水圏における窒素固定に関する微生物学的研究I.

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Microbiological Studies on Nitrogen Fixation in Aquatic Environments—I. On the Distribution of Nitrogen Fixing Bacteria in Fresh Water Regions*

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In aquatic environments, nitrogen fixation is one of the most important processes in nitrogen cycle, on the account that the process is responsible for the recovery of nitrogen loss caused by denitrification and that the productivity of the water region is sometimes restricted by the nitrogen budget of the region.

In the first step of the study, the authors have intended to obtain the information about the distribution of nitrogen fixing bacteria in various water regions, M. P. N. method with the liquid media containing sucrose and non-nitrogenous compound being used for the enumeration of the bacteria.

This paper is concerned primarily with the occurrence and abundance of nitrogen fixing bacteria in some fresh water regions. In Lake Biwa and Lake Yunoko, the number of the bacteria was somewhat small in the water i.e. 10^{-1}–10^{2} cells/ml, while, 10^{2}–10^{4} cells per 1 g of the bacteria were detected in the bottom sediments of the lakes.

Nitrogen, one of the essential components of livings, is quite small in its content in aquatic environments. Besides in the form of dissolved gas, nitrogen in hydrosphere is known to be present in the forms of dissolved and particulated organic matter and also of inorganic compounds such as ammonia, nitrite and nitrate.

Fig. 1 shows the outline of the transformation processes of nitrogenous compounds in aquatic environments. In the first step of the mineralization process, organic compounds liberate ammonia which may be oxidized by some groups of chemoautotrophic bacteria to form nitrate via nitrite. Nitrate is also reduced by the action of bacteria to nitrite, further to molecular nitrogen. The denitrification process is considered to be responsible for the remarkable loss of nitrogen from hydrosphere.

Therefore, the process of nitrogen fixation, recovery process of lost nitrogen pool in aquatic environments, is one of the most important process in nitrogen bugedt.

Some of aquatic microorganisms including bacteria have been known to have the

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ability of fixing dissolved nitrogen gas in water, however, little information is available about the bacterial nitrogen fixation in hydrosphere.

The present paper is attempted to reveal the distribution of nitrogen fixing bacteria in fresh water environments.

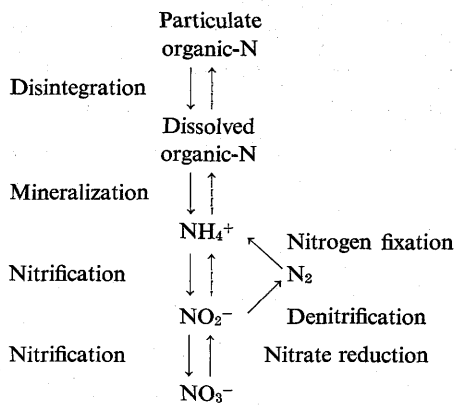

Fig. 1. Nitrogen cycle in aquatic environments

Methods

Enumeration of bacterial number. The M.P.N. method was used for the enumeration of nitrogen fixing bacteria and total heterotrophic bacteria. The medium for counting the number of nitrogen fixing bacteria was as follows: Sucrose 20.0 g, KH_2PO_4 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, NaCl 0.01 g, $FeSO_4$ 0.001 g, $MnSO_4$ 0.001 g, $Na_2MoO_4 \cdot 2H_2O$ 0.01 g, Distilled water 1,000 mL, pH 7.0–7.5. The following medium was used for counting the number of total heterotrophic bacteria: Bacto-casitone 5.0 g, Beef extract 3.0 g, KH_2PO_4 0.1 g, Fe–EDTA 6 mg, KNO_3 0.5 g, $NaHCO_3$ 0.2 g, $CaCl_2$ 0.02 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, Distilled water 1,000 mL, pH 7.0–7.5. KH_2PO_4 was added into the medium aseptically after separately autoclaved. These media were autoclaved at 15 pounds/inch^2 for 15 minutes. The inoculation was carried out after decimal dilutions in five tubes per each dilution. Cultivation was done at 20°C for 2–4 weeks aerobically or anaerobically. In the anaerobic incubation, inoculated media were put into the anaerobic jar and air in it was replaced with nitrogen gas. After the incubation period, the presence or absence of the bacteria in each tube was estimated from the turbidity of the culture media, and the M.P.N. (Most Probable Number) per 1 mL of the water or 1 g of the mud was calculated from the number of positive tubes in each dilution.

Analysis. pH value of the samples was measured by the use of pH meter with glass electrode (Toa Electronics Ltd., Model HM-5A). Dissolved oxygen in water samples was analyzed by using Winkler's method and C.O.D. value was estimated from the amount of $KMnO_4$ consumed under alkaline reaction. Ammonia was determined by the method of RICHARDS and KLETSCH[1]. Nitrite was determined by the use of Griess-Romjn reagent. Nitrate was determined by the method of STRICKLAND and PARSONS[2]. Inorganic phosphate was determined by the method of MURPHY and RILEY[3]. The content of sugars was determined by the anthrone method[4] and represented as the value of sucrose equivalent.

Water regions studied. *Lake Biwa*, the largest lake in Japan, belongs to a typical

oligotrophic lake with the maximum depth of about 104 m and the surface area of 674.4 km^2. Shiozu Bay is located in the north part of Lake Biwa. It is a small bay with the maximum depth of 60 m, the length of 8.5 km and the width of 4.0 km. Four stations were set up in Lake Biwa as shown in Fig. 2.

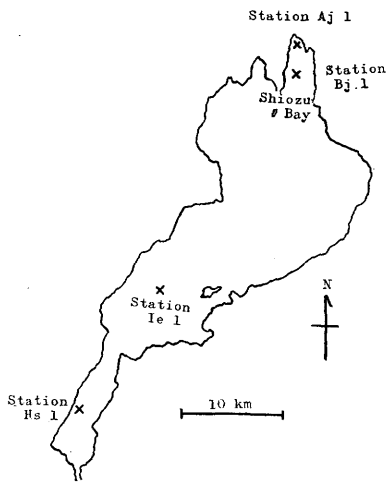

Fig. 2. The stations set up in Lake Biwa.

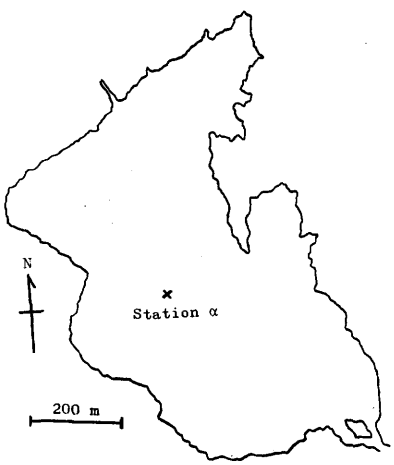

Fig. 3. The station set up in Lake Yunoko.

Lake Yunoko is a small lake locating to the north part of Tochigi Prefecture. It is a eutrophic lake with the surface area of 0.33 km^2, the shore line of 2.8 km and the maximum depth of about 12.5 m. Station α (depth 12.0 m) was set up in the center of the lake as shown in Fig. 3.

Collection of water and mud samples. The water samples were collected by the use of the J–Z sampler[5] on November 7, 1967 from Stations Aj 1 and Bj 1, on May 8, November 16, 1968 and January 27, 1969 from Station Hs 1 and on July 22, 1969 from Station Ie 1 in Lake Biwa and on August 26, 1967 from Station α in Lake Yunoko. The bottom mud was collected by the use of Eckman Birge sampler at each station of Shiozu Bay also by the K–K core sampler[6] at the other stations of Lake Biwa and Lake Yunoko. Immediately after the collection, the water and mud samples were submitted to the analysis.

Results and Discussion

Lake Biwa. The station Hs 1 is in the offing of Otsu Hydrobiological Station, Kyoto University. The depth at Station Hs 1 ranges from 3 m to 4 m. The transparency at the station usually falls in 1–2 m. The water is well-oxygenated, occasionally supersaturated. There is no vertical change in water temperature at the shallow station, Station Hs 1. The water of 2 m layer on May 8, 1968 contained abundant suspended matter, which amounted to 4.12 mg dry weight per 1 liter of the water and about 36.4 % of it was

lost on ignition. It indicated that the biomass in the water is abundant at that time. The Station Ie 1 was set up near the center of the lake. The quality of the water was shown in Table 1. The station depth at Station Ie 1 is 70–80 m and the transparency is usually around 6.5 m. The stable thermal stratification with a clinolimnion at the layer of 30–50 m was observed during summer. During winter, the mixing of lake water from the surface to the bottom was observed, and the water temperature was usually constant vertically. The contents of ammonia and nitrite are as small as about 1 μg atom/L, while the nitrate content gradually increased with the water depth from 1 μg atom/L in the surface water to 10 μg atoms/L in the water of 70 m layer during summer. Station Aj 1 was set up in the center of the shallow inlet at the north part of Shiozu Bay. The depth at the station is about 2.0 m, while the transparency is about 1.1 m. The pH, water temperature and dissolved oxygen of the surface water were 7.0, 15.2°C and 6.54 mL/L (92% of saturation), respectively on November 7, 1967. Station Bj 1 was set up in the center of Shiozu Bay. The depth at the station was about 40 m and the transparency was about 5.2 m which is similar to the values measured at the other stations in Lake Biwa. The pH, water temperature and dissolved oxygen of the surface water were 7.5, 15.7°C and 7.28 mL/L (104% of saturation), respectively on November 7, 1967. A stable thermal stratification was also observed at Station Bj 1 during summer.

The results in Table 2 showed the counts of nitrogen fixing bacteria and total heterotrophic bacteria at different stations in the lake. The number of total heterotrophic bacteria was usually about 10^3–10^5 cells/mL, while nitrogen fixing bacteria were very few, ranging from 10^{-1} to 10^2 cells/mL, or sometimes lower than the value. The number of nitrogen fixing bacteria in the water at Station Ie 1 was very few, i.e. about 10^{-1} cells/mL or less at each depth of the water column. The number of nitrogen fixing bacteria grown anaerobically was almost as same as that of aerobic ones. On the contrary, nitrogen fixing bacteria were abundant in the water of Shiozu Bay. Nitrogen fixing bacteria in the water at Station Hs 1 were usually in the range of 10^0–10^1 cells/mL.

Table 1. Quality of the water in Lake Biwa.

	Depth of water (m)				
	0	10	30	50	70
Water temperature (°C)	27.1	23.1	23.0	8.8	8.0
NH_4–N (μg atoms/L)	0.34	0.42	0.17	1.82	0.34
NO_2–N (μg atoms/L)	0.12	0.43	0.09	0.03	0.59
NO_3–N (μg atoms/L)	1.02	1.74	4.86	8.10	10.6
Sugars (μg/L)	0	0.37	0.94	0.75	5.84
PO_4–P (μg atoms/L)	0	0	2.37	0	3.95

Station Ie 1 (Depth 80 m), July 22, 1969.
Transparency 6.5 m, Atomsphere temperature 30.0°C.

Table 2. Distribution of nitrogen fixing bacteria in the water of Lake Biwa.

Date	Station (Depth)	Transparency (m)	Depth of water (m)	Water temp. (°C)	Total heterotrophs (cells/mL)	Nitrogen fixing bacteria (cells/mL)
11/7, 1967	Bj 1 (40 m)	5.2	0	15.7	4.9×10^4	4.0×10^2
	Aj 1 (2 m)	1.1	0	15.2	1.7×10^5	3.3×10^2
7/22, 1969	Ie 1 (80 m)	6.5	0	27.1	4.0×10^4	0.2
			10	23.1	4.5×10^4	<0.2
			30	23.0	$<2.0\times10^4$	<0.2
			50	8.8	2.0×10^4	<0.2
			70	8.0	2.0×10^4	<0.2
5/8, 1968	Hs 1 (4 m)	1.3	2	17.2	$*7.8\times10^3$	$*1.7\times10$
11/16, 1968		1.5	2	13.4	5.4×10^4	7.9
					$*3.3\times10^3$	*1.7
1/27, 1969		2.2	2	5.4	4.9×10^3	4.0×10
					$*1.1\times10^4$	$*2.7\times10$

* cultivated under anaerobic condition.

Table 3. Distribution of nitrogen fixing bacteria in the bottom mud of Lake Biwa.

Date	Station (Depth)	Depth of mud (cm)	Total heterotrophs (cells/g mud)	Nitrogen fixing bacteria (cells/g mud)
11/7, 1967	Bj 1 (40 m)	0–2	5.4×10^5	2.1×10^3
	Aj 1 (2 m)		———	2.1×10^4
5/8, 1968	Hs 1 (4 m)	0–4	4.6×10^6	6.4×10^2
			$*3.5\times10^6$	$*4.6\times10^3$
		21–24	$*7.8\times10^4$	$*7.9\times10^2$
11/16, 1968		0–4	1.1×10^6	4.9×10^2
			$*9.5\times10^5$	$*9.2\times10^2$
		21–25	$*1.3\times10^4$	$*7.0\times10^2$
1/27, 1969		0–4	4.9×10^5	4.6×10^3
			$*2.2\times10^6$	$*3.3\times10^3$
		21–25	7.8×10^3	2.0×10^2
			$*1.4\times10^4$	$*7.9\times10^2$

* cultivated under anaerobic condition.

The quality of the bottom mud in the surface layer at Station Hs 1 on May 8, 1968 was as follows: mud temperature 15.0°C, pH 6.81, dry matter 26% of wet mud, C/N ratio 12.9, loss on ignition 11.9% of dry matter. The quality of the 22–25 cm layer of the bottom mud was as follows: pH 7.11, dry matter 40.7% of wet mud, C/N ratio 15.4.

The values of C/N ratio indicated that the degree of the decomposition of organic matter proceeds gradually with the increase in the depth of mud. As to the seston (suspended matter) collected in the water of 2 m layer at the station, C/N ratio and the loss on ignition of suspended matter were 6.5 and 36.4% of dry matter, respectively. The content of organic matter was quite abundant in the suspended matter but small in the sedimented matter as indicated in the value of the loss on ignition. It seemed likely that the decomposition which may be closely related with the number of total heterotrophic bacteria as well as nitrogen fixing bacteria is carried out mainly on the surface mud.

The results in Table 3 indicated the occurrence of nitrogen fixing bacteria in the bottom mud in Lake Biwa. It can be observed that total heterotrophic bacteria in the surface layer of the bottom mud are usually abundant, i.e. 10^5–10^6 cells/g and nitrogen fixing bacteria are at the level of 10^2–10^4 cells/g. The count of nitrogen fixing bacteria decreased slightly with the depth of mud. The number of nitrogen fixing bacteria grown anaerobically was almost similar to that of aerobically grown ones.

Lake Yunoko. The qualities of the water and the bottom mud were shown in Table 4. Nitrite and inorganic phosphate are slightly low at each layer of water. The contents of ammonia and nitrate in the water were fairly abundant. The large amounts of sulfur compounds are detected in the water and the bottom mud in Lake Yunoko. The contents of sulfide and ammonia gradually increase with the depth in summer. The bottom water and the bottom mud are found to have reductive properties.

Table 4. Quality of the water and the bottom mud in Lake Yunoko.

	Depth of water (m)				Depth of mud (cm)	
	1	5	8	11	0–2	10–12
Temperature (°C)	17.8	13.6	12.6	11.9	12.0	——
pH	7.5	6.8	6.6	6.7	6.8	6.4
Moisture (%)	——	——	——	——	66.4	65.0
NH_4–N (μg atoms/L or g)	2.37	1.75	3.73	10.6	——	——
NO_2–N (μg atoms/L or g)	0	0	0	0.06	0	0
NO_3–N (μg atoms/L or g)	0.06	5.50	4.88	1.29	——	——
PO_4–P (μg atoms/L or g)	0.30	0.44	0.07	0	0.04	0.85
C.O.D. (O_2 mg/L or g)	2.16	0.64	8.08	1.60	20.8	13.5

Station α (Depth 12 m), August 26, 1967.

The results in Table 5 indicated that nitrogen fixing bacteria are present as many as about 10^1 cells/mL at the each depth of water. Nitrogen fixing bacteria in the mud were also abundant, i.e. about 10^3 cells/g in the surface layer and about 10^2 cells/g in the 10–12 cm layer of the mud.

The correlation between the number of nitrogen fixing bacteria and the C.O.D. value was observed in Lake Yunoko. However, clear relationship was not found between

Table 5. Distribution of nitrogen fixing bacteria in the water and the bottom mud of Lake Yunoko.

Sample	Depth of water or mud	Total heterotrophs (cells/mL or g)	Nitrogen fixing bacteria (cells/mL or g)
Water	1 m	6.4×10^{3}	8.1×10
	5	7.0×10^{3}	2.0×10
	8	1.7×10^{3}	6.1×10
	11	2.2×10^{4}	2.0×10
Mud	0–2 cm	5.4×10^{6}	3.8×10^{3}
	10–12	1.6×10^{6}	4.7×10^{2}

Station α (Depth 12 m), August 26, 1967.

the number of nitrogen fixing bacteria and the other environmental factors in Lake Biwa and Lake Yunoko.

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