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# Detection and enumeration of microorganisms that are lethal to harmful phytoplankton in coastal waters

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**Abstract:** The microplate MPN method was developed for detecting and enumerating algicidal microorganisms lethal to harmful marine phytoplankton which do not make lawns on agar media. To examine the accuracy of the microplate MPN method, the "killer" bacterium *Cytophaga* sp. J18/M01 was added to a concentration of $6.2\times10^3$ cells $ml^{-1}$, determined by DAPI staining and epifluorescence microscopy, to a water sample collected in the Harima-Nada Sea. The number of *Cytophaga* present was checked using the microplate MPN method with *Chattonella antiqua* as the susceptible organism. The value obtained, $8.1\times10^3$ cells $ml^{-1}$, was comparable to the previously calculated value, indicating the feasibility of this method for enumerating *C. antiqua* "killers". However the number of "killer" microorganisms is occasionally underestimated in some water samples using this method, possibly because some co-occurring microorganisms seem to inhibit the algicidal activity of the "killers". Using water samples collected from northern Hiroshima Bay at the end of and after a bloom of *Heterosigma akashiwo*, the numbers of microorganisms algicidal to *H. akashiwo* and *C. antiqua* were enumerated by the microplate MPN method. The number of *H. akashiwo* "killers" was in the order of $10^3\,ml^{-1}$ which was much higher than the number of *C. antiqua* "killers" (in the order of $10^0\,ml^{-1}$ or less), suggesting that *H. akashiwo* killers are specifically associated with the occurrence of *H. akashiwo* red tides. The microplate MPN method will allow investigations on the ecology of microorganisms lethal to marine phytoplankton in coastal environments.

**Key words:** red tide, *Chattonella*, *Heterosigma*, killer bacteria, viruses

## Introduction

The incidence of harmful phytoplankton blooms, or red tides, has increased in frequency recently as a long-term trend on a global scale (Anderson 1989; Smayda 1990). Harmful phytoplankton blooms have caused mass mortalities of cultured fish and bivalves (Okaichi 1989; Matsuyama et al. 1995), and shellfish poisonings (Hallegraeff 1993) in the coastal seas of the world. There is an urgent need for the development of techniques for predicting and reducing the impacts of harmful phytoplankton blooms.

A possibility is that algicidal (killer) bacteria and viruses could be useful tools in reducing the impact of harmful phytoplankton blooms. Algicidal marine bacteria that kill bloom-forming phytoplankton have been isolated from the coastal sea of Japan (Sakata 1990; Imai et al. 1991, 1993a, 1995; Fukami et al. 1992; Mitsutani et al. 1992; Yoshinaga et al. 1995). It is now thought that these killer bacteria may terminate harmful phytoplankton blooms and regulate phytoplankton population dynamics in coastal environments. Viruses are also killers of marine phytoplankton (Suttle et al. 1990). Viruses and virus-like particles are rather commonly observed in eukaryotic algae (reviewed by van Etten et al. 1991). For example, virus-like particles have been detected in the cells of bloom-forming phytoplankton such as *Emiliania huxleyi* (Lohmann) Hay et Mohler (Haptophyceae), *Heterosigma akashiwo* (Hada) Hada ex Hara et Chihara (Raphidophyceae), *Aureococcus anophagefferens* Sieburth et al. (Chrysophyceae), and *Phaeocystis pouchetii* (Hariot) Lagerheim (Haptophyceae) at the final stage of blooms, and their significance has been suggested in the rapid termination of blooms in coastal seas (Sieburth et al. 1988; Bratbak et al. 1993; Nagasaki et al. 1994a, b; Milligan & Cosper 1994; Brussaard et al. 1996; Jacobsen et al. 1996).

Algicidal agents in freshwater ecosystems are usually investigated by means of the soft-agar overlayer technique using phytoplankton capable of making a lawn on agar media (Safferman & Morris 1963; Daft et al. 1975; Yamamoto 1978). However, there are few species of marine phytoplankton, especially harmful flagellates, capable of growing on agar media (Nagasaki & Imai 1994). It has therefore been very difficult to detect and enumerate marine microorganisms that kill harmful marine phytoplankton. The development of a method to do this is indispensable for studies on marine algicidal microorganisms. We have developed a method (the microplate MPN method) for the detection and enumeration of these "killer" microorganisms by applying the MPN method using a liquid medium. In the present paper we describe the microplate MPN method, and discuss the accuracy and limitations of this method and the important role of "killer" microorganisms in terminating harmful phytoplankton blooms in coastal seas.

## Materials and Methods

### Procedure of the microplate MPN method for the detection and enumeration of algicidal microorganisms

Figure 1 shows the procedure of the microplate MPN method for the detection and enumeration of microorganisms lethal to harmful marine phytoplankton in seawater samples collected from coastal waters. Various species of fragile marine phytoplankton such as *Chattonella antiqua* (Hada) Ono (Raphidophyceae), *Heterosigma akashiwo*, and *Gymnodinium mikimotoi* Miyake et Kominami ex Oda can be used as the test organisms in this method. The method is described using the harmful red tide forming flagellate *C. antiqua*. An axenic clone culture of *C. antiqua* (NIES-1, provided by the National Institute for Environmental Study) was grown in a modified SWM-3 culture medium (Chen et al. 1969; Itoh & Imai 1987) at 22°C and at a light intensity of about 50 $\mu$mol $m^{-2}$ $sec^{-1}$ with a 14 h light 10 h dark photocycle. The culture conditions were basically identical throughout this study. A preculture of *C. antiqua* grown in 25 ml of the modified SWM-3 medium (ca. $2\times10^4$–$7\times10^4$ cells $ml^{-1}$) was diluted with 100 ml of the same medium, and subsequently 0.5-ml aliquots of the diluted *C. antiqua* culture were inoculated into the wells of a disposable sterilized tissue culture microplate (48 wells, Costar). Seawater samples were first filtered through a 0.8-$\mu$m pore size

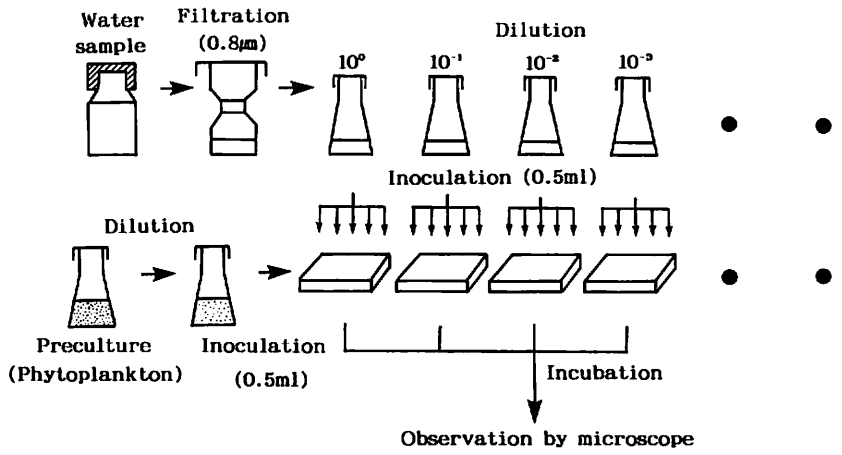


**Fig. 1.** Procedure for the microplate MPN method for the enumeration of microorganisms lethal to harmful phytoplankton from seawater samples.

Nuclepore filter in order to eliminate particles larger than bacteria (Imai 1984; Imai et al. 1993a). After serial 10-fold dilutions with filtered and autoclaved seawater, 0.5-ml aliquots at each dilution level were inoculated into the 48 wells of the microplate containing 0.5 ml of *C. antiqua* culture in each well. The inoculated microplates (initial *C. antiqua* concentrations, ca. $2\times10^3$–$7\times10^3$ cells $ml^{-1}$) were tightly sealed, to prevent evaporation, and incubated for 15 d or more under the conditions described above. Wells in which more than 99% of the phytoplankton cells were ruptured were scored as positive. A microplate inoculated with a 0.8 $\mu$m-filtered and autoclaved seawater sample was used as a control. The most probable number (MPN) of microorganisms lethal to *C. antiqua* in each water sample was calculated with a microcomputer (Nishihara et al. 1986; Koch 1994).

Enumeration of the "killer" bacterium *Cytophaga* sp., artificially added to seawater samples

Samples of surface water were collected from the pier of Nansei National Fisheries Research Institute (NNFRI), located in northern Hiroshima Bay (Fig.2), on 5 August 1994, and at Stn NH3 in northern Harima-Nada Sea (Fig.2) on 23 August 1994. Hiroshima Bay and Harima-Nada Sea are both located within the Seto Inland Sea of Japan. Water samples collected at Stn NH3 were chilled at 5°C and transported to the NNFRI. The samples reached the NNFRI in the morning of the next day after collection. Water samples were then filtered through 0.8-$\mu$m Nuclepore filters. The total number of bacterial cells in filtered water samples were counted by DAPI (4'6-diamidino-2-phenylindole) staining and epifluorescence microscopy (Porter & Feig 1980; Imai 1984, 1987). The viable number of bacteria in the filtered samples were enumerated by the MPN method using a culture medium containing 0.5-g Tripticase peptone and 50-mg yeast extract in 1-liter seawater (Ishida et al. 1986; Imai 1987).

The gliding bacterium *Cytophaga* sp. J18/M01 (Imai et al. 1991, 1993a) was used as the algicidal agent acting against *Chattonella antiqua*. The bacterium had been maintained in culture using *C. antiqua* as the nutrient source. The "killer" bacterium *Cytophaga* sp. J18/M01 preys upon *C. antiqua* and commonly reaches densities of more than $10^8$ cells $ml^{-1}$ when *C. antiqua* cells are supplied in concentrations of the order of $10^4$ cells $ml^{-1}$ (Imai et al. 1993a). A portion (0.25 ml) of this bacterial culture was added to 25 ml of *C. antiqua* culture

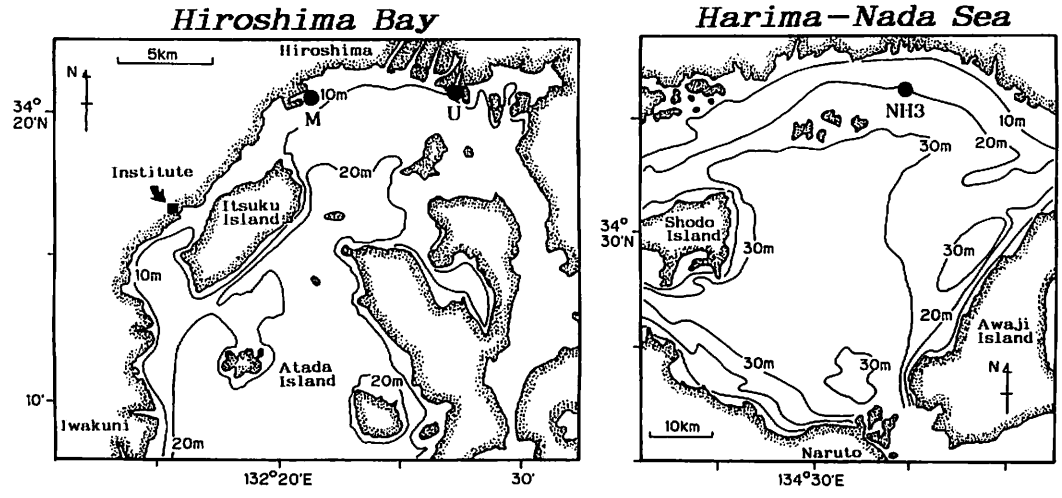


**Fig. 2.** Sampling stations in the northern Hiroshima Bay and Harima-Nada Sea in the Seto Inland Sea.

($>5\times10^4$ cells $ml^{-1}$), and incubated for 3 d at 22°C in the light. The cell densities of the bacterium usually reached $5\times10^8$ cells $ml^{-1}$ or more. The bacterial cultures were then diluted with the 0.8 $\mu$m-filtered seawater samples, and the concentrations of "killer" bacterium were adjusted to the order of $10^3$ cells $ml^{-1}$. The artificial samples were thus prepared, and the numbers of the "killer" bacterium *Cytophaga* sp. J18/M01 were enumerated by the microplate MPN method (Fig. 1). As controls, water samples filtered through Nuclepore filters of 0.8-$\mu$m pore size were processed by the microplate MPN method without the addition of *Cytophaga* sp. J18/M01.

Enumeration of "killer" microorganisms in seawater samples collected during and after a red tide of *Heterosigma akashiwo*

In June of 1992, a red tide of *Heterosigma akashiwo* occurred in the northern part of Hiroshima Bay (Fig. 2). Surface water samples were collected at Stn M on 11 June towards the end of the bloom, and at Stn U on 18 June after the termination of the red tide. Water samples were then filtered through 0.8-$\mu$m Nuclepore filters, and the number of microorganisms algicidal to *H. akashiwo* was enumerated for each sample using the microplate MPN method (Fig. 1). An axenic strain of *H. akashiwo*, isolated from Hiroshima Bay by I. Imai in 1989 (Imai et al. 1993b), was used as the assay organism. Concurrently, microorganisms lethal to *Chattonella antiqua* were enumerated for both surface water samples by the same method using *C. antiqua* as the assay organism.

## Results

Figure 3 shows examples of the algicidal effects on *Chattonella antiqua* and *Heterosigma akashiwo* of the bacterium *Cytophaga* sp. J18/M01. Since both *C. antiqua* and *H. akashiwo* are "naked" species, their cells were entirely ruptured by the attack of the bacterium. Such algicidal activity patterns are common for "killers" in natural water samples.

To evaluate the accuracy of the microplate MPN method (see Fig. 1), the bacterium *Cy-*

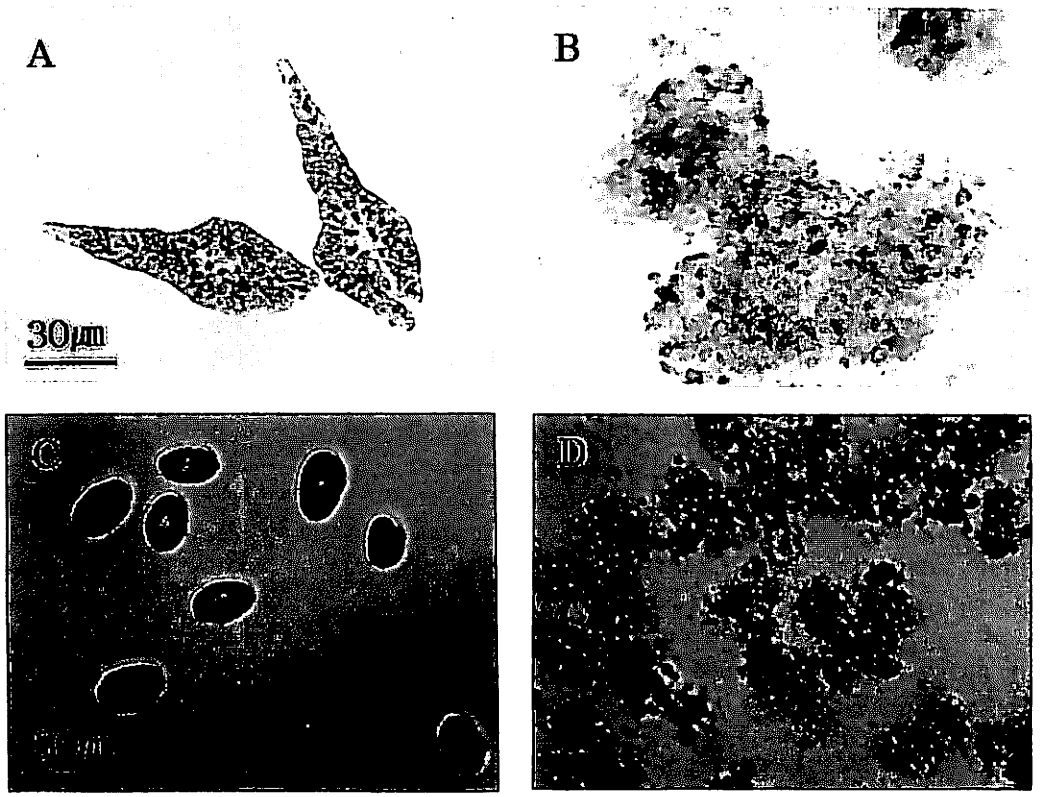


**Fig. 3.** Microscopical observations on algicidal activity of the "killer" bacterium *Cytophaga* sp. J18/M01 towards *Chattonella antiqua* and *Heterosigma akashiwo*. Organisms were combined and incubated for three days. A: Live cells of *C. antiqua*. B: Ruptured cells of *C. antiqua* killed by the bacterium. C: Live cells of *H. akashiwo*. D: Ruptured cells of *H. akashiwo* killed by the bacterium.

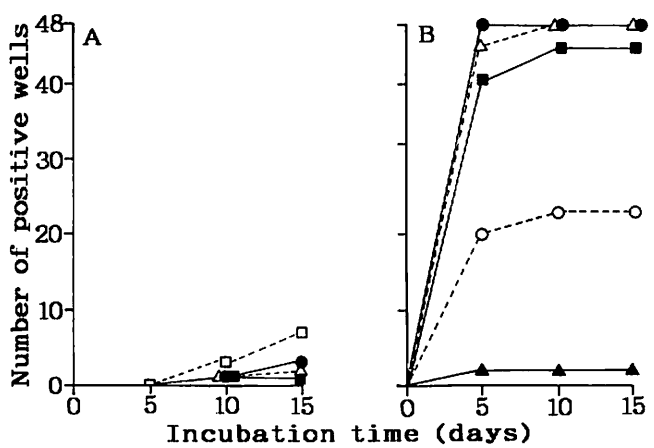


**Fig. 4.** Temporal changes in the number of wells that tested positive, (**A**) indicating the existence of "killers" at each dilution level for naturally occurring "killers" in the water sample and for (**B**) *Cytophaga* sp. J18/M01 artificially added to the sample at a concentration of $6.2\times10^3$ cells $ml^{-1}$. Surface water sample was collected at Stn NH3 on 23 August 1994, filtered through a 0.8-μm pore size Nuclepore filter, and was processed according to the microplate MPN method. Dilution levels: $10^0$ (□), $10^{-1}$ (●), $10^{-2}$ (△), $10^{-3}$ (■), $10^{-4}$ (○), $10^{-5}$ (▲).

*tophaga* sp. J18/M01 was artificially added to natural seawater, and the number of the "killer" bacterium was enumerated by this method. A water sample was collected from Stn NH3 in the northern Harima-Nada Sea on 23 August 1994, and the concentration of the "killer" bacterium was adjusted to $6.2\times10^3$ cells $ml^{-1}$. The density of this bacterium was then re-enumerated by the method. Figure 4 shows temporal changes in the number of wells testing positive (indicating the existence of and algicidal activity of *Cytophaga* sp. J18/M01) at each dilution level. In the microplates at lower dilution levels ($10^{-1}$ and $10^{-2}$), all 48 wells were scored as positive. The number of wells testing positive decreased at higher dilution levels. Table 1 summarizes MPN value results for *Cytophaga* sp. J18/M01 as enumerated by the microplate MPN method. The density value of $8.1\times10^3$ cells $ml^{-1}$ so obtained was comparable to the real concentration of $6.2\times10^3$ cells $ml^{-1}$ initially added. The total density of bacteria in the water sample from Stn NH3 was $4.3\times10^6$ cells $ml^{-1}$, and the density of viable bacteria was $2.3\times10^5$ cells $ml^{-1}$. The number of microorganisms lethal to *C. antiqua* that originally existed in this water sample was estimated to be 0.53 $ml^{-1}$.

Figure 5 shows the temporal changes in the number of wells testing positive at each dilution level in the experiment using the water sample collected from the pier of the NNFRI (Fig. 2) on 5 August 1994. The "killer" bacterium was added to a concentration of $5.8\times10^3$ cells $ml^{-1}$ into seawater filtered through a 0.8-$\mu$m Nuclepore filter. The total and viable numbers of bacteria in the filtered sample were $3.4\times10^6$ cells $ml^{-1}$ and $3.3\times10^5$ cells $ml^{-1}$, respectively. The number of microorganisms lethal to *C. antiqua* that originally existed in this water sample was estimated to be 2.1 $ml^{-1}$. The number of wells testing positive in each 48-well microplate for the dilution levels ($10^{-1}$, $10^{-2}$, $10^{-3}$, and $10^{-4}$) was 29, 45, 46, and 13, respectively. Thus not all the wells tested positive, even at the lower dilution levels.

Table 2 shows the number of microorganisms lethal to *H. akashiwo* and *C. antiqua* as enumerated by the microplate MPN method using natural water samples collected at Stn M towards the end of a *H. akashiwo* red tide, and at Stn U after the termination of the *H. akashiwo* red tide in June of 1992. The dominant phytoplankton species in the sample from Stn M was *H. akashiwo* ($2.0\times10^4$ cells $ml^{-1}$). Microorganisms lethal to *H. akashiwo* were numerous ($>7.7\times10^3$ $ml^{-1}$), but those of *C. antiqua* were much more scarce (0.9 $ml^{-1}$). In the water sample from Stn U, the dominant phytoplankton species was the dinoflagellate *Prorocentrum triestinum* Schiller ($6.2\times10^3$ cells $ml^{-1}$) with *H. akashiwo* being less abundant (67 cells $ml^{-1}$). However, the density of *H. akashiwo* "killers" was rather high ($1.2\times10^3$ $ml^{-1}$), while

**Table 1.** Comparison of estimated concentration (MPN) of the "killer" bacterium *Cytophaga* sp. J18/M01, enumerated by the microplate MPN method, with the cell concentration initially added to the surface water sample collected at Stn NH3 in the Harima-Nada Sea, on 23 August 1994. The total and viable counts of natural bacteria, and the *Chattonella antiqua* "killer" naturally occurring in the water sample are also shown.

| | Concentration ($ml^{-1}$) |
|---|---|
| *Cytophaga* sp. J18/M01 | |
| Density initially added (DC*) | $6.2\times10^3$ |
| Density estimated (MPN**) | $8.1\times10^3$ |
| Bacteria in the sample | |
| Total bacteria (DC*) | $4.3\times10^6$ |
| Viable bacteria (MPN**) | $2.3\times10^5$ |
| *C. antiqua* killer (MPN**) | $5.3\times10^{-1}$ |

* DC: Direct count. ** MPN: Most probable number.

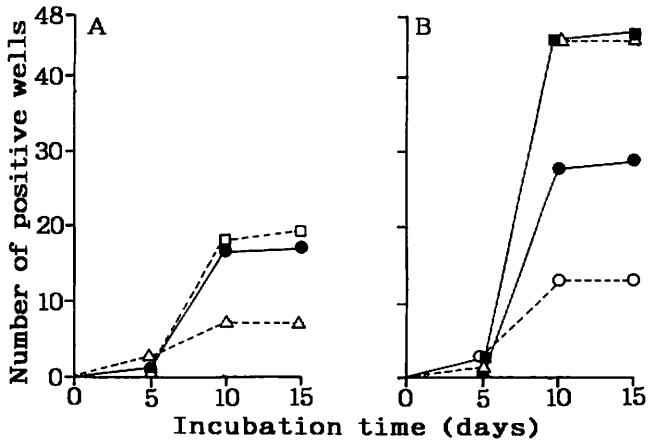


**Fig. 5.** Temporal changes in the number of wells that tested positive, (**A**) indicating the existence of "killers" at each dilution level for natural "killers" in the water sample and for (**B**) *Cytophaga* sp. J18/M01 artificially added to the sample at a concentration of $5.8\times10^3$ cells $ml^{-1}$. Surface water sample was collected from the pier of the NNFRI on 5 August 1994, filtered through a 0.8-$\mu$m pore size Nuclepore filter, and was processed according to the microplate MPN method. Dilution levels: $10^0$ (□), $10^{-1}$ (●), $10^{-2}$ (△), $10^{-3}$ (■), $10^{-4}$ (○), $10^{-5}$ (▲).

"killers" of *C. antiqua* were much more scarce ($1.2\,ml^{-1}$). *Chattonella antiqua* was not observed in either sample. In summary, high concentrations of *H. akashiwo* "killers" were found to be associated with the *H. akashiwo* bloom.

## Discussion

In freshwater ecosystems, the soft-agar layer technique is commonly employed as a useful tool for investigating algicidal agents, because many freshwater species are able to form a lawn on agar media (Safferman & Morris 1963; Mitsutani et al. 1987). On the other hand, with the exception of some diatom species, most species of marine phytoplankton are unable

**Table 2.** Numbers of *Heterosigma akashiwo* "killers" and *Chattonella antiqua* "killers" in water samples collected at Stn M on 11 June 1992 towards the end of a *H. akashiwo* red tide, and at Stn U on 19 June 1992 after the *H. akashiwo* red tide. The number of "killers" was estimated by the microplate MPN method. Dominant phytoplankton species and cell densities are also shown.

| Sampling site and date | Density ($ml^{-1}$) | | Dominant phytoplankton species (cells $ml^{-1}$) |
|---|---|---|---|
| | *H. akashiwo* "killers" | *C. antiqua* "killers" | |
| Stn M<br>11 June 1992 | $>7.7\times10^3$ | 0.9 | *Heterosigma akashiwo* ($2.0\times10^4$)<br>*Prorocentrum triestinum* ($2.2\times10^3$) |
| Stn U<br>19 June 1992 | $1.2\times10^3$ | 1.2 | *Prorocentrum triestinum* ($6.2\times10^3$)<br>*Heterosigma akashiwo* ($6.7\times10^1$) |

to grow on agar media (Yamada et al. 1985; Sakata 1990; Nagasaki & Imai 1994). Investigations on agents lethal to harmful marine phytoplankton have therefore been few due to a lack of suitable methods, especially in the case of fragile species such as raphidophycean flagellates and dinoflagellates. The present microplate MPN method enables the detection and enumeration of microorganisms that are lethal to harmful marine phytoplankton. Additionally, different species of "killer" microorganisms can be detected and isolated by changing the phytoplankton species used in the assay. Naked and/or fragile phytoplankton species are more suitable for this method, because their death is easily detected through the rupture of their cells (see Fig. 3).

When the "killer" bacterium *Cytophaga* sp. J18/M01 was added to the surface water sample taken at Stn NH3 and re-enumerated by the microplate MPN method using *Chattonella antiqua*, the MPN value obtained for the "killer" microorganisms so obtained was $8.1\times10^{3}\,ml^{-1}$ which is comparable to the concentration of $6.2\times10^{3}\,ml^{-1}$ which was initially added (see Fig. 4 and Table 1). The sample from Stn NH3 contained viable bacteria at a concentration of $2.3\times10^{5}$ cells $ml^{-1}$ and the total bacterial count was $4.3\times10^{6}$ cells $ml^{-1}$. In water samples such as that taken from Stn NH3 on 23 August 1994, the microplate MPN method can be employed as an effective tool allowing the enumeration of "killer" microorganisms.

According to the 1991 report on the development of technology for the prevention of red tides (Imai & Mitsutani, unpublished data), Fukami et al. (1996) also employed microplates to enumerate bacteria inhibitory to the diatom *Skeletonema costatum* (Greville) Cleve and to the red tide dinoflagellate *Gymnodinium mikimotoi*, although they did not calculate the MPN. They assessed the number of inhibitory bacteria statistically by assuming that the number of wells showing growth inhibition followed the Poisson distribution. Calculating the MPNs of "killer" microorganisms using at least 3 microplates (microplate MPN method) presumably offers a higher degree of accuracy than that of values estimated with a single microplate based on the Poisson distribution.

With respect to the water sample collected from the pier at the NNFRI on 5 August 1994, the result was rather complicated (see Fig. 5). Not all the wells tested positive, even at the lower dilution levels. This result suggests the possibility of the existence of microorganisms (bacteria and/or viruses) in the water sample collected from the pier at the NNFRI on 5 August 1994 that inhibited the algicidal activity of *Cytophaga* sp.J18/M01 towards *C. antiqua*. If the concentration of "killer" microorganisms is thus calculated, the MPN value would be an underestimate. Research on microorganisms that inhibit the algicidal activity of "killer" bacteria is needed in order to clarify their effects not only on the microplate MPN method but also on the occurrence and termination of red tides.

The number of microorganisms lethal to *Heterosigma akashiwo* was much higher than those lethal to *C. antiqua* in the water samples collected from Stns M and U in northern Hiroshima Bay in June of 1992 (see Table 2). *Heterosigma akashiwo* "killers" appear to be associated with the occurrence of *H. akashiwo* red tides, and they also appear to be species-specific. Although *C. antiqua* belongs to the same Class of Raphidophyceae as *H. akashiwo*, it is not affected by *H. akashiwo* "killers". In Uranouchi-Cove, a small inlet in Kochi Prefecture, Fukami et al. (1996) investigated the effects of bacterial assemblages on the succession of phytoplankton species from *S. costatum* to *H. akashiwo* from April 1993 through January 1994. Their results suggested that bacteria affected the growth of phytoplankton in seawater and that the bacterial effects were species-specific. Accordingly, "killer" microorganisms are presumed to play an important role in the termination of red tides composed of a single

species, and in the species succession of phytoplankton.

Even after filtration through a 0.8-$\mu$m Nuclepore filter, water samples generally retain both bacteria and viruses. If a Nuclepore filter with a pore size of 0.2 $\mu$m is used, most bacteria are eliminated and the effects of "killer" viruses during periods of harmful phytoplankton blooms can therefore be investigated using the microplate MPN method. In general, viruses kill phytoplankton with a high species-specificity (Safferman & Morris 1964; Stewart & Brown 1969; Stewart & Daft 1977; Suttle et al. 1990). Virus-like particles were found inside cells of *H. akashiwo* in the final stages of *H. akashiwo* red tides in northern Hiroshima Bay in 1992 and 1993 (Nagasaki et al. 1994a, b), but not in 1994 (Nagasaki et al. 1996). On the other hand, several hundred bacteria lethal to *H. akashiwo* were isolated during the course of *H. akashiwo* blooms in 1994 and 1995 in samples from northern Hiroshima Bay using the microplate MPN method (Kim et al., unpublished data). Several important questions remain to be answered: to what extent do "killer" bacteria and viruses affect the population dynamics of harmful marine phytoplankton, and/or which is more important in controlling their dynamics? The microplate MPN method described here will allow the study of the presumably significant ecological role that "killer" microorganisms play in coastal waters.

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