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A Modified MPN Method for Counting Oligotrophic Bacteria by using Naturally Occurring Organics

Fukami, Kimio,*1 Satoshi Ohara,*1 Yuzaburo Ishida,*1
and Aldo A. Mariazzi*2

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A MPN method was modified to determine the number of oligotrophic bacteria in fresh water. A series of MPN media were prepared with natural lake water by either autoclaving or filter-sterilization, and with diluted peptone solution (0.5 mg/*l*) as a reference. Bacterial growth-positive test tubes were judged directly by epifluorescence microscopic counting. The bacterial counts using the medium of filter-sterilized *in situ* lake water was significantly higher than those obtained by autoclaved *in situ* lake water or peptone media. The difference in counting values between autoclaving and filter-sterilization was much larger when natural lake water media were prepared after several days' incubation under light condition. In this period, the organic carbon concentration (as COD, chemical oxygen demand) increased more than twice, mainly due to the excretion by phytoplnakton. These results suggest that naturally occurring dissolved organic matter, which was much suitable for the growth of autochthonous bacteria (oligotrophs) in the corresponding period, becomes one of the best media for counting the number of oligotrophic bacteria. Seasonal fluctuation of bacterial counts obtained by the present method showed a good correlation to that of chl. a, suggesting that the natural population of heterotrophic bacteria was influenced by phytoplankton through its excreted organic carbon.

In aquatic environments, free-living heterotrophic bacteria must usually utilize dissoved organic carbon (DOC) as one of the most important substrates for growth. Input of organic matter from terrestrial areas via rivers, the extracellular release of *in situ* phytoplankton, and solubilization of particulate matter during the decomposition process are mainly considered as an origin of DOC in fresh and sea water.

Hellebust[1] reported that phytoplankton released as much as 30% of photoassimilated carbon to extracellular environment. In eutrophic areas a contribution of excerted organic carbon by phytoplankton (EOC) would be up to 10% of total DOC because there are various sources of dissolved organic matters. In oligotrophic areas, however, the percentage of phytoplankton EOC in total DOC is much higher and the organic materials released by microalgae are the most important substrates for aquatic bacteria.[2-4] Chrost[5] reported that the aquatic bacteria have taken up more actively the phytoplankton-EOC than simple substances such as glucose, acetate and others.

For the enumeration of the number of "viable" heterotrophic bacteria in the oligotrophic area, the Most Probable Number (MPN) method using peptone and/or yeast extract of low concentration (0.5–5 mg/*l*) for liquid media has been used.[6] These organic substrates, however, are artificial ones and they may not be necessarily the suitable substarates for growth of the natural community of heterotrophic bacteria. Melchiorri-Santolini and Cafarelli[7] mentioned that MPN using filtered natural lake water medium gave 3 times higher counts than that using conventional concentrated peptone-yeast extract medium (ZoBell's 2216E). However, as they used the autoclaved lake water for preparation of MPN medium, it is possible that the most labile fraction of dissolved organic matter in natural lake water medium had been degraded by heat treatment.

The purpose of this paper is to describe that the media containing non-heated natural organic matter (*i.e.* EOC) are more preferable to the artificial organic matter media to count the

*1 Department of Fisheries, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan (深見公雄, 小原 賢, 石田祐三郎: 京都大学農学部水産微生物学研究室)

*2 Instituto de Limnologia, Facultad de Ciencias Naturales y Museo, Paseo del Bosque, 1900 La Plata, Argentina.

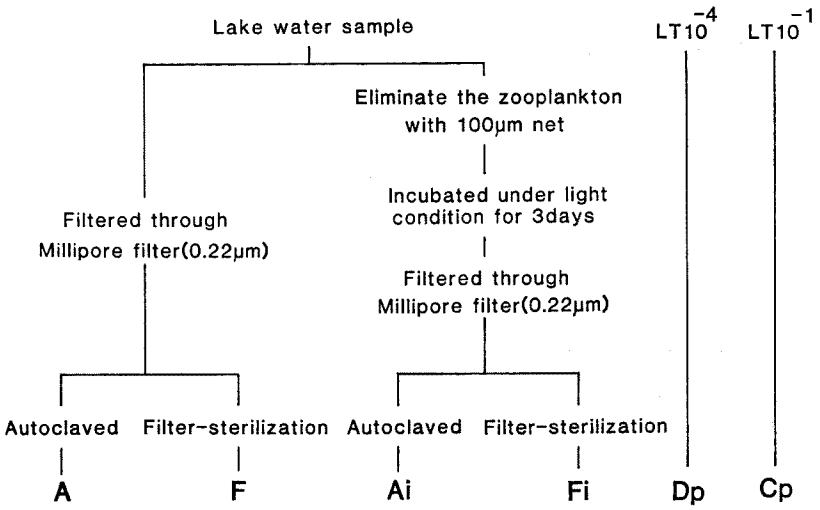

Fig. 1. Scheme of the procedure for the preparation of 6 types of MPN meia.

number of viable heterotrophic bacteria (oligotrophs) in the aquatic environments by MPN method, and to discuss the role of phytoplankton-EOC as the substrates of bacterial growth.

Materials and Methods

Lake water samples were collected with well-rinsed Van Dorn sampler from 6 m depth of one station (Stn. Ie) in the northern Lake Biwa.[6] The depth of 6 m is the mean chl. a maximum layer of this station in summer season.

Lake water samples collected were used for the preparation of MPN media. Six types of the media for MPN were prepared in accordance with the scheme as shown in Fig. 1. Lake water samples were filtered through 0.22 μm Millipore filters. A part of the filtered lake water was autoclaved. This is referred to as A. Another part of the filtered sample was refiltered aseptically through a 0.22 μm filter (Millex-GV, Millipore) attached to a sterilized plastic syringe without autoclaving and this filter-sterilized sample is named F. Some other part of lake water sample was filtered through 100 μm nylon net for elimination of larger zooplanktonic consumers, and then was incubated under light condition (9000 lux of 14: 10 LD cycle at 20°C) for 3 days. After that, the sample was filtered through 0.22 μm Millipore filter and then prepared the media by either autoclaving (Ai) or filter-sterilization (Fi). As a reference, diluted $LT10^{-4}$ medium (Dp, trypticase peptone 0.5 mg and yeast extract 0.05 mg in 1 *l* of aged lake water) and concentrated $LT10^{-1}$ (Cp,

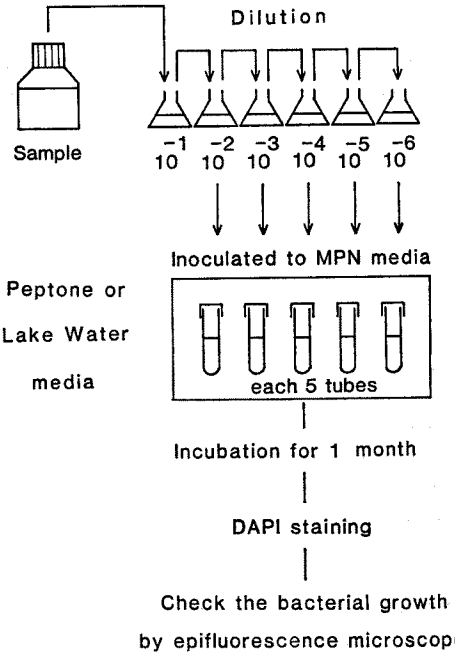

Fig. 2. Scheme of the procedure for the determination of bacterial number. Extinction dilution method (MPN, most probable number) with 5 replicate test tubes was employed.

1000 times concentrated of $LT10^{-4}$) were also prepared as described in a previous paper.[8] All the glass bottles and test tubes were previously combusted at 450°C for several hours to remove the organic contamination.

Within 1 week after the preparations for 6 types

of liquid media for MPN described above, lake water samples for an inoculum were collected at the same station and depth, and were taken back immediately to the laboratory. After appropriate decimal dilution they were inoculated to a series of 5 replicate test tubes of each medium (Fig. 2). Water samples of minimum dilution step were fixed with filtered neutral formalin (final conc. 2%) and simultaneously inoculated to media as blanks. The bacteria-inoculated media for MPN were incubated at near *in situ* temperature for 1 month and then the MPN values were estimated. In the case of Cp, the turbidity of bacterial growth was detected. However, as nutrient concentrations of another 5 media were so low (less than few mg-C/*l*) that the bacteria, even if they grew, did not show any turbidity, bacterial growth was checked by epifluorescence microscopic observation after DAPI staining.[8] The test tubes with more than 5 times of blank count besides more than 3×10^4 cells/m*l* were judged as bacterial-growth positive.

The concentrations of chl. a were determined by the method of SCOR/UNESCO.[9] The chemical oxygen demand (COD) was determined by a conventional standard method by the oxidation with $KMnO_3$.[10]

Results and Discussion

The station Ie, investigated in the present study, is the middle part of the northern Lake Biwa. Water depth of this station is about 70 m. Recently the water is mesotrophic although it had been rather oligotrophic until 10 years ago. Blooms of a diatom *Asterionella formosa* Hassall, chrysophyceae *Uroglena americana* (Calkins) Lemmermann,[11] and a green alga *Staurastrum dorsidentiferum* var. *ornatum* Gronbl, were usually observed from spring to early summer, and the transparency decreased to less than 2 m in these period.

Figure 3 shows the seasonal variation of MPN counts obtained by 6 different media at the 6 m layer of the sampling station. MPN counts of A (an autoclaved natural lake water medium) were sometimes much less than those of Dp (a low concentration of peptone medium). However, when natural lake water media were prepared by filter-sterilization (F), the counts were remarkably higher. The maximum MPN count was often obtained by Fi, which was prepared by filter-sterilization of lake water after 3 days light incubation. The chemical oxygen demand (COD), which is considered an index of labile dissolved organic carbon, of Fi increased twice to four times greater than that of F in the process of light incubation. The concentration of chl. a increased simultaneously about 50% during the incubation. These results suggest that the increase of dissolved organic carbon was due to the excretion of organic matter by photoassimilating phytoplankton.

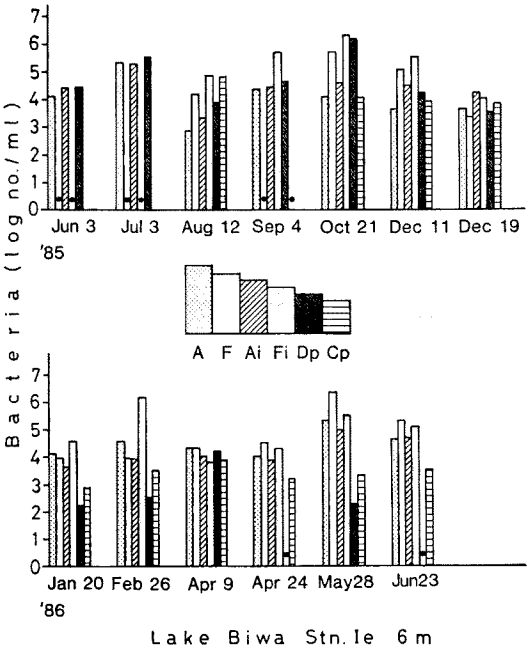

Fig. 3. Changes in MPN counts of bacteria in lake water collected at 6 m layer of Stn. Ie in Lake Biwa obtained by 6 different types of media. A, autoclaved lake water; F, filter-sterilized lake water; Ai, autoclaved lake water incubated under light condition; Fi, filter-sterilized lake water under light condition; Dp, diluted peptone solution (0.5 mg/*l*); Cp, concentrated peptone soution (0.5 g/*l*). See Fig. 1 for the details. *: data not available.

From spring to early summer in 1986, MPN counts by F (filter-sterilized *in situ* lake water medium) showed the maximum number. Moreover, MPN count by Dp was relatively low. In this period, the concentrations of chl. a were generally high and COD values of *in situ* lake water were considerablly high (Fig. 4). These results were explained by the fact that the concentration of dissolved organic carbon in these period was higher because of the phytoplankton blooming and the bacteria which can utilize relatively high concentration of organic substrate were predominant, resulting in that the MPN by F medium showed

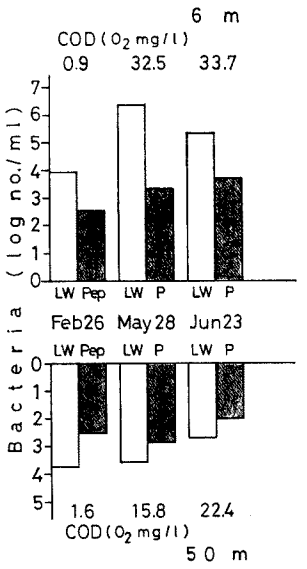

Fig. 4. Comparison in MPN counts of bacteria between by natural lake water medium (LW: open column) and by peptone medium adjusted to COD value similar to that of natural lake water at each month (P: shadowed column) at Stn. Ie in Lake Biwa. Upper, 6 m layer; bottom, 50 m layer. LW coincides with F medium in Figs. 1 and 3.

the maximum number.

According to a previous report,[7] MPN using autoclaved filtered natural lake water medium usually gave higher counts, 3 times greater as a mean value, than those using peptone-yeast extract medium (ZoBell's 2216E). In the present study, the counts using autoclaved lake water medium (A) were rather lower than those using peptone medium (Dp) in 1985 (Fig. 3). However, the filter-sterilized lake water medium (F) gave significantly higher counts than those in the case of autoclaved (A). The discrepancy is due to that they used a quite high concentration peptone solution (ZoBell's 2216E) for medium, and that they detected the bacterial growth in test tubes by counting bacteria stained with classical erythrosin under phase contrast microscopy. In the present study we used the peptone medium with the concentration as low as *in situ* lake water (0.5 mg/*l*). Moreover, DAPI-staining epifluorescence microscopic method was used to check the bacterial growth. This method is very accurate and bacterial cells, even low number, can be distinguished from other small particles by its shape and color, resulting in that we could detect more numbers of bacterial growth-positive tubes

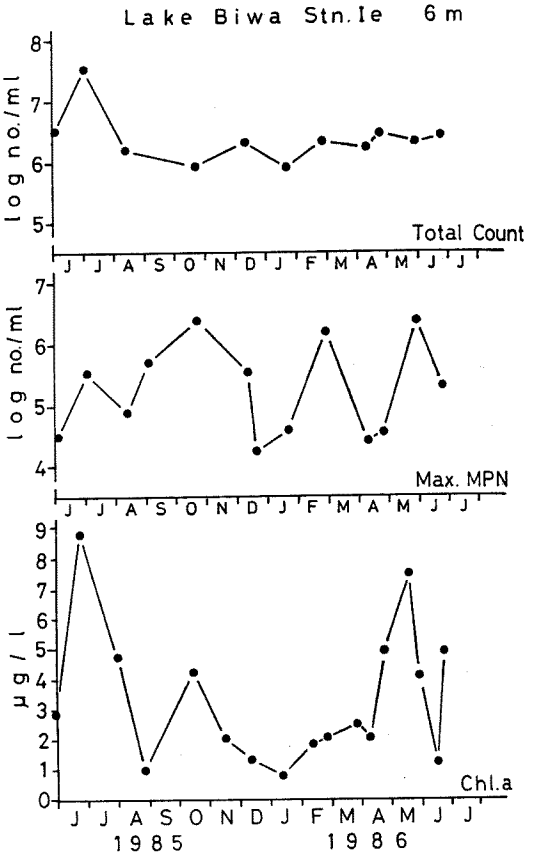

Fig. 5. Seasonal fluctuations of total count of bacteria (upper), maximum MPN counts by among 6 kinds of media (middle), and the concentration of chl. a (bottom) in lake waters at 6 m layer of Stn. Ie in Lake Biwa.

and get the higher MPN.

From these results of the MPN counts, it is suggested that naturally occurring organic substrates are superior to the artificial substrates of peptone and/or yeast extract for the growth of *in situ* bacteria (oligotrophs) and the dissolved organic matter in natural lake water is labile to the heat. The speculation is supported by the fact that COD values of lake water decreased drastically after autoclaving (about one third to one several tenth).

There still remains a possibility that the differences in bacterial MPN counts are simply due to the differences in the concentration of organic substrates. To check this point, bacterial counts by using two types of MPN media, which are the filter-sterilized *in situ* lake water medium and the peptone medium adjusted to COD similar to that of the lake water medium, were compared. The

result clearly shows that MPN counts by the natural lake water medium are much higher than those by the artificial peptone medium with the same COD value (Fig. 4). The difference of bacterial counts often reached nearly 3 orders of magnitude.

Another problem is the existence of "ultramicrobacteria". Li and Dickie[12] reported that there were extremely small size of bacteria passing through a 0.2 μm Nucleopre filter in seawater but could be removed by filtering the samples with a cellulose membrane filter of 0.22 μm. In the present study, water samples were prefiltered through a 0.22 μm Millipore filters and again were filtered through a 0.22 μm Millipore filter. In this system, lake water samples passed through the 0.22 μm filter twice. These filtered lake water media without bacterial inoculum were incubated simultaneously and checked the growth of ultramicrobacteria. Bacterial growth was not observed in the filtered water of northern Lake Biwa, but usually in the filtered water of southern Lake Biwa which is polluted. This problem is discussed in the other paper now in preparation.

Figure 5 shows the seasonal variations of total counts of bacteria enumerated by DAPI-staining under the epifluorescence microscopy (upper), the maximum MPN count of bacteria among the 6 different kinds of MPN media (middle), and the concentration of chl. a (bottom). Although there is no relationship between total counts and chl. a concentrations, maximum MPN counts showed relatively good correlation to chl. a, that is, high bacterial counts were obtained when the concentration of chl. a increased. In addition, the maximum MPN counts sometimes showed the same level as total direct counts. These results strongly suggest that the natural community of heterotrophic bacteria (mostly oligotrophs) utilizes *in situ* dissolved organic matter more effectively than the artificial substrate (*i.e.* peptone) for their growth, and that the DOC in *in situ* lake water mainly derives from the excretion of phytoplankton.

From the results of the present study, we can conclude that MPN method using filter-sterilized *in situ* water as a medium is one of the most suitable methods to enumerate the number of "viable" heterotrophic bacteria, especially for the algal depending oligotrophs.

Acknowledgements

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