay, in medium supplied with extract. Bull. Plankton Soc. Japan **19:** 13–21.

IIZUKA, S. 1972. *Gymnodinium* type-'65 red tide occurring in anoxic environment of Omura Bay. Bull. Plankton Soc. Japan **19:** 22–33. (in Japanese with English abstract)

IIZUKA, S. 1976. Succession of red tide organisms in Omura Bay, with relation to water pollution. Bull. Plankton Soc. Japan **23:** 31–43. (in Japanese with English abstract)

IIZUKA, S. 1979. Maximum growth rate of natural population of a *Gymnodinium* red tide. p. 111–114. *In* D. L. TAYLOR and H. H. SELIGER (eds.), Toxic Dinoflagellate Blooms. Elsevier North-Holland, New York.

IIZUKA, S. and IRIE, H. 1966. The hydrographic conditions and the fisheries damages by the red tide occurred in Omura Bay in summer 1965-II. Bull. Fac. Fish. Nagasaki Univ. (21): 67–101. (in Japanese with English abstract)

IIZUKA, S. and IRIE, H. 1969. Movement of red water plankton in the year of no red water occurrence in the case of Omura Bay in 1966. Bull. Fac. Fish. Nagasaki Univ. (27): 19–37. (in Japanese with English abstract)

IIZUKA, S. and NAKAJIMA, T. 1975. Response of red tide organisms to sulphide. Bull. Plankton Soc. Japan **22:** 27–32. (in Japanese with English abstract)

ISHIMARU, T., TAKEUCHI, T., FUKUYO, Y. and KODAMA, M. 1989. The selenium requirement of *Gymnodinium nagasakiense*. p. 357–360. *In* T. OKAICHI, G. C. ANDERSON and T. NEMOTO (eds.), Red Tides: Biology, Environmental Science, and Toxicology. Elsevier Sci. Publ., Amsterdam.

IWASAKI, H. 1965. Nutritional studies of the edible seaweed *Porphyra tenera* I. The influence of different B_{12} analogues, plant hormones, purines and pyrimidines on the growth of *Conchocelis*. Plant Cell Physiol. **6:** 325–336.

IWASAKI, H. 1973. The physiological characteristics of neritic red tide flagellates. Bull. Plankton Soc. Japan **19:** 104–114. (in Japanese with English abstract)

IWASAKI, H. 1979. Physiological ecology of red tide flagellates. p. 357–393. *In* M. LEVANDOWSKY and S. H. HUTNER (eds.), Biochemistry and Physiology of Protozoa, Vol. 2. Academic Press, New York.

NISHIMURA, A. 1982. Effects of organic matters produced in fish farms on the growth of red tide algae *Gymnodinium* type-'65 and *Chattonella antiqua*. Bull. Plankton Soc. Japan **29:** 1–7. (in Japanese with English abstract)

PROVASOLI, L., MCLAUGHLIN, J. J. A. and DROOP, M. R. 1957. The development of artificial media for marine algae. Arch. Mikrobiol. **25:** 392–428.

TAKAYAMA, H. and ADACHI, R. 1984. *Gymnodinium nagasakiense* sp. nov., a red-tide forming dinophyte in the adjacent waters of Japan. Bull. Plankton Soc. Japan **31:** 7–14.

岩崎英雄・金　昌勲・土屋正隆：渦鞭毛藻 *Gymnodinium nagasakiense* TAKAYAMA et ADACHI の増殖特性

1984年，三重県五ケ所湾に出現した赤潮海水から *Gymnodinium nagasakiense* を分離し，ミクロピペット洗浄法によって得られた無菌のクロン株を用いて，その増殖特性を調べた。本種は水温 20–25°C，塩分25–26‰，130 μE/m^2/sec 以上の光強度，および pH 8.0 で最高の増殖を示した。*G. nagasakiense* は無機および有機の窒素源，リン源をともに利用する能力を有し，低濃度でもよく増殖した。また，多くの有機物も利用され，その増殖の活性化に役立ったが，暗所では増殖を維持できなかった。可溶態の鉄，マンガンは 200 μg/l の濃度で増殖を著しく促進した。ビタミン B_{12} は本種に必須の生長因子であり，チアミンは B_{12} との共存で増殖を増大させた。本種に対する B_{12} の臨界濃度は 10 ng/l で，ビタミン B_{12} 類似物に対する反応特性は *Escherichia coli* 113-3 に近かった。B_{12} 類似物のベンズイミダゾール・コバラミンと5-メチルベンズイミダゾール・コバラミンは B_{12} と同程度の増殖を与えた。また，抗生物質のペニシリンは B_{12} の存在下で増殖を促進した。（514 津市上浜町1515　三重大学生物資源学部）