Growth Potentials of Red Tide Phytoplankters in Coastal Seawater by AGP Assay

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The method for the preparation of algal inocula for AGP (algal growth potential) assay was studied, and the growth potentials and growth limiting nutrients of red tide phytoplankters in coastal seawater were determined. The red tide phytoplankters *Heterosigma akashiwo*, and *Skeletonema costatum* were used as assay organisms. When using AGP assay the growth potentials were found to be dependent on the preparation of the algal inoculum. Accurate growth potentials were obtained only when sufficiently nutrient-starved cells were inoculated into the samples. Insufficient starvation of the cells resulted in an overestimation. Based from the results of the assay methods, the growth potentials of Osaka Bay water were about 10^4 cells/ml for *H. akashiwo* and about 10^5 cells/ml for *S. costatum*. Moreover, the water was found to have a potential to develop to the density of red tide for both organisms. The growth limiting nutrients of the water for both red tide organisms were determined to be nitrogen and phosphorus.

In the eutrophied seawater of Japan, such as the Seto Inland Sea, red tides have occurred frequently for about twenty-five years without any evidence of improvement. Reducing the nutrient supply flowing into the water is an important factor necessary to prevent the outbreak of red tides. Algal growth potential (AGP) is useful to estimate the eutrophication and the growth potential of water, and to ascertain algal growth limiting factors. The growth potential by algal assay depends on the assay organism, the physiological condition of the inoculating organism, and the size of the inoculum. In batch culture methods, only starved cells should be inoculated because algae can stock any excess of nutrients above their immediate needs.

Materials and methods

Assay organisms

Red tide phytoplankters, *Heterosigma akashiwo* (Hada) Hada strain NIES-6 (Raphidophyceae) and *Skeletonema costatum* (Greville) Cleve strain NIES-324 (Bacillariophyceae) were used for the assay. Both strains were isolated originally from red tide water in Osaka Bay and were transferred from the National Institute for Environmental Studies (NIES). Axenic clonal cultures were utilized in all experiments.

AGP assay

AGP was estimated from the maximum growth yields using the batch method under axenic conditions. The cultures were incubated at 22 °C with fluorescent light of 3,000-4,000 lux under a light/dark cycle of 14h/10h. Growth was determined by counting the cell density every 2 or 3 days.

Preparation of inoculum for accurate AGP assay

Nutrient-limited media for pre-culture and the number of times it is necessary to starve the algal inoculum for an accurate AGP assay were determined. The influence of vitamin B_{12} content in the medium for pre-culture on AGP was also tested because both assay organisms require vitamin B_{12} and could accumulate the vitamin. The accuracy of AGP was evaluated to ascertain whether the maximum growth yields decreased proportionally with the dilution ratios of nutrients in seawater models. Two kinds of seawater models with 4 different concentrations were used. In the nutrients-limited sea water model all nutrients were diluted and in the vitamin B_{12}-limited one, vitamin B_{12} was diluted and the level of the other nutrients was held constant. Composition of the solution for both pre-culture and seawater models with full-nutrient concentration were based on f/2 medium (Guillard & Ryther,1962) for *H. akashiwo* and ASP_2NTA medium (Provasoli *et al.*,1957) for *S.costatum* and the concentration of nutrients were modified to the levels necessary for saturating their growth. The nutritional requirements and media for pre-culture or starvation of algal inoculum are shown in Tables 1 and 2.

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Growth potentials of Osaka Bay water

Osaka Bay is located in the east part of the Seto Inland Sea, Japan, and eutrophied because of receiving a large amount of industrial and domestic drainage. Recently, red tides by *Skeletonema, Heterosigma, Chattonella,* and *Gymnodinium* have occurred in Osaka Bay. Water samples were collected from the surface water at St.17 and 19 in Osaka Bay (Fig.1) on September 8th and December 8th, 1987. Water samples were filtered through HA Millipore filters (pore size: 0.45μm) and were kept frozen until the time of assays. The growth potentials were deter mined using water samples sterilized by filtering with GS Millipore filter (pore size: 0.22μm).

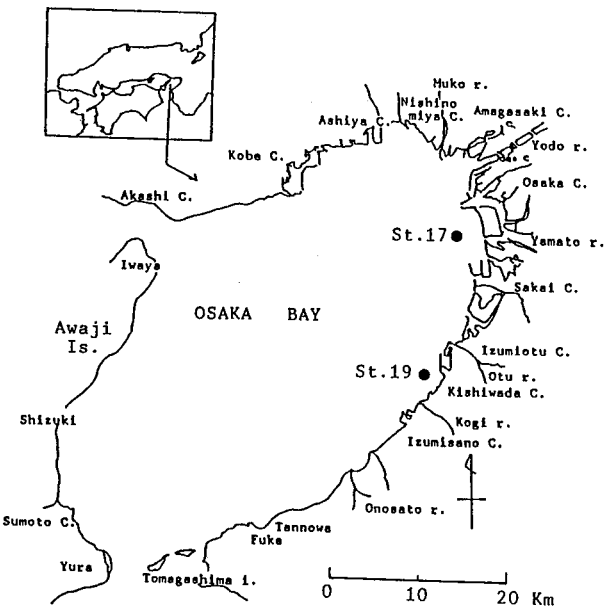

Fig.1 Location of sampling sites in Osaka Bay.

Results

Inoculum preparation of H. akashiwo for accurate assay

The evaluation of algal preparation for accurate assay using *H. akashiwo* is shown in Table 3. In this experiment, the size of the inoculum adopted was 1/20 of the volume of the seawater models because the smaller inoculum prolonged the period for the maximum growth yields. From the result, it was found that one time starvation gave an overestimation for the nutrients-limited and the vitamin B_{12}-limited seawater models with lower concentrations. Likewise, in the case of two times starvation, if vitamin B_{12} was included in the pre-culture medium AGP was also overestimated. On the other hand, the 1/100-V medium (vitamin B_{12}-free and 100 times diluted medium) gave an underestimation in lower concentrations. Only two times starvation with 1/10-V medium (vitamin B_{12}-free and 10 times diluted medium) gave an accurate estimation for both seawater models with all concentrations. Therefore, the inoculum starved two times with 1/10-V medium was used for the AGP assay of Osaka Bay water.

Inoculum preparation of S. costatum for accurate assay

The evaluation of inoculum preparation using *S. costatum* is shown in Table 4. In the case of one time starvation, a smaller inoculum (1/100 and 1/200) gave an underestimation

Table 1. Nutrient requirements for maximum growth and composition of media for the pre-culture of *H.akashiwo**

Nutrients (conc.)	Requirement	Composition of media for the pre-culture					
		1/1+V	1/10+V	1/100+V	1/1-V	1/10-V	1/100-V
NO_3-N(mg/l)	14	14	1.4	0.14	14	1.4	0.14
PO_4-P (mg/l)	0.93	0.93	0.093	0.0093	0.93	0.093	0.0093
Fe (mg/l)	0.56	0.56	0.056	0.0056	0.56	0.056	0.0056
f/2 metals(ml/l)	--	1	0.1	0.01	1	0.1	0.01
B_{12} (ng/l)	20	20	2.0	0.2	0	0	0

* f/2 medium was used as a basal medium.

Table 2. Nutrient requirements for maximum growth and composition of media for the pre-culture of *S. costatum**

Nutrients (conc.)	Requirement	Composition of media for the pre-culture					
		1/1+V	1/10+V	1/100+V	1/1-V	1/10-V	1/100-V
NO_3-N (mg/l)	1.6	1.6	0.16	0.016	1.6	0.16	0.016
PO_4-P (mg/l)	0.5	0.5	0.05	0.005	0.5	0.05	0.005
Si (mg/l)	10	10	1.0	0.1	10	1.0	0.1
Fe (mg/l)	--	0.5	0.05	0.005	0.5	0.05	0.005
PII (ml/l)	--	30	3.0	0.3	30	3.0	0.3
S3 (ml/l)	--	10	1.0	0.1	10	1.0	0.1
B_{12} (ng/l)	5	5	0.5	0.05	0	0	0

* ASP_2NTA medium was used as a basal medium.

Table 3. Evaluation of algal inoculum preparation of *H. akashiwo* for accurate AGP assay of nutrient- and vitamin B_{12}-limited seawater model

Medium of pre-culture*	Number of times starved	Size of inoculum	Evaluation of AGP**							
			Nutrient-limited model				B_{12}-limited model			
			1	0.1	0.02	0.01	1	0.1	0.02	0.01
1/1+V	1	1/20	A	+	+	+	A	A	+	+
1/1-V	1	1/20	A	+	+	+	A	A	+	+
1/10+V	1	1/20	A	+	+	+	A	A	+	+
1/10-V	1	1/20	A	+	+	+	A	A	+	+
1/100+V	1	1/20	A	+	+	+	A	A	+	+
1/100-V	1	1/20	A	+	+	+	A	A	+	+
1/1+V	2	1/20	A	+	+	+	A	A	+	+
1/1-V	2	1/20	A	A	A	A	A	-	-	-
1/10+V	2	1/20	A	+	+	+	A	A	+	+
1/10-V	2	1/20	A	A	A	A	A	A	A	A
1/100+V	2	1/20	A	A	A	+	A	A	A	+
1/100-V	2	1/20	A	-	-	-	A	A	-	-

* See Table 1.

** Evaluated for two models with four concentrations. The signs of A, +, and - represent the accurate estimation, overestimation, and underestimation, respectively.

in higher concentrations but a larger inoculum (1/20) gave an overestimation in lower concentrations. As was also true of *H. akashiwo*, if the pre-culture medium contained vitamin B_{12} the AGP was overestimated. In the case of *S. costatum*, also two times starvation with 1/100-V medium gave an accurate estimation for both models and was adopted for the AGP assay of Osaka Bay water.

Growth potentials of Osaka Bay water for H. akashiwo

Growth potentials were measured using the above pre-culture method for original and nutrients-supplemented Osaka Bay water. Nutrients supplemented to the water were nitrate (N), orthophosphate (P), Fe-EDTA (Fe), and vitamin B_{12} (B_{12}), singly or in various combinations. The concentration of supplements corresponded to the level for saturation of their growth. From the result shown in Fig. 2, the growth potentials for original water were found to be $1.74\text{-}3.58\times10^4$ cells/ml. These potentials exceeded the lower limit of cell density for red tide by this alga, about 10^3 cells/ml. Therefore, it revealed that the water of Osaka Bay has a potential for developing into *Heterosigma* red tides. From the effect of nutrient supplement, singular addition of nitrogen, vitamin B_{12}, or iron gave no growth enhancement, but the growth yields enhanced 1.6-4.5 times as many as that of original water by the addition of phosphorus except for the sample of St.19 on Dec.8th. Growth enhancement was remarkable by the supplement in combination of nitrogen and phosphorous. Consequently, the growth of *H. akashiwo* is limited with nitrogen and phosphorus in the water of Osaka Bay. The first growth limiting nutrient of the water is estimated to be phosphorus and the second one to be nitrogen except for the water of St.19 on Dec.8th.

Table 4. Evaluation of algal inoculum preparation of *S. costatum* for accurate AGP assay of nutrient- and vitamin B_{12}-limited seawater model

Medium of pre-culture*	Number of times starved	Size of inoculum	Evaluation of AGP** Nutrient-limited model 1	0.1	0.02	0.01	B_{12}-limited model 1	0.1	0.02	0.01
1/1+V	1	1/20	A	+	+	+	A	+	+	+
1/1-V	1	1/20	A	+	+	+	A	+	+	+
1/10+V	1	1/20	A	+	+	+	A	+	+	+
1/10-V	1	1/20	A	+	+	+	A	+	+	+
1/50-V	1	1/200	-	A		+	-	-		A
1/100+V	1	1/20	A	+	+	+	A	+	+	+
1/100-V	1	1/20	A	+	+	+	A	+	+	+
1/100-V	1	1/100	-	A		A	-	-		A
1/100-V	1	1/200	-	A		+	-	-		-
1/1+V	2	1/20	A	+	+	+	A	+	+	+
1/1-V	2	1/20	A	+	+	+	A	A	A	A
1/10+V	2	1/20	A	+	+	+	A	+	+	+
1/10-V	2	1/20	A	A	+	+	A	A	+	+
1/100+V	2	1/20	A	A	A	+	A	A	+	+
1/100-V	2	1/20	A	A	A	A	A	A	A	A

* See Table 2.
** Evaluated for two models with four concentrations. The signs of A, +, and - represent the accurate estimation, overestimation, and underestimation, respectively.

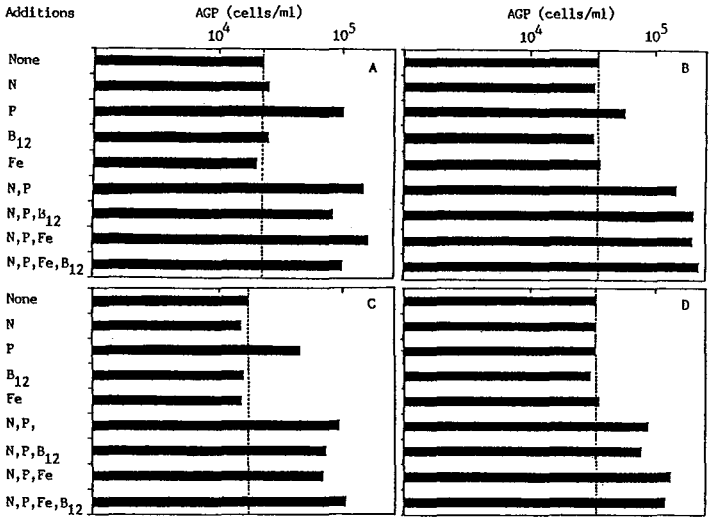

Fig. 2 Effects of various nutrient addition on AGP of Osaka Bay water for *H. akashiwo*. Concentration of nutrient added: N(NO_3-N),14 mg/l;P(PO_4-P), 0.93 mg/l;B_{12}(vitamin B_{12}),20 ng/l;Fe(Fe-EDTA),0.56 mg/l. Date of sampling:A(St.17) & B(St.19),Sep.8,'87;C(St.17) & D(St.19),Dec.8,'87.

Growth potentials of Osaka Bay water for S. costatum

The growth potentials of Osaka Bay water for *S.costatum* and the influence of nutrient supplement (nitrate, orthophosphate, vitamin B_{12}, Fe-EDTA, and silicate) on the potentials are shown in Fig.3. The growth potentials of the water were $2.53\text{-}4.59\times10^5$ cells/ml. From the result, the water was found to have a potential for developing into *Skeletonema* red tide. The growth potentials were not affected by the singular supplement of nitrogen, silicate, vitamin B_{12}, or Fe-EDTA, and were enhanced remarkably by the addition in combination of nitrogen and phosphorus.

Influences of dilution of the water on growth potentials

The effect of dilution of Osaka Bay water with artificial sea water on growth potentials of *H. akashiwo* is shown in Table 5. The growth potentials for diluted water generally decreased with

dilution ratios;the potentials of the 1/2-diluted water were 46.2-60.2 % of those of the original water. In the higher-diluted water (1/10-1/50), the potentials were found to exceed the ones expected with the dilution of sample water. One of the reasons for the unreasonable growth may be an excessive inoculum as compared with the low nutrient concentration in the diluted sample water.

Discussion

The growth response of phytoplankton under batch culture generally depends on both initial cell density and cellular nutrient content: low density and low cellular content generally prolong the period of the lag phase. Therefore, a large inoculum is needed to avoid the delay of growth for AGP assay.

From the present study,the growth potentials by the use of AGP assay were found to be dependent on the preparation of algal inoculum. The accurate growth potentials were obtained when sufficiently nutrient-starved cells were inoculated into the sample,even if the size of inoculum was relatively large: about $2x10^3$ cells/ml of the initial density for *H. akashiwo* and $7.7x10^3$ cells/ml for *S. costatum*. Nevertheless, insufficiently-starved cells gave an overestimation of AGP.

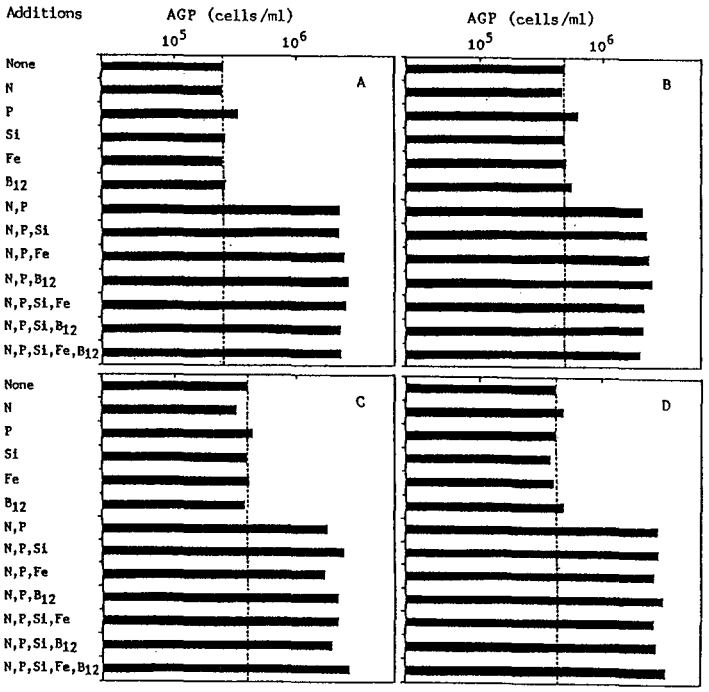

Fig.3 Effects of various nutrient addition on AGP of Osaka Bay water for *S. costatum*. Concentration of nutrient added:N(NO_3-N),1.6 mg/l;P(PO_4-P), 0.5 mg/l;Si(SiO_3-Si),10 mg/l;Fe(Fe-EDTA),0.5 mg/l;B_{12}(vitamin B_{12}),5.0 mg/l. Date of sampling:A(St.17) & B(St.19),Sep.8,'87;C(St.17) & D(St.19) Dec.8,'87.

Table 5. Effects of dilution on AGP of Osaka Bay water for *H. akashiwo*

Dilution ratio (%)	St.17				St.19			
	Sep.8,1987		Dec.8,1987		Sep.8,1987		Dec.8,1987	
	cells/ml	%	cells/ml	%	cells/ml	%	cells/ml	%
1/1(100)	23300	100	23300	100	20000	100	23300	100
1/2 (50)	10800	46.2	14000	60.2	9910	49.5	11700	50.1
1/3 (33)	6110	26.2	8280	35.6	5580	27.8	4470	19.2
1/4 (25)	5240	22.5	5670	24.4	2630	13.1	2680	11.5
1/5 (20)	5080	21.8	6250	26.9	2960	14.8	4470	19.2
1/10(10)	4470	19.2			2630	13.1	4470	19.2
1/20 (5)	3410	14.6			1160	5.8	2140	9.2
1/50 (2)	3170	13.6			910	4.6	0	0
1/100(1)	2530	10.8			0	0	0	0

Table 6. Growth yields of *H. akashiwo* and limiting nutrients for its growth in Osaka Bay water estimated from the minimum celluar content

Nutrient**	Minimum cellular content pg/cell	St.17 Sep.8,1987		St.17 Dec.8,1987		St.19 Sep.8,1987		St.19 Dec.8,1987	
		Conc.in water*** µg/l	Yields cells/ml	Conc.in water*** µg/l	Yields cells/ml	Conc.in water*** µg/l	Yields cells/ml	Conc.in water*** µg/l	Yields cells/ml
IN	20.2	91.9	$4.55x10^3$*	238	$1.18x10^4$	57.0	$2.83x10^3$*	270	$1.33x10^4$
IP	2.29	44.7	$1.52x10^4$	33.4	$1.13x10^4$	23.2	$7.88x10^3$	24.1	$8.20x10^3$*
B_{12}	$8.9x10^{-5}$	$1.01x10^{-2}$	$1.13x10^5$	$<0.5x10^{-3}$	$<5.6x10^3$*	$1.21x10^{-2}$	$1.36x10^5$	$5.9x10^{-3}$	$6.63x10^4$

* Growth limiting in the original water.
** IN:inorganic nitrogen,IP:inorganic phosphorus,B_{12}:vitamin B_{12}.
*** Concentration of dissolved form.

Table 7. Growth yields of *S. costatum* and limiting nutrients for its growth in Osaka Bay water estimated from the minimum cellular content

Nutrient**	Minimum cellular content pg/cell	St.17 Sep.8,1987		St.17.Dec.8,1987		St.19 Sep.8,1987		St.19.Dec.8,1987	
		Conc.in water*** µg/l	Yields cells/ml	Conc.in water*** µg/l	Yields cells/ml	Conc.in water*** µg/l	Yields cells/ml	Conc.in water*** µg/l	Yields cells/ml
IN	0.54	91.9	$1.70x10^5$*	238	$4.41x10^5$	57.0	$1.07x10^5$*	270	$5.00x10^5$
IP	0.14	44.7	$3.19x10^5$	33.4	$2.39x10^5$	23.2	$1.66x10^5$	24.1	$1.72x10^5$*
B_{12}	$2.8x10^{-6}$	$1.01x10^{-2}$	$3.6x10^6$	$<0.5x10^{-3}$	$<1.8x10^5$*	$1.21x10^{-2}$	$4.3x10^6$	$5.9x10^{-3}$	$2.1x10^7$

* Growth limiting in the original water.
** and *** See foot note of Table 6.

Based from the results of assay methods, the growth potentials of Osaka Bay water were about 10^4 cells/ml for *H. akashiwo* and 10^5 cells/ml for *S. costatum*. These growth potential levels for *S. costatum* are about 10 times higher than those for *H. akashiwo*. They are consistent with the difference of nutrient levels for their growth. It is evident that the water in Osaka Bay has a potential to develop to the density of a red tide for both organisms. Moreover, the growth limiting nutrients in the water were nitrogen and phosphorus, but not vitamin B_{12}, iron, or silicon. The growth yields estimated by dividing the concentration of the nutrients (inorganic nitrogen and phosphorus, and vitamin B_{12}) in original water by minimum cellular content of each nutrient (Nishijima and Hata,1984;Watanabe *et al.*, 1982; Nishijima *et al.*,1990) are shown in Tables 6 and 7. From the Tables, the growth yields limiting in the original water of Osaka Bay were estimated to be about 5-8x10^3 cells/ml for *H. akashiwo* and about 1-2x10^5 cells/ml for *S. costatum*. The growth yields estimated for *S. costatum* are much the same as growth potentials of the water by AGP assay, but those for *H. akashiwo* are smaller than the growth potentials of the water. The discrepancy for *H. akashiwo* suggests that their minimum cellular content of nitrogen and phosphorus could be overestimated. Consequently, nutrients should be reduced to one-tenth to decrease the growth potentials to a lower level than the red tide density.

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