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# Growth Suppression of the Marine Phytoplankton *Alexandrium catenella* and *Tetraselmis* sp. caused by Ultrafiltrated Seawater collected from Coastal Area on the Mouth of Funka Bay

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Filtrable pathogens infecting the phytoplankton *Alexandrium catenella* and *Tetraselmis* sp. were screened from coastal seawater on the mouth of Funka Bay, Hokkaido, Japan from 1993 to 1994. Growth suppression against these phytoplankton species was observed in the seawater samples collected during September and October 1993. The growth of *A. catenella* was suppressed from 40 to 45%, and that of *Tetraselmis* sp. was suppressed from 20 to 30%. Re-inoculation of the culture filtrate of growth-suppressed phytoplankton after passing the culture through a 0.22-$\mu$m filter also caused growth suppression of the fresh culture. However, these effects disappeared after several treatments including heating at 50°C for 30 min, exposure to acidic conditions below pH 5.0, passing through a 0.05-$\mu$m filter, and Proteinase K and RNase treatment. Cell free extracts of the growth-suppressed phytoplankton caused the same extent of growth suppression. Electron microscopic observation of *A. catenella* cells that were lead to the growth suppression revealed that the cells were severely damaged, whereas no virus-like particles or bacterial cells were observed. Growth suppression was observed in a fresh culture of *A. catenella* and an axenic culture of *Gymnodinium mikimotoi* by the growth suppressed *Tetraselmis* sp. culture filtrate, and the *A. catenella* culture filtrate affected the growth of *Tetraselmis* sp. and an axenic culture of *G. mikimotoi*. However, the growth suppression or inhibition was not observed in fresh cultures of *Prorocentrum micans, P. minimum, A. tamarense, G. mikimotoi, Chattonella antiqua, C. marina*, and *Heterosigma akashiwo*. These results suggested that unique filterable pathogens might be found in the seawater samples.

**Key words:** phytoplankton, *Alexandrium catenella, Tetraselmis* sp., growth suppression, filtrable pathogens

Recently, there have been many reports of the abundance of viruses or virus-like particles in marine environments.[1-4] These particles have been observed from $10^6$ to $10^9$ per ml of seawater in various ocean environments and are recognized to be an important microbial community in marine ecosystems. Virus-like particles have been observed in 12 of the 14 classes of marine phytoplankton, suggesting that some viruses might affect their host's reproduction.[5-7] However, there are few reports of marine viruses affecting phytoplankton growth and causing lysis in phytoplankton cells. The manner in which viruses influence the productivity of the marine phytoplankton is not well understood.

Virus particles have also been observed in phytoplankton cells that form huge blooms in the ocean. The viruses infecting marine phytoplankton might control and suppress the blooms. To date, viruses that cause cell lysis of *Micromonas pusilla* (red tide phytoplankton),[8,9] *Emiliania huxleyi* (white water phytoplankton),[10] *Aureococcus anophagefferens* (brown tide phytoplankton),[11] and *Heterosigma akashiwo* (red tide phytoplankton)[12] have been isolated. Since virus particles were observed to be abundant in wild type *Heterosigma akashiwo* cells particularly at the rapid decreasing state of the red tide,[13] viral infection associated with the disappearance of huge phytoplankton blooms has recently become a focus of research and intensive work in the field has been performed by Nagasaki *et al.* (1994).[13]

We also screened filterable pathogens affecting growth of marine phytoplankton from seawater samples, and a growth suppression was observed in two phytoplankton species. Here we report the screening results and the characteristics of the unique growth suppression agents.

## Materials and Methods

*Strains and Culture of Phytoplankton*

Axenic cultures of *Tetraselmis* sp. FK-1, *Alexandrium catenella* TN-7, *Gymnodinium mikimotoi* G-303, *A. tamarense* OF151, *A. catenella* OF071, *Chattonella marina* NIES-3, C. *antiqua* NIES-1, *Heterosigma akashiwo* NIES-4, and xenic cultures of *Prorocentrum micans* Ka-13, *P. minimum* Ka-14, *G. mikimotoi* Ka-34 were used in this study. All strains were cultured in f/2 medium[14] under cool white light irradiation at about 45.6 $\mu$mol photon $m^{-2}$ $s^{-1}$ with a 14:10 LD cycle. The *Tetraselmis* sp. FK-1 was cultured at 15°C, and the other phytoplankton species were cultured at 20°C, respectively.

Cell number of all cultures was counted using a hemocytometer (Erma) after the addition of a one-tenth

volume of 10% formalin and was corrected from the dilution rate caused by the addition of formalin.

*Ultrafiltration and Pretreatment of the Seawater Sample*

Seawater samples were collected at Usujiri, a coastal area on the mouth of Funka Bay, Hokkaido, Japan from June 1993 to February 1994. Each 10-liter sample was ice-chilled and transported to our laboratory for experiments. The seawater was filtrated through a 47-mm-diameter glass-fiber filter (Whatman GF/C) and 0.45 $\mu$m pore-size membrane filter (Millipore HA; 47-mm-diameter) held in place by stainless steel filter holders, and followed by ultrafiltration using a Millipore Pellicon cassette system with a polysulfone membrane (PTTK: 30,000 molecular weight) for the concentration of high-molecular materials in the seawater. The sample preparation was according to Suttle *et al.* (1991).[15] The seawater was concentrated to 50 m*l* volume using this method. The seawater concentrate was sterilized with a 0.45-$\mu$m pore-size membrane filter. The seawater concentrate was stored in the dark at 4°C until use (within 4 days). Samples of the concentrate were dispensed in 2-m*l* aliquots into 2.5 m*l* tubes and kept at −80°C until use.

*Assay for Growth Inhibition of* A. catenella *and* Tetraselmis *sp. Inoculated the Seawater Concentrate*

A 1-m*l* aliquot of the seawater concentrate was inoculated to 10 m*l* of each culture of *A. catenella* TN-7 and *Tetraselmis* sp. FK-1, which were pre-incubated for 2 and 4 days in f/2 medium, respectively. The cell numbers of *A. catenella* TN-7 and *Tetraselmis* sp. FK-1 culture were counted every 2 and 4 days, respectively, during the assay. The growth inhibitory effect of the seawater sample was assessed by comparison with the growth of a control culture inoculated with only sterilized natural seawater. Duplicate cultures were performed.

When a growth suppression of phytoplankton was observed in the culture inoculated with the seawater concentrate, the cultured medium was filtrated using a 0.45 or 0.22-$\mu$m pore-size filter, and the filtrate was inoculated into a fresh culture of *A. catenella* TN-7 and *Tetraselmis* sp. FK-1. Then the reproducibility was measured. This process was repeated at least 2 times using the 0.45-$\mu$m pore-size filter, and 2 times using the 0.22-$\mu$m pore-size filter. 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;

$$\text{Growth suppression rate (\%)} = \{1 - (EN/CN)\} \times 100\ (\%).$$

And growth suppression rate less than 10 % was defined as negative growth suppression.

*Characterization of the filterable growth suppression agents*

As the growth suppression agents were filterable, it was considered that the main body might be viruses or ultramicrocells.[16] Therefore, we tentatively characterized the filterable growth suppression agents according to the general viral characterization.[17]

*1) Size Estimation by Membrane Filtration*

The size of phytoplankton growth suppression agents in the culture filtrate was estimated by passing the filtrate through various pore-size membrane filters. These filters were Millipore filter-0.45-$\mu$m(HA), 0.22-$\mu$m(GS), 0.10-$\mu$m(VC), and 0.05-$\mu$m(VM). Each filtrate was inoculated in fresh culture as mentioned above, and the growth of phytoplankton was compared to a control culture.

*2) Heat Stability*

The culture filtrate was dispensed in 2-m*l* aliquots into sterilized tubes and the tubes were placed in water baths maintained at 50°C, 60°C and 70°C for 30 min. Another tube was autoclaved (121°C for 15 min). Each tube was rapidly chilled on ice, and the remaining activity of growth suppression was measured in the manner mentioned above. An unheated culture filtrate was used as a positive control.

*3) Nuclease Sensitivity*

Each of 10 $\mu$*l* aliquots of DNase I (Pharmacia; 1 mg/m*l*) and RNase A (Pharmacia; 1 mg/m*l*) were added to 1 m*l* of the culture filtrates and the mixtures were incubated at 37°C for 30 min. These samples were inoculated to fresh cultures of *A. catenella* TN-7 and *Tetraselmis* sp. FK-1 and the growth suppression effect was observed, respectively. Both nucleases showed no inhibitory effects of the growth of *A. catenella* TN-7 and *Tetraselmis* sp. FK-1 at the same concentration described above.

*4) Proteinase K Sensitivity*

Aliquots of 10 $\mu$*l* of Proteinase K (Wako; 1 mg/m*l*) were added to 1 m*l* of the culture filtrates and the mixtures were incubated at 37°C for 1 h. These samples were inoculated to fresh cultures of *A. catenella* TN-7 and *Tetraselmis* sp. FK-1 and changes in the phytoplankton growth suppression effect were observed. Proteinase K indicated no inhibitory effects of the growth of *A. catenella* TN-7 and *Tetraselmis* sp. FK-1 at the same concentration described above.

*5) Ether Sensitivity*

One m*l* of diethyl ether was mixed with 4 m*l* of the culture filtrates. These mixtures and the control tubes containing f/2 medium were allowed to stand at 15 or 20°C respectively for 16 h. The ether was then removed using vacuum aspiration, and these samples were then inoculated to fresh cultures of *A. catenella* TN-7 and *Tetraselmis* sp. FK-1. The change in the phytoplankton growth suppression effect in each sample was observed.

*6) Stability under Acidic Condition*

The pH of the culture filtrate was adjusted to pH 3 and 5 by the addition of 0.1 N HCl or NaOH. After a 3-h incubation at 15 or 20°C, the pH of samples was re-adjusted to pH 8 by the addition of 0.1 N HCl or NaOH. The samples were inoculated to a fresh culture of *A. catenella* TN-7 and *Tetraselmis* sp. FK-1, and the change in the growth suppression was observed. The pH of the f/2 medium was changed in the same way, and then used as a control.

*7) Sensitivity for Ultraviolet (UV) Irradiation*

The culture filtrates were dispensed in 2-m*l* aliquots into Petri dishes and exposed to UV irradiation at room temperature using an UV killing lamp (National, GL15). UV dosage was set to 2,000 $\mu$W measured by an illuminator (Topcon UVR-245) and irradiated periods. Then each sample was filtrated through a 0.45-$\mu$m (HA) filter before inoculation into a fresh culture as mentioned above, and the growth of phytoplankton was compared to a control culture.

*The Growth Suppression Effect of the Cell Free Extract*

The cells of the growth-suppressed *A. catenella* TN-7 and *Tetraselmis* sp. FK-1 were collected by centrifugation (400 × g) for 15 min at 4°C from 150 ml of the growth-suppressed phytoplankton cultures. The cells were then washed 2 or 3 times with f/2 medium and suspended in 2m*l* of f/2 medium. The cells of each phytoplankton were disrupted by ultrasonication (Otake, 30s by 6 times under 100W) on ice. After removal of cell debris by centrifugation (15 min at 400 × g), the cell free extract was obtained and filter-sterilized with a 0.45 $\mu$m pore-size membrane filter. The cell free extracts were inoculated into fresh cultures of *A. catenella* TN-7 and *Tetraselmis* sp. FK-1. Changes in the growth suppression effect were then observed. The cell free extract prepared from uninfected cells was used as a control.

*Electron Microscopy*

The growth-suppressed *A. catenella* TN-7 cells were collected by centrifugation (15 min at 400 × g) at 20 days after inoculation of the agent. The cells were resuspended in sterilized natural seawater, and fixed with 5% glutaraldehyde (2.5% final concentration in sterilized natural seawater) for 2 h. Then the cells were washed with sterilized natural seawater and distilled water, and post-fixed with 2% osmium tetroxide (1% final concentration in distilled water) for 2 h. The cells were dehydrated in a graded ethanol series, then embedded in Spurr and sectioned. Ultrathin sections were stained with 4% uranyl acetate and lead citrate, then observed using a transmission electron microscope (Hitachi H-7000) at the acceleration voltage of 75 kV. Ultrathin section of the normal cells were prepared and observed as a negative control.

*Host Range*

The growth suppression effect by the culture filtrates which suppressed growth in *A. catenella* TN-7 and *Tetraselmis* sp. FK-1 was examined for the following 11 phytoplankton species: *A. catenella* TN-7 and *Tetraselmis* sp. FK-1, xenic culture of *Prorocentrum micans* Ka-13, xenic culture of *P. minimum* Ka-14, xenic culture of *Gymnodinium mikimotoi* Ka-34, axenic culture of *G. mikimotoi* G-303, axenic culture of *A. tamarense* OF-151, axenic culture of *A. catenella* OF-071, *Chattonella marina* NIES-3, *C. antiqua* NIES-1, and *Heterosigma akashiwo* NIES-4.

## Results

*Growth Suppression of* A. catenella *and* Tetraselmis *sp. by Seawater Sample*

Results of the screening of filterable pathogens that affected on the growth of *A. catenella* and *Tetraselmis* sp. in the coastal seawater on the mouth of Funka Bay are shown in Table 1. Growth suppression of *A. catenella* and *Tetraselmis* sp. was only observed in cultures inoculated with the seawater samples collected from September to October 1993 and concentrated using ultrafiltration. Growth suppression of *A. catenella* and *Tetraselmis* sp. by the seawater concentrate was observed during the late growth phase of each phytoplankton. The maximum cell numbers of *A. catenella* and *Tetraselmis* sp. in f/2 medium were approximately $1.5 \times 10^4$ cells/m*l* and $1.0 \times 10^6$ cells/m*l* at 18 and 9 days culture, respectively, while that of the cultures inoculated with the seawater concentrate were $8.0 \times 10^3$ cells/m*l* and $8.4 \times 10^5$ cells/m*l*, respectively (data not shown.). The growth suppression rate of *A. catenella* and *Tetraselmis* sp. by the seawater concentrate ranged from 44.0 to 45.5% and from 22.8 to 27.8%, respectively (Table

**Table 1.** Results in screening of algal growth suppression by inoculation of seawater concentrate collected at Funka Bay

| Sampling Date<br>Date | 1993<br>Jun.28 | Jul.26 | Sep.24 | Oct.28 | Nov.26 | 1994<br>Jan.26 | Feb.28 |
|---|---|---|---|---|---|---|---|
| Water temperature (°C) | 14.6 | 12.9 | 17.5 | 13.4 | 11.7 | 4.4 | 4.4 |
| Suppression effect against: | | | | | | | |
| *Alexandrium catenella* TN-7 | | | | | | | |
| Concentrated seawater | − (0.0%)[b] | − (0.0%)[b] | + (45.5%)[b] | + (44.0%)[b] | − (0.0%)[b] | − (0.0%)[b] | − (0.0%)[b] |
| Culture filtrates[a] | | | + (40.0%)[c] | + (40.0%)[c] | | | |
| *Tetraselmis* sp. FK-1 | | | | | | | |
| Concentrated seawater | − (0.6%)[b] | − (1.2%)[b] | + (27.8%)[b] | + (22.6%)[b] | − (0.0%)[b] | − (0.2%)[b] | − (2.4%)[b] |
| Culture filtrates[a] | | | + (30.9%)[c] | + (23.8%)[c] | | | |

+ Suppression was observed.
− No effect.
[a] Culture filtrate passed through HA filter 2 times and GS filter 2 times.
[b] Growth suppression rate (%) of concentrated seawater.
[c] Growth suppression rate (%) of culture filtrate through filter 4 times.

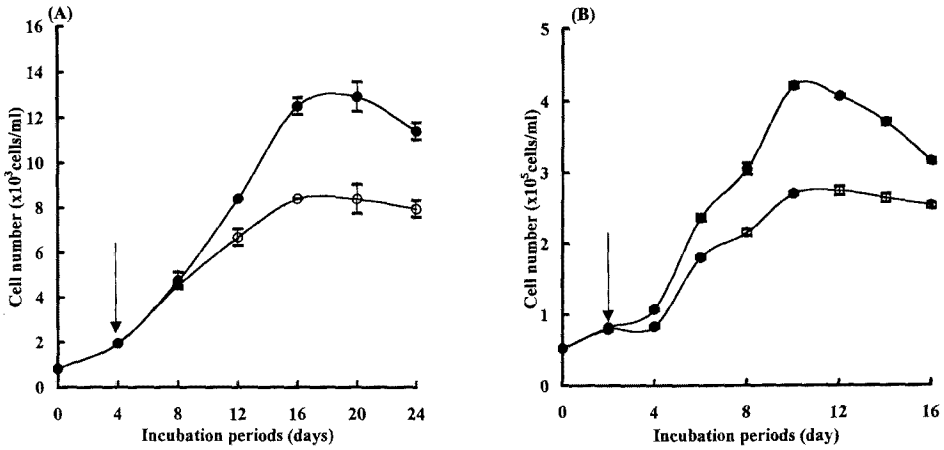


**Fig. 1.** Representative growth profiles of *Alexandrium catenella* TN-7 and *Tetraselmis* sp. FK-1 in f/2 medium inoculated with the growth suppression agents.

(A) *A. catenella* inoculated with the growth suppression agent (ACO2893). (B) *Tetraselmis* sp. inoculated with the growth suppression agent (TSS2493). Closed symbols indicate control culture, and open symbols indicate the experimental culture inoculated with the culture filtrate. Downward directed arrows indicate the inoculation time of the culture filtrate. Error bars are standard deviations of triplicate determination.

**Table 2.** Characteristics of the filterable-transferable growth suppression agents of *Tetraselmis* sp. and *A. catenella*

| Treatment | Growth suppression rate (%) | | | |
|---|---|---|---|---|
| | TSS2493 | TSo2893 | ACS2493 | ACO2893 |
| Passed through | | | | |
| 0.45 μm filter | 32.7 | 30.4 | 48.3 | 45.2 |
| 0.22 μm filter | 32.4 | 29.0 | 45.2 | 45.2 |
| 0.10 μm filter | 23.9 | 17.2 | 41.9 | 38.7 |
| 0.05 μm filter | 2.5 | 1.1 | 12.9 | 9.7 |
| Heating at | | | | |
| 50°C for 30 min | 4.5 | 7.9 | 14.7 | 5.6 |
| 60°C for 30 min | 2.0 | 7.9 | 14.7 | 5.6 |
| 70°C for 30 min | 7.4 | 5.7 | 8.8 | 5.6 |
| 121°C for 15 min | 5.3 | 5.7 | 2.9 | 2.8 |
| No heating | 33.6 | 33.2 | 52.9 | 30.6 |
| DNase treatment | 26.0 | 24.0 | 37.1 | 37.1 |
| RNase treatment | 4.3 | 2.8 | 5.7 | 5.7 |
| No treatment | 26.8 | 26.4 | 37.1 | 37.1 |
| Protease K treatment | 3.7 | 3.6 | 7.3 | 0.0 |
| No treatment | 34.1 | 34.2 | 43.9 | 40.5 |
| Ether treatment | 39.2 | 36.1 | 36.0 | 40.0 |
| No treatment | 39.2 | 37.6 | 36.0 | 40.0 |
| Stability at | | | | |
| pH 3 | 3.4 | 2.7 | 3.4 | 3.4 |
| pH 5 | 2.7 | 4.1 | 3.4 | 6.9 |
| pH 8 | 25.2 | 25.9 | 31.0 | 31.0 |
| UV irradiation | | | | |
| No irradiation | 33.9 | NT | NT | 47.5 |
| $5.0\times10^{2}$ μW·s/cm² | 33.9 | NT | NT | 43.8 |
| $1.0\times10^{3}$ μW·s/cm² | 30.6 | NT | NT | 47.5 |
| $5.0\times10^{3}$ μW·s/cm² | 5.2 | NT | NT | 2.5 |
| $1.0\times10^{4}$ μW·s/cm² | 2.6 | NT | NT | 2.5 |
| $5.0\times10^{4}$ μW·s/cm² | 2.6 | NT | NT | 2.5 |

NT: Not test.

1). The spent culture in which growth suppression occurred was passed through a 0.45-μm (HA) filter, then the culture filtrate was inoculated into fresh cultures of *A. catenella* and *Tetraselmis* sp., respectively. The serial transfer of the filtrates passed through 0.45-μm filter was performed 2 times. Growth suppression of *A. catenella* and

*Tetraselmis* sp. was also observed in the cultures inoculated with the culture filtrate showing growth suppression. The growth suppression rate was the same as in the culture inoculated with concentrated seawater (Table 1). Serial transfer of the culture filtrate that caused the growth suppression effect was repeated 2 times after filtration by using a 0.22-$\mu$m (GS) filter instead of the 0.45-$\mu$m filter. Growth suppression with the culture filtrates after the serial transfer was also observed without any reduction of the growth suppression rate (Fig. 1 and Table 1), suggesting that the growth suppression agents of these phytoplankton were transferable.

*Characteristics of the Transferable Growth Suppression Agents*

The four transferable and filtrable growth suppression agents were designated as ACS2493, ACO2893, TSS2493, and TSO2893, respectively, according to the host and sampling date. These agents which suppressed phytoplankton growth were tentatively characterized by estimating the size, heat stability, diethyl ether sensitivity, and proteinase and nuclease susceptibility. The results are summarized in Table 2. Growth suppression was observed in the cultures of *A. catenella* and *Tetraselmis* sp. inoculated with the filtrate that passed through a 0.45 or 0.22-$\mu$m filter without reduction of growth suppression rate. After passing the filtrate through a 0.10-$\mu$m filter, the growth suppression effect was slightly reduced. But passing through a 0.05-$\mu$m filter remarkably decreased the growth suppression (Table 2). The growth suppression effect was also remarkably reduced by heat treatment at more than 50°C for 30 min; the rate was one sixth that of the unheated control culture (Table 2), but no change in growth suppression was observed by heat treatment at temperatures less than 40°C (data not shown). The growth suppression agents were incubated with DNase, RNase or Proteinase K, and each sample was inoculated to a fresh culture of *A. catenella* and *Tetraselmis* sp. The growth suppression effects were lost after RNase treatment, but were not affected by DNase treatment (Table 2). And the growth suppression effects were also lost after Proteinase K treatment (Table 2). Addition of DNase, RNase or Proteinase K to the phytoplankton cultures did not influence the growth of *A. catenella* or *Tetraselmis* sp. Diethyl ether treatment did not reduce the growth suppression effect for *A. catenella* and *Tetraselmis* sp. (Table 2). The growth suppression agents were inactivated under acidic conditions at pH 3 and pH 5, respectively, but treatment at pH 8 did not change the effect (Table 2). The growth suppression agents in culture filtrates after UV irradiated treatment were inactivated with UV dosage at more than $5 \times 10^3$ $\mu W \cdot s / cm^2$ (Table 2).

*The Growth Suppression Effect by Cell Free Extract*

As for cell free extracts of each phytoplankton of which culture filtrate suppressed the growth of *A. catenella* (30.3%) and *Tetraselmis* sp. (21.8%), the growth suppression effect was observed, though cell free extracts of each control culture did not show the effect.

*Electron Microscopic Observation of the* A. catenella *Cells*

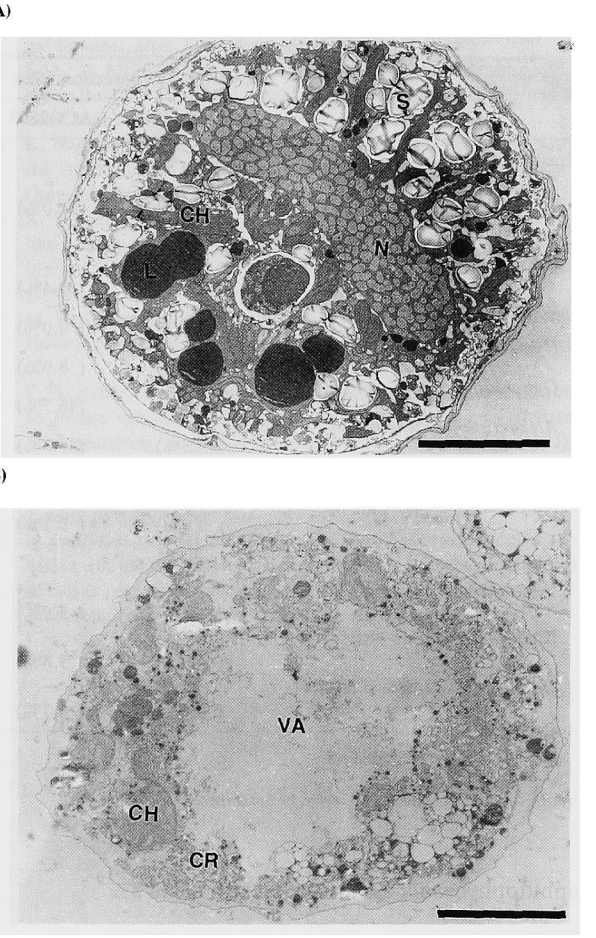


**Fig. 2** Ultrastructure of *Alexandrium catenella* TN-7 cells causes the growth suppression.
(A) Normal cell of *A. catenella* TN-7. (B) *A. catenella* TN-7 cell inoculating the growth suppression agent. Scale bars: 5 $\mu$m. N: nucleus; CH: chloroplast; S: starch; L: lipid; CR: crystal structure; VA: vacuole.

An electron microscopic observation of the growth-suppressed *A. catenella* at 20 days after inoculation of the agent clearly revealed severely damages of the cell wall structure, cell shape, vacuole formation, the disappearance of the nucleus, swelling of the chloroplast structure, and the appearance of a crystal structure (Fig. 2-B), when compared with the ultrastructure of the normal cell (Fig.2-A).

*Growth Suppression Effect against Other Species of Phytoplankton*

The culture filtrate which suppressed the growth of *A. catenella* (ACO2893) affected the growth of *Tetraselmis* sp., *G. mikimotoi* G303, *A. catenella* OF-071, and the original host *A. catenella* TN-7. The culture filtrate that suppressed growth of *Tetraselmis* sp. (TSS2493) also had an effect on the growth of *A. catenella* TN-7, OF-071, *G. mikimotoi* G-303, and the original host *Tetraselmis* sp. FK-1. But both culture filtrates had no effect on a xenic strain of *G. mikimotoi* Ka-34. Growth suppression or lysis in the 4 species of Dinophyceae and 3 species of

**Table 3.** Growth suppression effect of the culture filtrate against 11 microalgae in f/2 medium

| Host | Growth suppression effect | |
|---|---|---|
| | TSS2493 | ACO2893 |
| Prasinophyceae | | |
| *Tetraselmis* sp. | + (39.1%) | + (41.0%) |
| Dinophyceae | | |
| *Prorocentrum micans* Ka-13 | − ( 4.9%) | − ( 1.4%) |
| *Prorocentrum minimum* Ka-14 | − ( 4.2%) | − ( 0.0%) |
| *Gymnodinium mikimotoi* Ka-34 | − ( 2.5%) | − ( 8.9%) |
| *Gymnodinium mikimotoi* G-303 | + (38.5%) | + (46.7%) |
| *Alexandrium catenella* TN-7 | + (35.3%) | + (38.7%) |
| *Alexandrium catenella* OF-071 | + (40.7%) | + (46.7%) |
| *Alexandrium tamarense* OF-151 | − ( 0.0%) | − ( 9.1%) |
| Raphidophyceae | | |
| *Chattonella antiqua* NIES-1 | − ( 7.7%) | − ( 5.9%) |
| *Chattonella marina* NIES-3 | − ( 0.0%) | − ( 3.8%) |
| *Heterosigma akashiwo* NIES-4 | − ( 2.2%) | − ( 3.7%) |

+: Suppression was observed.
−: No effect.
*Growth suppression rate(%) are given in Parentheses.

Raphidophyceae was not observed (Table 3).

## Discussion

We observed that 0.45 $\mu$m filtered fractions of concentrated seawater collected on the mouth of Funka Bay, Hokkaido Japan, from September to October 1993, reduced the cell yields of *A. catenella* and *Tetraselmis* sp. by 40–45% and 22–27% respectively. Serial transfer of the culture filtrates maintained the same rate of growth suppression (Table 1). The growth suppression of *Tetraselmis* sp. and *A. catenella* in the culture filtrate disappeared after one-tenth dilution, which corresponded to 2 serial transfers, using the end-point dilution method (data not shown). However, the growth suppression was still observed after more than 2 serial transfers (Table 1 and Fig.1). These results suggested that the growth suppression agents could multiply in a phytoplankton culture and were transferable. The effects were also observed after the culture filtrate was passed through a 0.22-$\mu$m filter. Moreover, growth suppression was remarkably reduced after passing the culture filtrate through a 0.05-$\mu$m filter, suggesting that the causative agents of growth suppression to *Tetraselmis* sp. and *A. catenella* might have a particle form. These results indicated a possibility of viruses or ultramicrocells[16] for the growth suppression agents. Further characterization was done according to the general viral characterization.[17] The growth suppression disappeared after Proteinase K treatment and UV irradiation (UV dosage: $>5\times10^3$ $\mu$m.s/cm$^2$) (Table 2). Therefore, it is thought that the growth suppression agents may be nucleic acid or protein complex agents. The growth suppression also disappeared after RNase treatment, but did not disappear after DNase treatment (Table 2). Although viral coat proteins protect viral RNA digestion by external RNase, it is interested that the growth suppression agents showed RNase sensitivity. And the growth suppression agents did not disappear after ether treatment (Table 2). It may be presumed that the agents do not have lipid-layered structure. Proteinase K, UV irradiation, and ether sensitivity suggested that the agents showed virus-like properties.

Furthermore, growth suppression effects were observed from cell free extract of the growth-suppressed *A. catenella* and *Tetraselmis* sp., suggesting that the growth suppression agents also existed within the cells. The ultrastructure of the growth suppressed *A. catenella* cells seemed to be damaged (Fig.2. B). Unfortunately, electron microscopic observation failed to catch the main body of the agents. Furthermore it is necessary to observe the ultrastructure of *A. catenella* cells on an early stage after inoculation of the growth suppression agent.

The agents suppressed the growth of *A. catenella* TN-7 and *Tetraselmis* sp. FK-1, and *G. mikimotoi* Ka-34. Moreover, the agents suppressed the growth of both of *A. catenella* TN-7 and OF-071, but the agents did not suppress the growth of *A. tamarence* OF-151 that is relative of *A. catenella*. Consequently, the agents suppressed the growth of xenic strains of dinophyceae except of *A. tamarence* OF-151 in this study. No effects were observed in the three species of raphidophyceae examined. These results suggest the agents have a wide host range (Table 3). The agents affected the growth of an axenic strain of *G. mikimotoi* G-303, but did not affect that of a xenic strain of *G. mikimotoi* Ka-34. A microbial population in the culture of *G. mikimotoi* Ka-34 may have reduced the growth suppression effect for *G. mikimotoi* Ka-34.

Finally, the growth suppression agents were detected from results of screening and were only characterized tentatively. It is possible that some agents were mixed in this sample, because a cloning procedure has not yet been determined. The agent's form and physiological condition in marine ecosystem is unclear from this work. Further work is needed to elucidate the actual condition of the agents. Moreover, it is necessary to continue the screening of the growth suppression agents at the same sampling site.

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