Microbiological Studies on Nitrogen Fixation in Aquatic Environments—VII. Some Ecological Aspects of Nitrogen Fixing Bacteria*

Akira KAWAI** and Isao SUGAHARA***

(Received October 23, 1971)

In connection with nitrogen fixation in aquatic environments, some ecological aspects of nitrogen fixing bacteria have been studied.

In various water regions, a close relationship between the number of anaerobic nitrogen fixing bacteria and that of nitrate reducing bacteria or denitrifying bacteria was observed. Considerable numbers of nitrogen fixing bacteria occurred not in free form but attached on to or in suspended particles in the water. Besides nitrogen fixing bacteria, the groups of the bacteria in the nitrogen cycle form a peculiar pattern of the microflora, which was illustrated as a diagram. The diagram obtained for the bottom sediments of open seas and the bays in which offshore waters flow was clearly different from that of eutrophized fresh water regions.

The life of organisms in aquatic environments is affected significantly by the various environmental factors, while it has direct effects on the circumstances in which the organisms live. Similar relationship may also be observed in the microorganisms which are responsible for each process involved in 'nitrogen cycle', which has an important role on the productivity of the aquatic environment. Then, the population size as well as the activity of nitrogen fixing bacteria in the water might be affected by biotic and abiotic environmental factors, and consequently, the occurrence and the abundance of the bacteria responsible for nitrogen metabolism may form a peculiar pattern for each water region although the standing crop of nitrogen fixing bacteria is different with the region. The present paper deals with some ecological aspects of occurrence of nitrogen fixing bacteria in various water environments.

Methods

Besides nitrogen fixing bacteria and total heterotrophs, the number of each group of the bacteria which have the ability of performing the following processes in the nitrogen cycle was counted in the water and bottom sediments of various water regions; ammonifica-

---

* Special Project Research supported by the Ministry of Education 'Studies on the Dynamic States of Biosphere'. This study was carried out as part of JIBP Project. The outline of this article was presented at the annual meeting of the Japanese Society of Scientific Fisheries, Tokyo, Japan, April, 1969.

** The Research Institute for Food Science, Kyoto University, Kyoto, Japan. (河合 章: 京都大学食糧科学研究所)

*** Faculty of Fisheries, Prefectural University of Mie, Tsu, Mie, Japan. (菅原 庸: 三重県立大学水産学部)

tion, nitrate reduction, denitrification, nitrification (ammonia oxidation and nitrite oxidation). For the enumeration of ammonifying bacteria, nitrate reducing bacteria and denitrifying bacteria, the same culture medium that for total heterotrophs was used^{1,2)}, and the occurrence of these groups of the bacteria in the cultures was judged from the formation of ammonia, nitrite and nitrogen gas by the use of indophenol reagent, Griess-Romjñ reagent and Durham tubes, respectively. The number of each groups of the bacteria in the water and bottom sediments was represented as M.P.N. (Most Provable Number)¹⁾.

The medium for ammonia oxidizing bacteria was as follows; $(\text{NH}_4)_2\text{SO}_4$ 30 mg, KH_2PO_4 100 mg, EDTA-Fe 6 mg, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 50 mg, CaCl_2 20 mg, NaHCO_3 200 mg, Distilled water 1,000 mL, pH 7.0–7.2. The following medium was used for nitrite oxidizing bacteria; KNO_2 30 mg, KH_2PO_4 100 mg, EDTA-Fe 6 mg, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 50 mg, CaCl_2 20 mg, NaHCO_3 200 mg, Distilled water 1,000 mL, pH 7.0–7.2. A small amount of calcium carbonate was added into each culture tube, and KH_2PO_4 solution was autoclaved separately then added aseptically into the culture tubes for both of ammonia oxidizers and nitrite oxidizers. The occurrence of ammonia oxidizing bacteria was judged from the formation of nitrite or nitrate by the use of reduced strychnin reagent and that of nitrite oxidizing bacteria from the disappearance of nitrite and the formation of nitrate by the use of Griess-Romjñ reagent and reduced strychnin reagent. In the case of sea water samples, artificial sea water was substituted by distilled water for preparing the culture media.

Results and Discussion

Relationship between the number of nitrogen fixing bacteria and that of the bacteria which are responsible for nitrogen cycle in various water regions The outline of bacterial participation in the metabolic processes of nitrogenous compounds in aquatic environments was illustrated in Fig. 1. Proteolytic and ammonifying bacteria participate in the decomposition and the mineralization of organic nitrogenous compounds. The nitrification processes, ammonia oxidation and nitrite oxidation, are performed by the action of ammonia oxidizing bacteria such as *Nitrosomonas* and nitrite oxidizing bacteria such as *Nitrobacter*. On the other side, nitrate is reduced by the action of nitrate reducing bacteria to nitrite, further to molecular nitrogen by denitrifying bacteria.

The relationship between the number of nitrogen fixing bacteria which occur in the water and bottom sediments of various fresh water regions and that of nitrate reducers and denitrifiers was shown in Fig. 2. The correlation coefficient, r values between the two groups of the bacteria were also described in the figure. Close relationship was observed between the number of anaerobic nitrogen fixing bacteria and that of denitrifiers and nitrate reducers in fresh water environments. It indicates that the growth of the three

Fig. 1. Participation of various groups of bacteria in the nitrogen cycle in aquatic environments.

groups of the bacteria may be controlled by same environmental factors, presumably by the concentration of available organic matter in the water region. However, clear relationship was not observed between the number of nitrogen fixing bacteria and that of nitrifying bacteria or protein decomposing bacteria. On the other hand, the relationship above mentioned was not obtained in sea water regions, in which the amount of organic matter is considerably poor compared with in fresh water environments.

From the fact that *Azotobacter* occurs abundantly on the surface of marine algae, REINKE^{3,4)} suggested the symbiosis between *Azotobacter* and marine algae, in which the latter supply the former with carbohydrates as the source of energy and utilize the nitrogen fixed by the former. PSHENIN⁵⁾ found that there is some correlation between the number of *Azotobacter* in the water mass and that of large sized phytoplanktons, but not between the total bacterial count and the population of phytoplankton. According to his report, the main carbon source for the growth of *Azotobacter* in the sea water was apparently the moribund cells of large sized phytoplanktons and the particles of indigested food of zooplanktons.

Nitrogen fixing bacteria utilize dissolved nitrogen gas, which is derived either from dissolved air into the water or from nitrogen liberated from combined nitrogen as the result of bacterial denitrification. The solubility of molecular nitrogen in water is 29.4 mg/

Fig. 2. Relationship between the number of nitrogen fixing bacteria and that of nitrate reducing bacteria in the water and the mud of various fresh water regions.
○ in water; ● in mud.

L at 0°C , 23.1 mg/L at 10°C and 19.0 mg/L at 20°C , respectively.⁶⁾ Thus, the supply of nitrogen gas for nitrogen fixing bacteria seems to be enough in usual water regions where air is continually dissolved and penetrated, and the limiting factor which restricts the growth of nitrogen fixing bacteria might be the concentration of organic matter in the water.

In order to obtain some ecological information about the nutritional uptake of nitrogen fixing bacteria, the authors carried out an experiment as to the bacterial adhesion on the suspended matter present in water regions. The water samples collected from Pond I were treated with sonic oscillation (10 kc) for 0–120 seconds, then the bacterial counting was carried out. A portion of the sonicated water sample was filtrated through Millipore filter (pore size 0.8μ) before the bacterial counting. The results obtained were shown in Table 1. The increase in the number of nitrogen fixing bacteria as well as total hetero-

trophic bacteria suggests the destruction of the suspended particles on which the bacterial cells attached. The evidence for the liberation of the bacteria from the particles by the sonic treatment could not be obtained, because the bacterial counts after filtration were almost constant over the period of oscillation from 0 to 120 seconds although some of the bacterial cells might be killed by the sonic oscillation. From these facts, it may be likely that many nitrogen fixing bacteria occur together with the other heterotrophs on or in the suspended particles and utilize the component of the particles as the nutritional sources.

Table 1. Number of nitrogen fixing bacteria and total heterotrophs in the water after sonic oscillation.

Period of sonic oscillation (sec.)	Nitrogen fixing bacteria (cells/mL)		Total heterotrophs (cells/mL)	
	Original water	Filtered water*	Original water	Filtered water*
0	4.7	1.3	49,000	1,100
10	9.5	0.68	79,000	11,000
30	17	2.3	17,000	2,300
60	3.9	1.7	31,000	2,300
120	28	0.68	240,000	1,200

* filtered through sterilized Millipore filter (0.8 μ).

RODINA⁷⁾ reported that the particles of detritus in lakes are consisted of an organic core and overgrowing bacteria. According to his data, bacteria occur not only on the surface of detritus particles but they penetrate into the particles, and all the internal space of the particles are often filled up by bacterial cells. The total number of bacteria in detritus varies from 2.2×10^9 to 4.8×10^{10} cells per 1 g of wet weight as determined by the direct-count method. The detritus always contained ammonia producing bacteria and a few denitrifying bacteria. The majority of the detritus samples contained nitrogen fixing bacteria; *Clostridium pasteurianum* and *Azotobacter*. In some samples, a considerable number of *Azotobacter* was observed. Nitrifying bacteria were found only in few samples. Thus, it seemed that the detritus particles consist of a great variety of microbitope for bacteria.

The results obtained in this paper as to the adhesion of bacteria on the suspended particles seem to be in agreement with RODINA's observation.

Pattern of the microflora in bottom sediments in various water regions. From the authors' accumulated data, the number of the bacteria which are responsible for each of the biochemical reactions in nitrogen cycle in the water regions can be summarized as shown in Table 2. In general, a dense bacterial biomass is found in fresh water environments, and the bacterial population decreases gradually from coastal to offshore regions.

In order to demonstrate the bacterial ecosystem in which nitrogen fixing bacteria involve, the number of each biochemical groups of bacteria in the bottom sediments of several different regions with fresh water and sea water was illustrated as the diagram as

Table 2. Standing crops of the bacteria which are responsible for the processes in nitrogen cycle in various water regions.

Process in nitrogen cycle	Water (cells/mL)			Bottom sediments (cells/g)			
	Fresh water regions	Inland bays	Offshore regions	Fresh water regions	Inland bays	Offshore regions	Open seas
(Total heterotrophs)	$10^3 - 10^5$	$10^2 - 10^4$	$10^0 - 10^3$	$10^3 - 10^7$	$10^4 - 10^5$	$10^3 - 10^5$	$10^5 - 10^6$
Proteolysis	$10^1 - 10^4$	$10^1 - 10^3$	$10^{-1} - 10^2$	$10^2 - 10^6$	$10^3 - 10^5$	$10^0 - 10^4$	—
Ammonification	$10^2 - 10^5$	$10^2 - 10^4$	$10^0 - 10^3$	$10^3 - 10^7$	$10^4 - 10^5$	$10^3 - 10^5$	$10^5 - 10^6$
Nitrate reduction	$10^2 - 10^5$	$10^2 - 10^3$	$0 - 10^0$	$10^3 - 10^7$	$10^4 - 10^5$	$10^2 - 10^5$	$10^5 - 10^6$
Denitrification	$10^1 - 10^4$	$10^0 - 10^2$	$0 - 10^0$	$10^2 - 10^6$	$10^3 - 10^4$	$10^2 - 10^4$	10^2
Ammonia oxidation	$10^{-1} - 10^1$	$10^{-1} - 10^3$	—	$10^0 - 10^2$	$10^1 - 10^3$	$10^{-1} - 10^2$	$10^{-1} - 10^0$
Nitrite oxidation	$10^{-1} - 10^1$	$10^{-1} - 10^1$	—	$10^0 - 10^3$	$10^0 - 10^2$	$10^{-1} - 10^2$	$10^{-1} - 10^0$
Nitrogen fixation	$10^{-1} - 10^3$	$10^{-1} - 10^2$	$0 - 10^0$	$10^2 - 10^4$	$10^2 <$	10^1	$10^0 - 10^1$

Fig. 3. Diagram of the microflora in the bottom sediments of various water regions.

Direction (1) Ammonifying bacteria (2) Ammonia oxidizing bacteria
 (3) Nitrite oxidizing bacteria (4) Nitrate reducing bacteria
 (5) Denitrifying bacteria (6) Nitrogen fixing bacteria

The radius of the circle and the length of broken line show the number of total heterotrophs and each group of the bacteria in log scale, respectively.

shown in Fig. 3. The direction and the length from the center of the circle indicate the type and the number of the bacteria responsible for each reaction in nitrogen cycle. The

radius of the circle represents the number of total heterotrophs. A peculiar pattern of microflora was obtained for each water region. In open sea, the occurrence of nitrogen fixing bacteria as well as nitrifying bacteria was very poor i.e. 1–10 cells/g or less, and the diagram showed L-type in these area. On the other hand, the ratio of the number of nitrogen fixing bacteria to that of total heterotrophic bacteria was high in inland bays such as Maizuru Bay and Kumihama Bay as well as in fresh water regions which were comparatively eutrophized, and their diagram showed triangular forms.

References

- 1) A. KAWAI and I. SUGAHARA: This Bull., **37**, 740–746 (1971).
- 2) A. KAWAI and I. SUGAHARA: *ibid.*, **37**, 747–754 (1971).
- 3) J. REINKE: *Ber. deut. bot. Gesell.*, **21**, 371–380 (1903).
- 4) J. REINKE: *ibid.*, **21**, 481–483 (1903).
- 5) L. N. PSHEININ: in "Symposium on Marine Microbiology" (C. H. OPPENHEIMER ed.), 383–391, C. C. Thomas Publ., Springfield, Illinois (1963).
- 6) T. D. BROCK: *Principles of Microbial Ecology*, 9 pp., Prentice-Hall, Englewood Cliffs, New Jersey (1966).
- 7) A. G. RODINA: *Limnol. Oceanogr.*, **8**, 388–393 (1963).