びわ湖における偏性低栄養細菌群の分布

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Distribution of Obligately Oligotrophic bacteria in Lake Biwa*1

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The distribution and seasonal changes of obligate and facultative oligotrophs in the water of Lake Biwa were investigated by the MPN-^{14}C-glutamate method with LT10^{-4} medium (ca 0.6 mg organics in one liter of aged lake water) by means of ^{14}C-uptake techinique as a criterion for bacterial growth.

In unpolluted waters the obligate oligotroph was detected as a dominant population, but not in polluted waters. The proportion of the obligate and facultative oligotrophs to total heterotrophs was very variable in epilimnion, compared with that in hypolimnion. The large population of those oligotrophs in an unpolluted water was observed in late spring to late autumn. The obligate oligotroph has planktonic property and prefers amino acids to glucose. The definition of the oligotrophs was discussed further.

In lake and sea waters generally the concentration of organic matter is extremely lower than that of conventional medium for bacteria. Most of autochthonous heterotrophic bacteria, which adapt themselves to the aquatic environments since the advent of bacterial life, may not grow on richer media. Those bacteria which grow on extremely low nutrient media probably belong to obligate oligotroph.[1] It is assumed that difference between direct microscopic counts and viable counts by conventional media might be due to the presence of obligate oligotrophs, and dormant and dead cells.

In a previous paper ISHIDA and KADOTA[1] first reported a new extinction dilution method for the detection and counting of oligotrophic bacteria in lake water, in which $^{14}CO_2$ evolution from ^{14}C-organic substances was used as a criterion for growth. It was proved that the number of heterotrophic bacteria, especially obligately oligotrophic bacteria, in aged lake water medium added with ^{14}C-glutamate were several times higher than that of heterotrophic bacteria in a conventional medium.

In the present paper the seasonal changes, and vertical and horizontal distributions in oligotrophic bacteria are investigated by the NPN-^{14}C-substrate method with extremely low nutrient medium using ^{14}C-uptake in place of $^{14}CO_2$-evolution as a criterion for growth.

Materials and Methods

Sampling Area and Method

Water samples were collected at several stations of Lake Biwa (Fig. 1) by 5 *l* Van Dorn sampler. Within 3 h after water samples were collected and maintained in sterilized 1 *l* glass bottle in ice, a part of water samples were filtered through sterilized 5 μm mesh nylon net. The whole water and the particles fraction larger than 5 μm were inoculated in the media described as follows.

Media

The LT10^{-1} medium contained 0.5 g trypticase (BBL) and 0.05 g yeast extract (Difco) in 1 *l* of aged lake water. The diluted LT10^{-4} media was prepared to contain trypticase and yeast extract in the concentration of 1/1,000 of the original (LT10^{-1} medium). The aged lake water was prepared as follows: Lake water was collected from the 40 m depth in the Northern Lake Biwa, filtered through a Whatman GF/C glass fiber filter, and kept in the dark at room temperature for more than one month. Before use, the aged lake water was passed through 0.22 μm pore size membrane filter (Millipore corp.), to avoid subsequent absorption of ^{14}C-compounds to small biotic and abiotic

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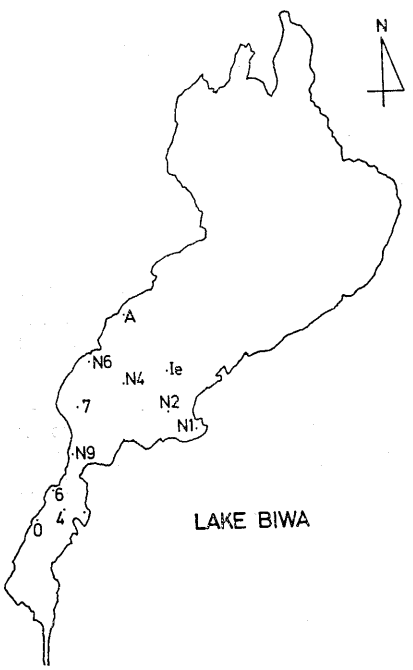


Fig. 1. Sampling area of Lake Biwa.

particles.

Procedure for Bacteria Counting

As previously described by ISHIDA and KADOTA,[1,2] bacterial growth in $LT10^{-1}$ medium was directly detected by turbidity (A). The most probable number (MPN) of bacteria was calculated by use of Hoskins' table[3] for 5 tubes. Detection of bacteria in tubes without turbid was performed by the same method (B) as described below.

Since bacterial growth in $LT10^{-4}$ medium can not be detected by turbidity, the following methods were used.

(A) Detection by turbidity in freshly prepared $LT10^{-4}$ broth: Duplicate tubes of $LT10^{-1}$ broth freshly prepared were inoculated with 0.5 m*l* aliquots from the 4 weeks old $LT10^{-1}$ MPN dilution series. After a 2 weeks incubation (20°C) the MPN value was calculated from the number of turbid tubes.

(B) Detection by uptake of ^{14}C-labelled organic compounds: Sterilized tubes* containing 1 m*l* of $LT10^{-4}$ medium and 0.1 m*l* of 200 mμCi/m*l* of L-[U-^{14}C] glutamic acid (specific activity, 275 mCi/m mol), [U-^{14}C] glucose (335 mCi/m mol) or L-[U-^{14}C] protein hydrolysate (57 mCi/atom) were inoculated with 1 m*l* of the water samples. The tubes were incubated at 20°C. After 2 weeks the ^{14}C-labelled cells in the culture tubes were retained on a 0.22 μm Millipore filter, and the filter was rinsed and dried, and was placed in a vial counting toluene fluor. MPN value was calculated from the number of the positive tubes obtained by ^{14}C-uptake. In the previous paper[1] $^{14}CO_2$ evolution-technique had been used. As the MPN count determined by ^{14}C-uptake was several times higher than that determined by $^{14}CO_2$ evolution when amino acids as ^{14}C-substrates were used, the ^{14}C-uptake technique was employed in this paper. A tube which gave radioactivity of at

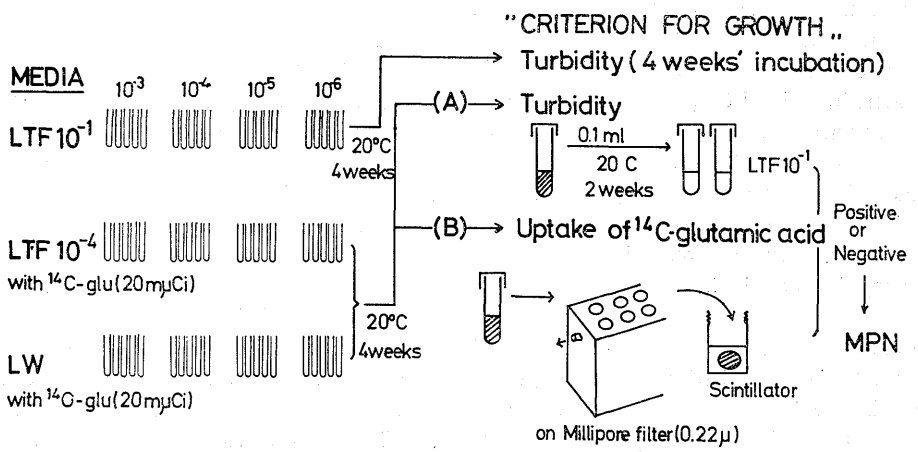


Fig. 2. Schema simply modified for enumeration and detection of oligotrophic bacteria.

* Since 1979, those procedures have been improved as follows: the lake water samples with successive 10-fold dilution were directely inoculated to the tubes containing $LT10^{-4}$ medium and 20 mμCi of L-[U-^{14}C] glutamic acid and incubated at 20°C for 4 weeks (Fig. 2).

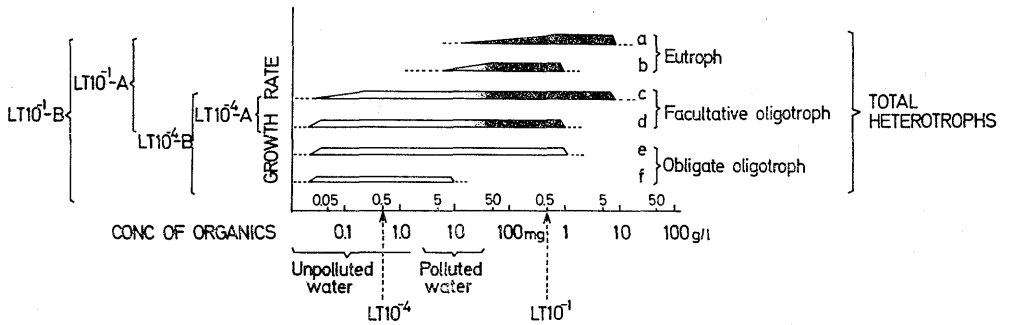


Fig. 3. Principal categories of heterotrophs in terms of the relationship between growth and nutrient concentration.

■ detection by turbidity or colony formation, □ no detection by turbidity or colony formation. LT10^{-4} (B)−LT10^{-4} (A)=obligate oligotroph (e, f), LT10^{-4} (B)=total oligotroph (c, d, e, f), LT10^{-1}+obligate oligotroph=total heterotroph (a, b, c, d, e, f).

least 3 times higher than that of the blank (less than 250 counts per 5 min) of milipore filter passing through only ^{14}C-glutamate-LT10^{-4} medium was regarded as positive.

A part of the lake water which was used for enumeration of heterotrophic bacteria was filtered through 0.22 μm Millipore filter within 3 h after sampling on each date, dispensed into test tubes with 20 mμCi of L-[U-^{14}C] glutamate and autoclaved. This medium was named the lake water medium (LW).

Plate counts were conducted by the spread plate technique using LT10^{-1} medium with 15 g of Bacto-agar per liter. The plates in duplicate were incubated at 20°C for two weeks.

It was premised in this study that the MPN-turbidity count using LT10^{-1} medium (A) would correspond to the number of both eutroph (a and b in Fig. 3) and facultative oligotroph (c and d in Fig. 3), and the MPN-^{14}C-substrate count using LT10^{-4} medium (B) would correspond to the number of both facultative oligotroph (c and d in Fig. 3) and obligate oligotroph (e and f in Fig. 3), which is obtained by subtracting (A) from (B) in LT10^{-4} medium, as illustrated in Fig. 3.

Procedure for Direct Counts

The scanning electron microscope was used for direct counts of the water samples. The water samples were fixed with gluteraldehyde (final conc. 2%) for one hour and passed through a nuclepore filter (12 mm diameter, 0.2 μm pore size) which was washed with a surfactant before use. After rinsing well with 0.2 μm filtered distilled water, the preparation was rinsed with increasing percentages of ethanol, and then dried. The specimen were coated with platinum and examined with JSM-P15 scanning electron microscope.

The data for concentration of dissolved organic substances as dissolved organic carbon (DOC) were kindly provided by Dr. O. MITAMURA.

Results and Discussions

Horizontal Distribution of Obligate Oligotroph and Total Heterotroph

The numbers of the obligate oligotroph (e and f in Fig. 3) and total heterotroph (a, b, c, d, e, f in Fig. 3) at eight different stations of unpolluted and polluted areas in Lake Biwa are shown in Table 1. The ratio of the obligate oligotroph to total heterotroph was about 50% at stations 7, Ie, N4 and N6 in unpolluted areas in the Northern Lake, and was about 15% at stations N1 and N2 in the east side of the Northern Lake, moderately polluted, while the obligate oligotroph was rarely or not detected at station N9 between Northern and Southern Lake and at station 0 in polluted area of the Southern Lake, in which there were large population of total heterotrophs. The data suggest that the obligate oligotroph has an advantage in unpolluted waters.

The results shown in Table 1 were obtained by using ^{14}C-glutamate as a tracer level substrate. The proportion of total oligotrophs (LT10^{-4}-[B]) which could use ^{14}C-protein hydrolysate and ^{14}C-glucose as compared to ^{14}C-glutamate were 40–70% (mean 86%) and 8–100% (mean 51%), respectively, as shown in Table 2. The data indicate that most of oligotrophs in Lake Biwa would prefer amino acids, especially glutamate to glucose. The data also suggest that glutamate and protein hydrolysate as a tracer level substrate are a more

Table 1. The number of heterotrophs counted by the MPN method with $LT10^{-1}$ and $LT10^{-4}$ media

Station (Sampling depth, m)		N9 (0.5)	O (0.5)	N1 (2)	N2 (0.5)	7 (5)	N4 (5)	N6 (5)	Ie (5)	N4 (30)
Date of sampling		5.18,'78	9.16,'77	5.4,'78	10.26,'78	11.24,'77	6.1,'78	10.26,'78	10.22,'77	5.11,'78
$LT10^{-1}$ MPN (ml^{-1})	A*1	1.6×10^6	5.4×10^5	1.7×10^5	1.3×10^5	5.4×10^4	4.9×10^4	1.7×10^4	1.3×10^4	1.7×10^3
	B*1	1.6×10^6	5.4×10^5	2.2×10^5	*3	9.2×10^4	1.1×10^5	*3	*3	4.9×10^3
$LT10^{-4}$ MPN (ml^{-1})	A*1	1.6×10^6	4.6×10^5	7.9×10^4	2.3×10^4	1.7×10^4	2.3×10^4	1.3×10^4	1.7×10^3	2.3×10^3
	B*1	3.5×10^5	5.4×10^5	1.3×10^5	4.9×10^4	9.2×10^4	4.9×10^4	4.9×10^4	2.4×10^4	4.9×10^3
Obligate oligotroph*2			8.0×10^4	5.0×10^4	2.6×10^4	7.5×10^4	2.6×10^4	3.2×10^4	2.2×10^4	2.6×10^3
% of total oligotroph		0	1	15	16	58	35	65	59	60
Total heterotroph*2		1.6×10^6	5.4×10^5	3.3×10^5	1.6×10^5	1.3×10^5	7.5×10^4	4.9×10^4	3.7×10^4	4.3×10^3
% of $LT10^{-1}$-B*1		100	100	150		141	68			88

*1 Growth in tubes was detected by (A) turbidity and by (B) ^{14}C-glutamate uptake.
*2 The number of the obligate oligotroph was calculated by subtracting (A) from (B) in $LT10^{-4}$ medium. The number of total heterotroph was calculated by adding the obligate oligotroph to (A) in $LT10^{-1}$ medium.
*3 No examination.

Table 2. Ratio of the MPN for oligotrophs by ^{14}C-glucose or ^{14}C-protein hydrolysate to the MPN by ^{14}C-glutamate

^{14}C-substrate	n	% to glutamate	Mean %
Protein hydrolysate	14	40–170	86
Glucose	14	8–100	51

suitable substrate than glucose for the MPN count of oligotrophs.

The MPN-^{14}C-substrate with $LT10^{-1}$ medium (B) was higher than the MPN-turbidity with $LT10^{-1}$ medium (A) in unpolluted area, and approximately coincided with the number of total heterotrophs. The fact strongly suggests a possibility that the MPN-^{14}C-glutamate with $LT10^{-1}$ medium (B) enumerates the bacterial populations of a, b, c, d, and e as shown in Fig. 3.

Vertical Distribution of Oligotrophs

At station N4, unpolluted area in the Northern Lake, vertical distribution of oligotrophic bacteria in water sample was observed in comparison with total hetertrophs number and direct microscopic counts, as shown in Fig. 4. Direct microcopic count ranged from 2.4×10^5 ml^{-1} to 3.9×10^5 ml^{-1} in the water column, and the ratio in number of total hetertrophs to direct microscopic count was 33%, 0.8%. 21%, 1.4% and 0.9% at the depth of 5 m, 10 m, 18 m (thermocline), 30 m and 40 m, respectively. At the depth of 10 m, in which the large amounts of *Pediastrum* sp., *Staurastrum dorsidentiferum* and some blue green algae were observed by scanning electron microscopy, the obligate oligotroph was not detected. It is assumed that those phytoplanktons gave some inhibitory effect on the obligate oligotroph population. At the thermocline the ratio of total oligotrophs to total heterotrophs was lowest, and eutrophs were predominant. The proportion of the obligate and facultative oligotrophs to total heterotophs was very variable in the epilimnion, as compared with that in the hypolimnion. The fact suggests that the oligotrophs are sensitive to chemical and biological changes in water, and then must be active in unpolluted water.

Seasonal Changes of Planktonic and Periphytic Oligotrophs

The populations of planktonic and periphytic oligotrophs were determined at station A from April 12 to December 25 in 1979, as shown in Fig. 5. From April 19 to May 23 the large blooms of *Uroglena americana* occurred three times. During

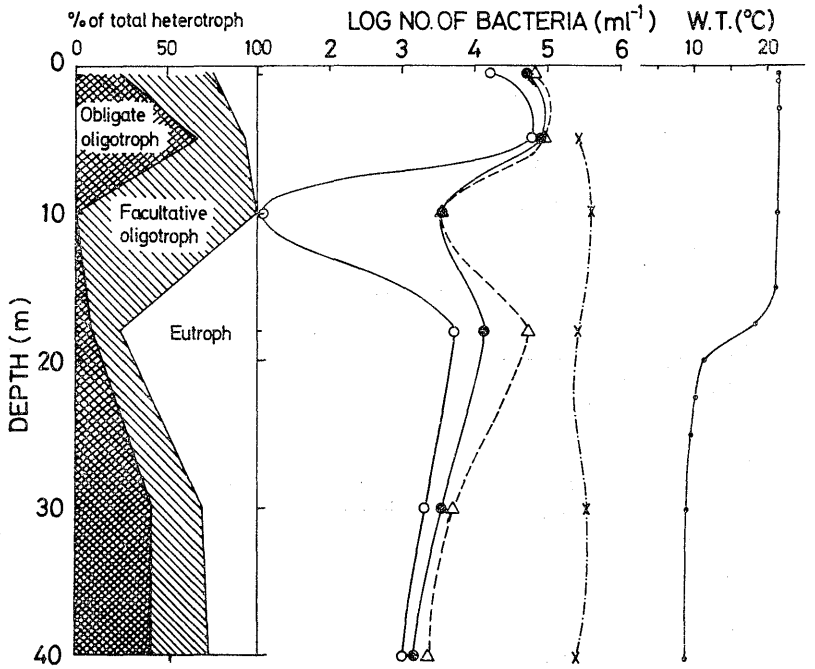


Fig. 4. Vertical distribution of obligate and facultative oligotrophs, total heterotrophs and total bacteria (direct counts with scanning electron microscopy) at station N4. (October 12, 1978).
○—○, obligate oligotroph; ●—●, facultative oligotroph; △---△, total heterotrophs; ×-·-×, total bacteria; ·—·, water temperature.

that period there were good correlations between *U. americana* blooms and DOC, and between DOC and periphytic oligotrophs, which were almost composed of the facultative oligotroph. In the particle fraction of water samples during the bloom any obligate oligotroph was not detected. From the result it is assumed that the facultative oligotroph may be closely participated to *U. americana* growth. This assumption is supported by the fact that we detected only the facultative oligotroph in *U. americana* colony which was washed enough with sterilized lake water (unpublished data). The data suggest that the obligate oligotroph has planktonic property and the facultative oligotroph tends to attach on particles in water.

By using LW medium the large number of the oligotrophs was obtained in filtrate and particle fractions of the water samples during a year, as well as by using $LT10^{-4}$ medium (Fig. 6). It is assumed from this data that the oligotrophs live in active state in Lake Biwa.

Comparison between MPN Count and Plate Count in $LT10^{-1}$ Media

Seasonal changes in the number of heterotrophic bacteria population by MPN and plate count methods at station A (3 m depth) in the Northern Lake are given in Fig. 7. In both filtrate and particle fractions of water samples the MPN count gave one to two order higher values than the plate count during a year. The data indicate that in enumeration of heterotrophs in the water samples of Lake Biwa the MPN method using $LT10^{-1}$ medium is far better than the plate count method. Furthermore the number of bacteria by the MPN method with $LT10^{-1}$ medium was compared with that by direct microscopic count by acridine orange epifluorescence microscopy[4] in the water samples collected on January 31 and February 21 in 1980. Direct count for the MPN count were 2.4×10^5 ml^{-1} for 2.4×10^4 ml^{-1} on January 31, and 2.1×10^5 ml^{-1} for 5.4×10^4 ml^{-1} on February 21. The results indicate that the MPN count with $LT10^{-1}$ medium, incubated at 20°C for 4 weeks, would be very useful for the enumeration of living heterotrophic bacteria in water of Lake Biwa, considering that the proportion of living heterotrophs was several tens % of direct microscopic counts in water samples taken from fresh water lakes and sea.[4-6]

In this study the number of obligate oligotroph

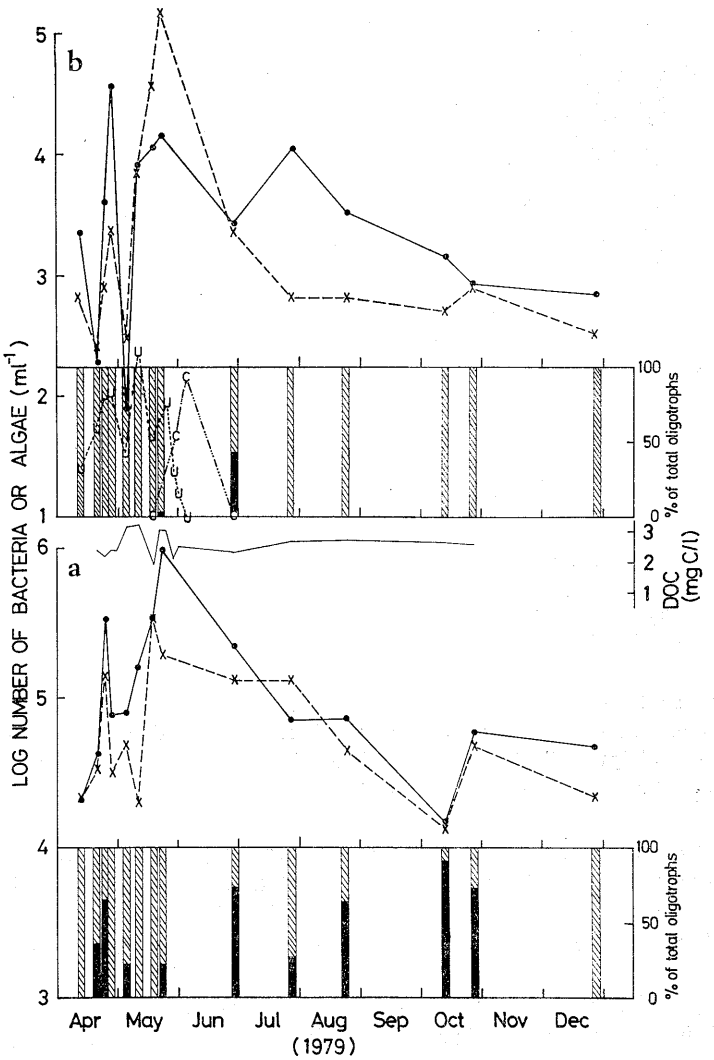
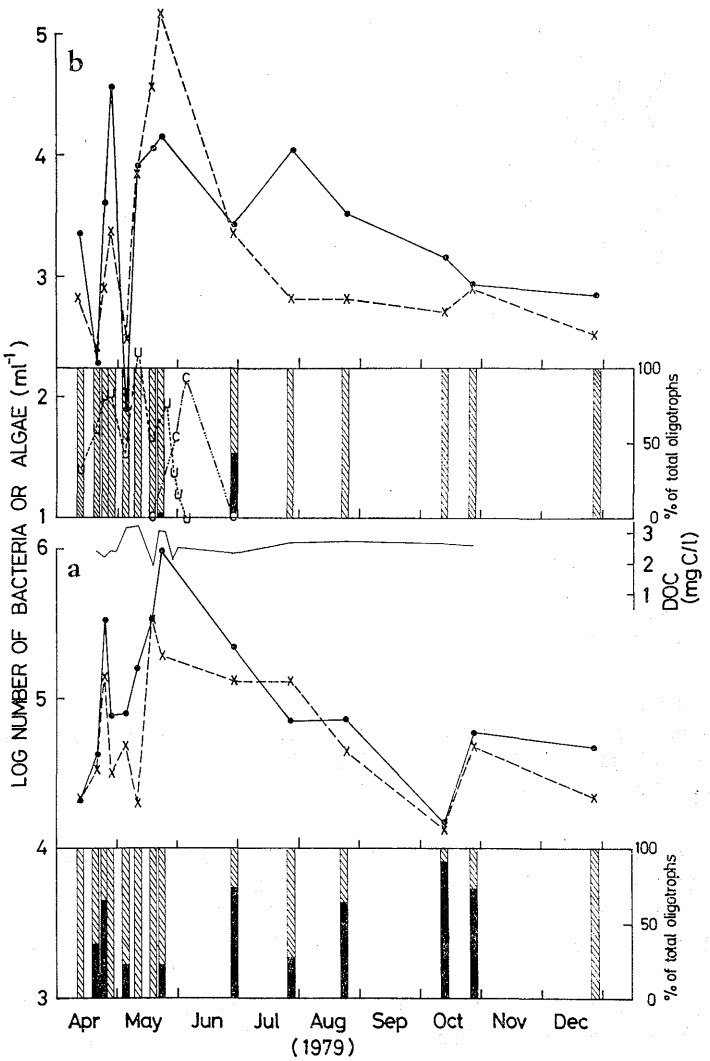


Fig. 5. Seasonal changes in number of obligate and facultative oligotrophs, total heterotrophs, two unicellular algae and DOC in filtrate (a) and particle (b) fractions of the water at station A. ●—●, total heterotrophs; ×---×, total oligotrophs; ▨, facultative oligotroph; ■, obligate oligotroph; U---U, *Uroglena americana*; C·····C, *Closterium aciculare*; ——, DOC.

(e, f) was obtained by subtraction of the number by LT10^{-4}-(A) from that by LT10^{-4}-(B), the number of total oligotrophs (c, d, e, f) was obtained as the number by LT10^{-4}-(B), and total heterotrophs (a, b, c, d, e, f) was obtained by adding the number by LT10^{-1}-(A) to obligate oligotroph number. Therefore, the oligotrophic bacteria* were defined as organisms that grow in liquid LT10^{-4} medium prepared with lake water of oligotrophic area or pelagic water of ocean, and the obligate oligotroph among the oligotrophs cannot grow in conventional rich media (ca 5 g/*l* of organic nutrients). KUZNETSOV[7] assumed that many of bacteria which fail to grow in the laboratory belong to oligocarbophilic species and require only trace amounts of organic substrates. Probably the oligocarbophiles which he assumed to be present in lake water correspond to the obligate oligotroph which was de-

* In the subcommitee meeting of the Japanese Society of Microbial Ecology, 1980, we defined oligotrophic microorganisms as organisms that grow in medium containing organic matter of 1 mg of C per liter. In this time the meeting dared not subdivide oligotrophs into obligate and facultative ones, because more kinds of oligotrophs will be isolated from waters and soils in near future.



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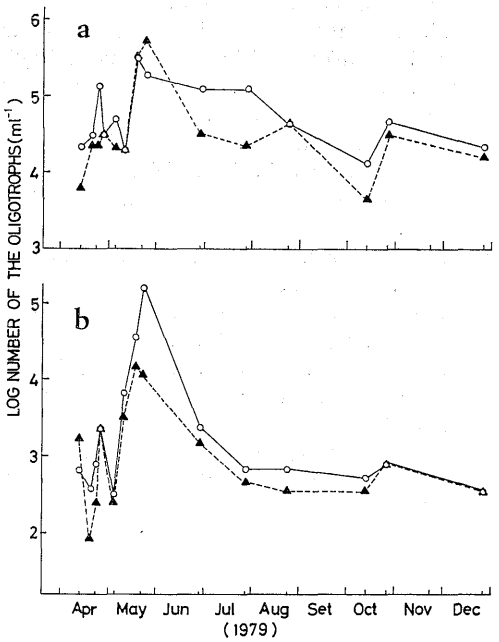

Fig. 6. Seasonal changes of the numbers of the oligotrophs counted by $LT10^{-4}$ medium and LW medium in filtrate (a) and particle (b) fractions of the water at station A.
○—○ by $LT10^{-4}$ medium; ▲---▲ by LW medium.

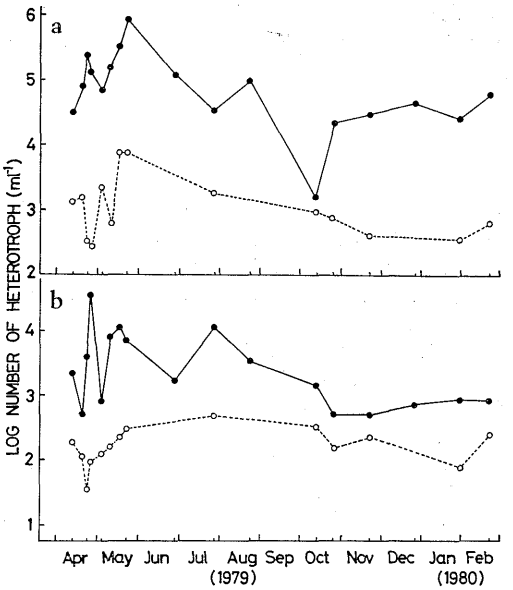

Fig. 7. Comparison between the MPN count and plate count in $LT10^{-1}$ media in filtrate (a) and particle (b) fractions of the water at station A.
●—● MPN count in $LT10^{-1}$ medium; ○—○ plate count in $LT10^{-1}$ medium with agar.

tected in this study by the MPN-^{14}C-glutamate with $LT10^{-4}$ medium. HIRSCH,[8] defined oligocarbophiles as organisms which are able to grow on media containing only minerals and to meet their carbon and energy requirement from hydrocarbons and other volatile substances in the air, and MOALEDJ[9,10] isolated *Vibrio, Coryneform, Hyphomicrobium, Caulobacter, Microcylus, Actinomycetes, Mycobacteria, Nocardia* etc. from Plußsee as oligocarbophiles defined by HIRSCH.[8] Those slow-growing bacteria were also isolated from Chesapeake Bay by MALLORY *et al.*[11] by use of plain agar medium, and were called oligotrophs. In order to count marine oligotrophic bacteria AKAGI *et al.*[12] proposed a useful membrane filter method. A glass-fiber filter was used as a substitute for agar and immersed in 17 mg C/*l* organic substrates. They defined oligotrophic bacteria as heterotrophs which formed microcolonies on the medium. According to YANAGITA *et al.*,[13] microorganisms that are able to grow in the presence of extremely low level of nutrilites and those requiring relatively high level of them were named tentatively oligotrophic bacteria and eutropic ones, respectively. In *sesu stricto* they defined the term oligotrophs as organisms that can grow only in the presence of a minor amount of nutrilites but not in the presence of a large amount, and facultative oligotrophs as organisms that can grow in a wide concentration range of nutrilites. Recently again KUZNETSOV[14] tentatively classifies as oligotrophic aquatic bactera those that develop at the first cultivation on media with the minimal content of organic matter of about 1–15 mg of C/*l* and that grow on such media at subsequent recultivations though they can grow on richer media. Diverse definitions are now used by aquatic microbiologists. The obligate oligotroph and the facultative oligotroph designated and detected by us may correspond to the definition by YANAGITA *et al.*,[13] but are different from the bacteria by MOALEDJ[9,10] and MALLORY *et al.*[11] Probably the oligotrophs are inherent to oligotrophic waters.

Several isolates of the obligate oligotrophs, which were predominant in the Northern Lake Biwa, show a relatively high growth rate (ca 0.09–0.15 h^{-1}), low maximum cell yield (ca $5-15\times10^5$ ml^{-1}) in $LT10^{-4}$ medium, and cannot grow in richer medium containing 5 g/*l* of organic nutrients. The more interesting characteristics of these obligate oligotrophs will be published in near future.

In any study concerning microbial ecology of

unpolluted lake (oligotrophic lake) the presence of the obligate oligotroph as dominant species should not be overlooked.

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