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Original Article

Characteristics of virus-like growth suppression agents against phytoplankton obtained from seawater at the mouth of Funka Bay, Hokkaido, Japan

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SUMMARY: Eighteen samples of virus-like growth suppression agents against the marine phytoplankton *Alexandrium catenella*, *Gymnodinium mikimotoi* and *Tetraselmis* sp., obtained from the coastal waters at the mouth of Funka Bay, Hokkaido, Japan, from 1993 to 1995, were characterized on size estimation, heat stability, nuclease sensitivity, proteinase K sensitivity, stability under acidic condition, titration, diethyl ether sensitivity, and ultraviolet (UV) sensitivity. All agents were affected by heating at 50°C for 30 min, exposure to acidic conditions below pH 5.0, passing through a 0.05 μm filter, RNase treatment, and irradiation of UV dosage at 5×10^4 μW/s per cm^2. The growth suppression effects of 11 agents from *Tetraselmis* sp. and *A. catenella* disappeared after proteinase K treatment, however, seven agents from *G. mikimotoi* were unaffected by this treatment. Furthermore, 11 agents against *Tetraselmis* sp. and *A. catenella* were collected from the bottom fraction by ultracentrifugation, while seven agents against *G. mikimotoi* were collected from the upper fraction, not from the precipitated fraction. These results suggest that at least two types exist in the virus-like agents showing growth suppression for phytoplankton.

KEY WORDS: ***Alexandrium catenella*, growth suppression, *Gymnodinium mikimotoi*, proteinase sensitivity, *Tetraselmis* sp., ultracentrifugation.**

INTRODUCTION

The existence of growth suppression agents for marine phytoplanktons *Alexandrium catenella* and *Tetraselmis* sp. was observed in seawater samples collected at the mouth of Funka Bay, Hokkaido, Japan, from September to October 1993.[1] It suggested that the growth suppression effect was caused by virus-like agents that were transferable and were 0.05–0.22 μm in size. During the screening of viruses infecting marine phytoplankton *A. catenella*, *Gymnodinium mikimotoi* and *Tetraselmis* sp. in 1994–95, the same effect was observed in a seawater sample collected only during September and October of that period. In the present study, further detailed characterization was provided for these growth suppression agents. Interestingly, proteinase K susceptibility and the ultracentrifugation profile are different among *A. catenella*, *Tetraselmis* sp. and *G. mikimotoi*'s growth suppression agents.

MATERIALS AND METHODS

Culture of phytoplankton

Axenic cultures of *Tetraselmis* sp. FK-1, *A. catenella* TN-7, and xenic cultures of *G. mikimotoi* Ka-34 were used in this study. All strains were cultured in f/2 medium[2] under cool white light irradiation at about 45.6 μmol photon/m^2 per s with a 14 : 10 Light-Dark cycle. The cell number of all cultures was counted using a hemocytometer (Erma, Tokyo, Japan) using the method of Onji *et al.*[1]

The phytoplankton growth suppression agents

In a previous study, four samples showing the growth suppression effect were found and tentatively characterized.[1]

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As further screening of viruses infecting 14 growth suppression positive samples (Fig. 1), 18 transferable growth suppression agents including the agents found in 1993 were used in the present study (Table 1). These agents were designated according to the host and sampling date (Table 1). These agents continued to propagate in the same phytoplankton used at the screening as the host. The agents were dispensed in 2 mL subsamples into 2.5 mL tubes and kept at −80°C before use.

The cell numbers in the control (CN) and that in experimental culture (EN) on the day of maximum growth in the control culture were measured, respectively. Then the growth suppression rate was calculated using the following formula: growth suppression rate (%) $=\{1(EN/CN)\}100$ (%). Growth suppression rate less than 10% was defined as negative growth suppression.

Characteristics of the transferable growth suppression agents

Characteristics of the transferable growth suppression agents were examined by the following traits: size estimation, heat stability, nuclease sensitivity, proteinase K sensitivity, stability under acidic conditions, titration, diethyl ether sensitivity, and ultraviolet (UV) sensitivity. These methods are described in a previous study.[1]

Titer was determined by serial dilution with sterilized f/2 medium. The dilution rates were 1/2, 1/5, 1/10 and 1/20. The titer was calculated from the highest dilution value at which growth suppression was observed.

Table 1 List of the growth suppression agents against phytoplankton strains used in this study

Host phytoplankton	Growth suppression agent	Date obtained
Tetraselmis sp. FK-1	TSS2493	24 Sep. 1993
	TSO2893	28 Oct. 1993
Alexandrium catenella TN-7	ACS2493	24 Sep. 1993
	ACO2893	28 Oct. 1993
	ACS1694	16 Sep. 1994
	ACS2894	28 Sep. 1994
	ACO1794	17 Oct. 1994
	ACO3194	31 Oct. 1994
	ACS1495	14 Sep. 1995
	ACS2995	29 Sep. 1995
	ACO3195	31 Oct. 1995
Gymnodinium mikimotoi Ka-34	GMS1694	16 Sep. 1994
	GMS2894	28 Sep. 1994
	GMO1794	17 Oct. 1994
	GMO3194	31 Oct. 1994
	GMS1495	14 Sep. 1995
	GMS2995	29 Sep. 1995
	ACO3195	31 Oct. 1995

Growth suppression effect of the ultracentrifuged fraction

The culture filtrates suppressed the growth of *A. catenella* TN-7, *G. mikimotoi* Ka-34 and *Tetraselmis* sp. FK-1 were ultracentrifuged (100 000 *g* for 4 h) and dispensed equally in four fractions from upper to bottom. Each fraction was inoculated to a fresh culture of *A. catenella* TN-7, *Gymnodinium mikimotoi* Ka-34 and *Tetraselmis* sp. FK-1, and the phytoplankton growth suppression effects of each fraction were observed.

Host range

The growth suppression effect of the culture filtrates of *A. catenella* TN-7, *G. mikimotoi* Ka-34 and *Tetraselmis* sp. FK-1 incubated with the agents were examined for the

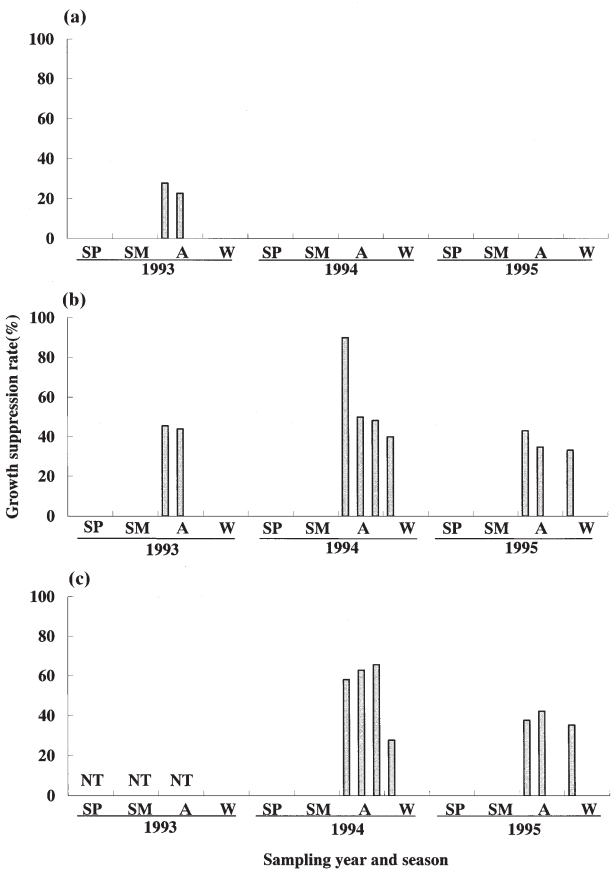

Fig. 1 Seasonal fluctuations of filtratable growth suppression agents for the marine phytoplanktons in the seawater sample collected from the mouth of Funka Bay, Hokkaido, Japan. (a) *Tetraselmis* sp., (b) *Alexandrium catenella*, (c) *Gymnodinium mikimotoi*. SP, spring season March to May; SM, summer season June to August; A, autumn season September to November; W, winter season December to February.

following 14 phytoplankton strains: *A. catenella* TN-7, *G. mikimotoi* Ka-34 and *Tetraselmis* sp. FK-1, xenic culture of *Prorocentrum micans* Ka-13, xenic culture of *P. minimum* Ka-14, xenic culture of *G. mikimotoi* Ka-34, axenic culture of *G. mikimotoi* G-303, axenic culture of *A. tamarense* OF151, axenic culture of *A. catenella* OF-071, *Chattonella marina* NIES-3, *C. antiqua* NIES-1, and *Heterosigma akashiwo* NIES-4, *Phaeodactylum tricornutum* FK-2, *Skeletolema costatum* NIES-323, *Chaetoceros sociale* NIES-377. *Tetraselmis* sp. FK-1, *Phaeodactylum tricornutum* FK-2, *Skeletolema costatum* NIES-323, *Chaetoceros sociale* NIES-377 were cultured at 15°C, while other phytoplankton strains were cultured at 20°C.

RESULTS

Characteristics of the growth suppression agents

Six traits (filterability, heat stability, pH stability, nuclease sensitivity, UV sensitivity, and ether sensitivity) of all agents tested in the present study were found to have the characteristics. Growth suppression was observed in the cultures of *A. catenella*, *G. mikimotoi*, and *Tetraselmis* sp. inoculated with the sample filtrates passed through a 0.45 or 0.22 μm filter. The filtrates passed through a 0.10 μm filter possessed a slightly decreased growth suppression effect, but those passed through a 0.05 μm filter did not show a remarkable suppression. Heating at more than 50°C for 30 min remarkably reduced the effects, but the effect was observed by heating at lower temperatures than 40°C. The growth suppression agents were inactivated under acidic conditions at pH 3, but the agents against *G. mikimotoi* showed partial resistance to pH 5 treatment. The growth suppression effects disappeared after RNase treatment, but the DNase treatment had no effect. The growth suppression agents were inactivated by UV irradiation with dosage at 5×10^4 μW/s per cm^2. Diethyl ether treatment did not reduce all the growth suppression effects. However, dilution end-point which the growth suppression effects produced in the culture filtrates of phytoplankton was slightly different, and among the agents disappeared at 1/10 for TSS2493, TSO2893, ACO2893, ACS2894, ACS1495, ACO3195, and GMS2995, and 1/20 for ACS2493, ACS1694, ACO1794, ACO3194, ACS2995, GMS1694, GMS2894, GMO1794, GMO3194, GMS1495, and GMO3195 (data not shown). Furthermore, proteinase K treatment revealed a significant loss of growth suppression in the culture filtrates from *Tetraselmis* sp. and *A. catenella*, but the agents against *G. mikimotoi* were not affected by proteinase K (Table 2). Addition of DNase, RNase or proteinase K to the phytoplankton cultures did not influence the growth of them. All characteristics with the exception of proteinase K susceptibility produced the same effect as described in a previous study.[1]

Table 2 Change of growth suppression rate of the growth suppression agents phytoplankton strains after proteinase K treatment

PGSA	Growth suppression rate (%)	
	Treated	Control
TSS2493	3.7	34.1
TSO2893	3.6	34.2
ACS2493	7.3	43.9
ACO2893	0.0	40.5
ACS1694	7.3	46.3
ACS2894	2.4	43.9
ACO1794	2.4	41.5
ACO3194	2.7	40.5
ACS1495	4.9	43.9
ACS2995	2.4	41.5
ACO3195	2.7	40.5
GMS1694	48.8	48.8
GMS2894	46.5	46.5
GMO1794	45.8	47.5
GMO3194	48.8	48.8
GMS1495	48.8	48.8
GMS2995	46.5	46.5
GMO3195	44.1	47.5

TSS2493 and ACO2893 affected the growth of *A. catenella* TN-7, *A. catenella* OF-071, *G. mikimotoi* G303, and *Tetraselmis* sp. FK-1, but had no effect on a xenic strain of *G. mikimotoi* Ka-34 and four species of Dinophyceae, three species of Raphidophyceae, and three species of Phaeophyceae (Table 3). ACO3194, GMO1794, ACS2995, and GMO3195 affected the growth of *G. mikimotoi* Ka-34, *G. mikimotoi* G-303, *A. catenella* OF-071, and *A. catenella* TN-7. However, growth suppression against Prasinophyceae *Tetraselmis* sp. FK-1, the four species of Dinophyceae, three species of Raphydophyceae, and three species of Phaeophyceae was not observed (Table 3).

Ultracentrifuged properties of the agents

When the growth-suppressed culture filtrates of *A. catenella*, *G. mikimotoi*, and *Tetraselmis* sp. were ultracentrifuged, growth suppression effect was observed in only the bottom fraction of the culture filtrate of *Tetraselmis* sp. and *A. catenella* (Fig. 2). In contrast, only the upper fraction in the culture filtrates of the growth-suppressed *G. mikimotoi* showed growth suppression activity (Fig. 3).

DISCUSSION

Many viruses have been observed to infect marine phytoplankton species, and isolated from seawater after ultrafiltration and from phytoplankton cells induced by UV-irradiation.[3–7] All the known virus-like particles

Table 3 Growth suppression effect of 14 species of phytoplankton inoculated with the growth suppression agents

Host	Growth suppression effect					
	TSS2493	ACO2893	ACO3194	GMO1794	ACS2995	GMO3195
Against:						
Prasinophyceae						
Tetraselmis sp. FK-1	+	+	–	–	–	–
Dinophyceae						
Prorocentrum micans Ka-13	–	–	–	–	–	–
Prorocentrum minimum Ka-14	–	–	–	–	–	–
Gymnodinium mikimotoi Ka-34	–	–	+	+	+	+
Gymnodinium mikimotoi G-303	+	+	+	+	+	+
Alexandrium catenella TN-7	+	+	+	+	+	+
Alexandrium catenella OF-071	+	+	+	+	+	+
Alexandrium tamarense OFX-151	–	–	–	–	–	–
Raphidophyceae						
Chattonella antiqua NIES-1	–	–	–	–	–	–
Chattonella marina NIES-3	–	–	–	–	–	–
Heterosigma akashiwo NIES-4	–	–	–	–	–	–
Phaeophyceae						
Phaeodactylum tricornutum FK-2	–	–	–	–	–	–
Skeletolema costatum NIES-323	–	–	–	–	–	–
Chaetoceros sociale NIES-377	–	–	–	–	–	–

+, Suppression was observed; –, no effect.

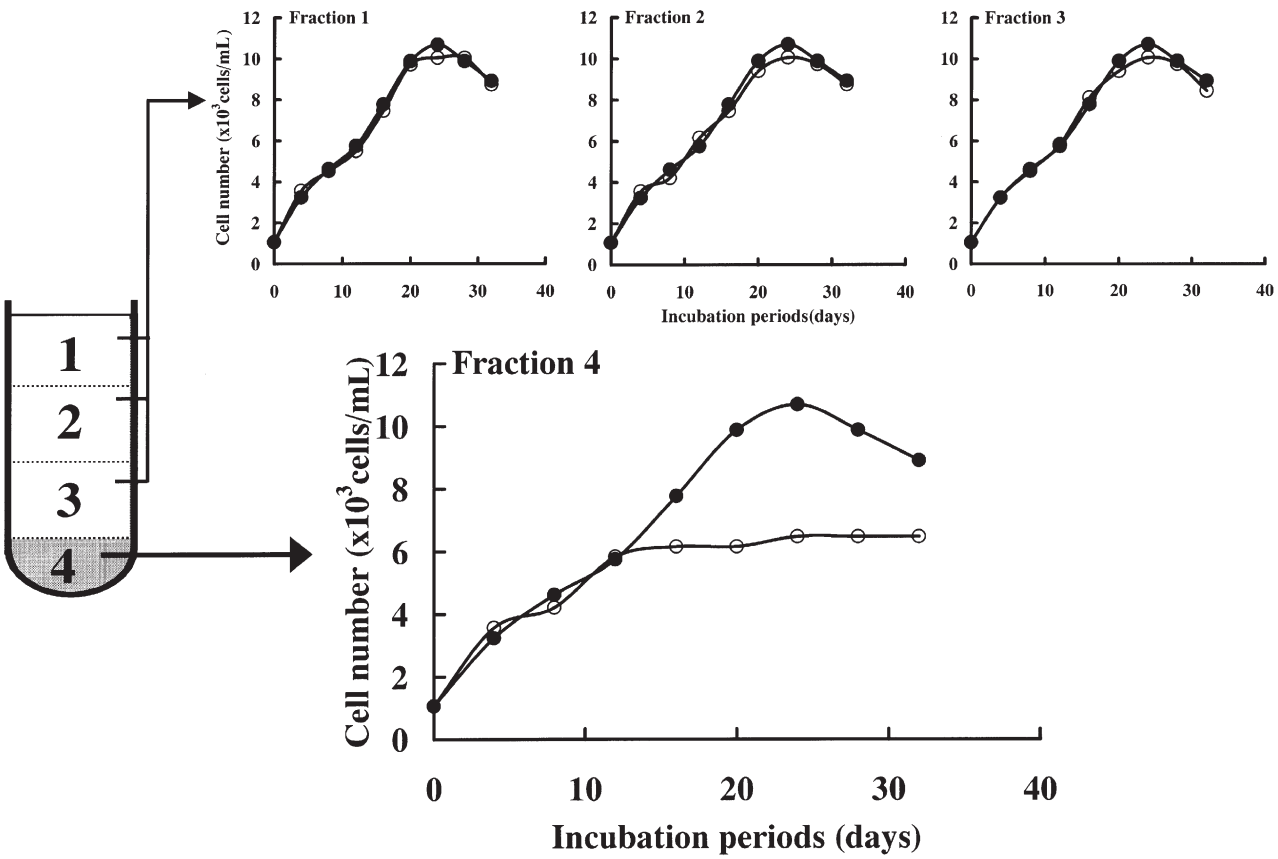

Fig. 2 Growth suppression effect of ultracentrifuged fraction of the culture filtrate of *Alexandrium catenella* TN-7. (●) Control culture inoculated with the filtrated culture of health phytoplankton; (○) experimental culture inoculated with the filtrated culture of the growth suppression against phytoplankton strains.

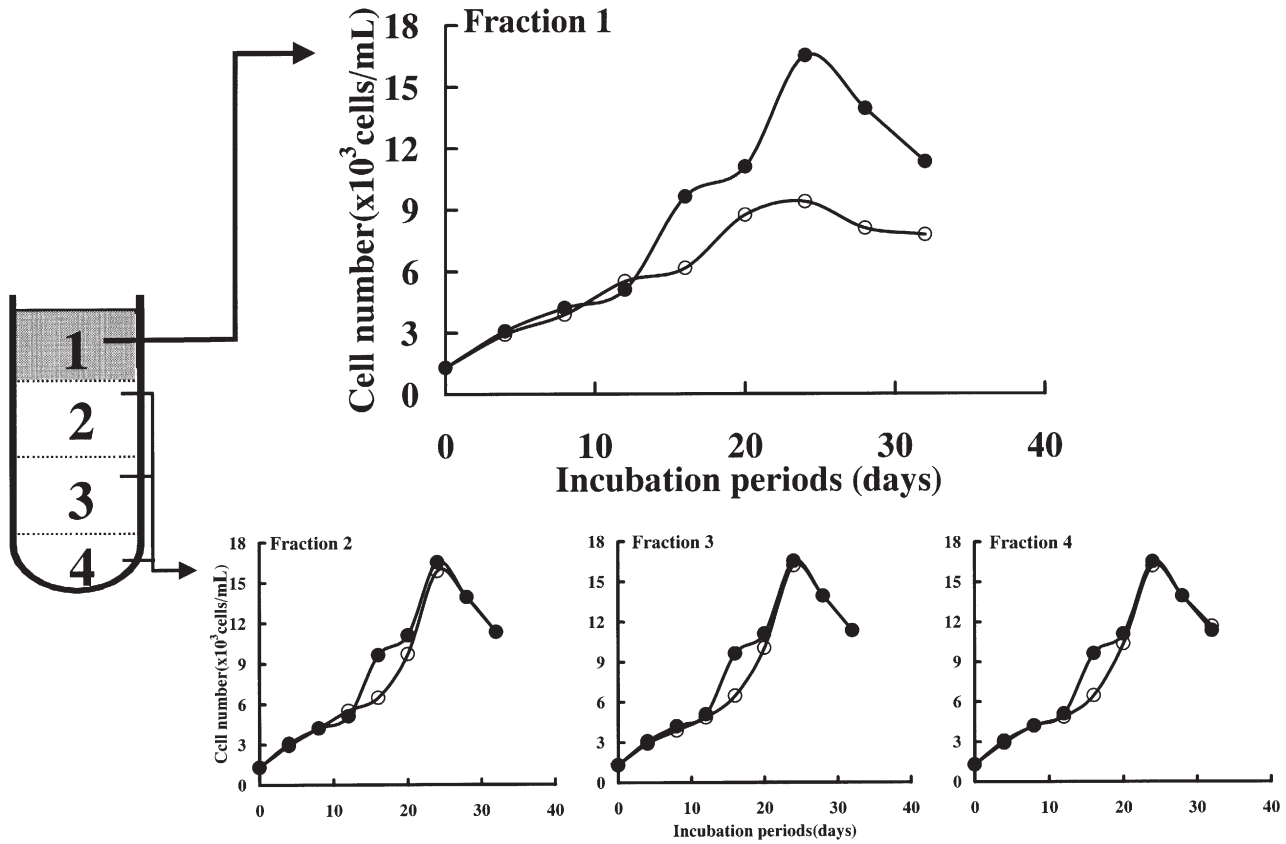

Fig. 3 Growth suppression effect of ultracentrifuged fraction of the culture filtrate of *Gymnodinium mikimotoi* Ka-34. (●) Control culture inoculated with the filtrated culture of health phytoplankton; (○) experimental culture inoculated with the filtrated culture of the growth suppression against phytoplankton strains.

from phytoplankton species were hexagonal shape, and recognized as DNA virus because of DAPI (4′,6-diamidino-2-phenylindole)-positive particles.

We characterized 18 growth suppression agents against three phytoplankton species, *Tetraselmis* sp., *A. catenella*, and *G. mikimotoi*, and all agents were affected by heating at 50°C for 30 min, exposure to acidic conditions below pH 5.0, diethyl ether treatment and were filterable through a 0.05 μm filter. Moreover, interestingly, the growth suppression effect of all agents was inactivated by an RNase treatment, and by UV irradiation with 5×10^4 μW/s per cm^2 dosage, but was not affected by DNase (Table 4). These results suggest that these growth suppression agents may be some nucleic acid complex agents that are RNA virus as described in a previous paper.[1] RNA virus for marine phytoplankton was never reported; therefore, a thorough investigation is needed to clarify these agents. Most plant viruses were known as RNA virus and the shape was rigid or flexible rod.[8] Comparing the characterization of known plant RNA viruses and the growth suppression agents against phytoplankton strains in the present study, further study is necessary.

Because these growth suppression titers were observed low, we attempted to concentrate these agents using ultracentrifugation (Figs 2,3). The growth suppression agents against *Tetraselmis* sp. and *A. catenella* were precipitated with an increase of titers (data not shown), but the agent against *G. mikimotoi* was not precipitated, and rather collected at the upper layer (Fig. 2). Table 4 summarizes the differentiated traits among 18 growth suppression agents. On properties of proteinase K susceptibility (Table 2), host range (Table 3) and the precipitation by ultracentrifugation (Figs 2,3), 18 growth suppression agents are differentiated in at least two groups. Furthermore, four samples showing proteinase sensitive-precipitated traits obtained in 1993 showed some differences in host range from the seven samples of proteinase sensitive-precipitated *A. catenella* growth suppression agent. Seven samples of ultracentrifugation-not-precipitated and proteinase-resistant *G. mikimotoi* growth suppression agents were unlikely to be a virus. As Diener first found a unique ultracentrifugation-not-precipitated pathogen and clarified the pathogen infections circular RNA molecule, viroid, the *G. mikimotoi* growth suppression agents seemed to have viroid-like properties rather than a virus.[9] The further characterization in the present study indicated that 11 samples showed virus-like traits and seven samples showed viroid-like traits.

Table 4 Characteristics of the growth suppression agents against phytoplankton strains

Host	PGSA	Characteristics*		
		Proteinase	Host	Ultracentrifugation
Tetraselmis sp. FK-1	TSS2493	Sensitive	*Tetraselmis* sp. FK-1,	Precipitated
	TSO2893	Sensitive	*A. catenella* TN-7,	Precipitated
Alexandrium catenella TN-7	ACS2493	Sensitive	*A. catenella* OF-071,	Precipitated
	ACO2893	Sensitive	*G. mikimotoi* G-303	Precipitated
A. catenella TN-7	ACS1694	Sensitive	*A. catenella* TN-7,	Precipitated
	ACS2894	Sensitive	*A. catenella* OF-071,	Precipitated
	ACO1794	Sensitive	*G. mikimotoi* Ka-34,	Precipitated
	ACO3194	Sensitive	*G. mikimotoi* G-303	Precipitated
	ACS1495	Sensitive		Precipitated
	ACS2995	Sensitive		Precipitated
	ACO3195	Sensitive		Precipitated
Gymnodinium mikimotoi Ka-34	GMS1694	Resistant	*A. catenella* TN-7,	Supernatant
	GMS2894	Resistant	*A. catenella* OF-071,	Supernatant
	GMO1794	Resistant	*G. mikimotoi* Ka-34,	Supernatant
	GMO3194	Resistant	*G. mikimotoi* G-303	Supernatant
	GMS1495	Resistant		Supernatant
	GMS2995	Resistant		Supernatant
	GMO3195	Resistant		Supernatant

*Common characteristics: $<0.10\,\mu m$ of the size, unstable at 50°C, tolerance of ether treatment, unstable at pH 3.0, resistance of DNase treatment, sensitivity of RNase treatment, sensitivity of UV irradiation ($5.0\times10^4\,\mu W\,s/cm^2$) and titration of 10^{-1}.

Further study is necessary in order to visualize the agents' body on referring the concentration and the purification techniques of isolated viruses against phytoplankton strains.

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