Distribution of Denitrifying Bacteria and Its Controlling Factors in Freshwater Environments

Takuo NAKAJIMA

Abstract

Distribution of denitrifying bacteria and its controlling factors were investigated in freshwater environments. Denitrifying bacteria were counted by use of PY-Nitrate liquid medium and the most probable number method. The population (10^0 - 10^3 cells·ml⁻¹) fluctuated more remarkably than that of aerobic heterotrophic bacteria in eutrophic pond and lakes. The ratio of the number of denitrifying bacteria to that of aerobic heterotrophic bacteria (D/Aeh) was between 0.0001 and 0.1 in those environments. In non-stratified eutrophic pond and lakes, the number of denitrifying bacteria tended to increase as the concentration of total nitrogen increased. In a stratified eutrophic lake, the D/Aeh ratio tended to increase as the concentration of dissolved oxygen decreased. Denitrifying bacteria in both water and attached substances in a polluted river tended to increase in number with the increase in BOD. The D/Aeh ratio in water and attached substances was higher than 0.01. Almost all denitrifying bacteria detected in the Himon-ya Pond, Lake Nakanuma and the River Tamagawa were gram-negative. *Pseudomonas* was the genus most frequently isolated from every type of water and attached substance.

1. Introduction

Many strains of denitrifying bacteria belonging to more than fifteen genera have been isolated by this time. They are facultative anaerobes which use nitrate (or nitrite) as a terminal electron acceptor in the absence of molecular oxygen. Denitrifying bacteria were reported to be heterotrophic except for one genus, *Thiobacillus* (PAYNE, 1973). According to KEENEY *et al.* (1971), the dominant genera of denitrifying bacteria were *Pseudomonas* and *Alcaligenes* in Wisconsin lake sediments. TERAJ (1979) reported that most denitrifying bacteria isolated from water of Lake Kizaki were classified as *Pseudomonas*.

Denitrifying bacteria in freshwater environments have been frequently counted. The ratio of the number of denitrifying bacteria to that of aerobic heterotrophic bacteria (D/Aeh) in water is usually on the order of 0.01 or less (DUTKA *et al.*, 1974; TERAJ, 1979; BELL *et al.*, 1980). However, only limited data are available in respect to the environmental factors which affect the distribution of denitrifying bacteria.

In this study, 1) factors affecting the distribution of denitrifying bacteria and 2) genera of these bacteria in freshwater environments are dealt with. The freshwater environments studied here were divided for convenience into three types, that is: 1) non-stratified eutrophic lakes, 2) stratified eutrophic lakes, and 3) rivers. A pond in Himon-ya Park (metropolitan Tokyo), Lake Suwa (Nagano Pref.), Lake Kasumigaura and Lake Kitaura (Ibaraki Pref.) were chosen as the representatives of type (1), Lake Nakanuma (Ibaraki Pref.) for type (2), and the River Tamagawa (metropolitan Tokyo) for type (3).

2. Description of the aquatic environments investigated

The Himon-ya Pond is located in a small park in metropolitan Tokyo. Its surface area is about 0.008 km² with a maximum water depth of 1.8 m, and it has little inflow except for rainwater. Lake Kasumigaura and Lake Kitaura are located in Ibaraki Prefecture. The former has an area of 178 km² (maximum depth, 7.0 m) and the latter has an area of 78.8 km² (maximum depth, 10.0 m). Lake Suwa (Nagano

Pref.) has an area of 14.5 km² with a maximum depth of 7.0 m. Blooming of *Microcystis* occurs in summer, but the anaerobic layer does not appear in the water bodies of these pond and lakes. Water samples were collected at the center of the pond and each lake.

Lake Nakanuma (Ibaraki Pref.) is a small eutrophic lake surrounded with paddy fields and has no constant inflow and outflow. Its area is 0.01 km² and water depth is 13.5 m (maximum). The anaerobic water layer appears below 8 m depth during the period of summer stratification. Blooming of *Microcystis* was not observed during the course of the present investigation. Samples were taken at the center of the lake (water depth, 13.5 m).

The River Tamagawa is located at the southwest part of Tokyo. Three stations were chosen for the investigations in the midstream. The river beds at these stations were covered with gravel. The river water was slightly polluted around St. 1 (Tamagawa-bashi) and heavily polluted with municipal sewage around St. 2 (Sekido-bashi) and St. 3 (between Futago-bashi and Maruko-bashi).

3. Methods

Sampling

Lake water samples were collected with a Van Dorn water sampler or with a glass syringe with two openings to which vinyl tubes were connected. Samples were maintained in an icebox until analyses. Sessile microbes in the River Tamagawa were collected from two different substrata, natural stones in the river and artificial substrata submerged in running water. All stones were picked up within a quadrat (50×50 cm) established on the river bed, and sessile microbes were stripped off from the stones with a wire-brush. In the case of artificial substrata, polyvinylchloride plates (5×5 cm) were used. Before immersion, surfaces of the plates were roughed with a rasp, and the plates were immersed horizontally in running water after affixing them to a metal frame at St. 3 (water

depth, about 50 cm; current velocity, 0.5–1 m·sec⁻¹). Microbial films on the surface of the plates were collected after the growth of sessile microbes was visually observed.

Enumeration and identification of bacteria

Water samples collected in sterilized test tubes for bacterial enumeration and identification were stored in an ice-box during transport to the laboratory and treated within several hours after sampling. Aerobic, heterotrophic bacteria and denitrifying bacteria were counted by the most probable number (MPN) method using peptone-yeast extract-nitrate medium (PY-Nitrate medium). The medium contained polypeptone, 5 g; yeast extract, 1 g; KNO₃, 1 g; and KH₂PO₄, 0.1 g in 1,000 ml of tap water adjusted to pH 7.0. After aliquots of each appropriate dilution of the sample were inoculated to five tubes, they were incubated at 20°C for 1 month. The cultures were observed for turbidity (counting the number of aerobic, heterotrophic bacteria) and gas accumulation in inverted vials within the tubes (counting the number of denitrifying bacteria). Denitrifying bacteria were isolated from the cultures for the bacterial counts. From the test tubes at highest dilution for observation of gas production, portions of the culture were transferred into PY-Nitrate medium in screw-capped test tubes for enrichment of denitrifying bacteria. A portion of each culture was transferred again to the fresh medium when vigorous gas production was observed. After three or four successive transfers, each sample was spread on the 1/10 strength nutrient agar plates. All colonies with a different appearance were picked up and the isolates were examined for purity and denitrifying ability. These isolates were identified at the generic level according to the scheme of SIMIDU (1974).

Chemical and physical analyses

Dissolved oxygen was measured by Winkler's method. BOD was measured after Standard Methods (APHA, AWWA, WPCF, 1975). Water samples for other chemical

analyses were filtered through Whatman GF/C filters. Particulate matter on the filter was dried at 60°C and determined for particulate organic carbon and nitrogen with a CHN coder (Yanagimoto Co.). Kjeldahl nitrogen and ammonium nitrogen were determined by the method described in IBP Handbook No. 8 (GOLTERMAN, 1969) and by the indophenol-hypochlorite method (SOLÓRZANO, 1969), respectively. The amount of dissolved organic nitrogen was calculated by subtracting ammonium nitrogen from Kjeldahl nitrogen. Nitrite and nitrate were determined by using sulphanilamide and N-(1-naphtyl) ethylenediamine dihydrochloride and by the same method after reducing nitrate to nitrite with a Cu-Cd column, respectively (KOBAYASHI and MORII, 1969).

4. Results

(1) *Distribution of denitrifying bacteria and its controlling factors in the non-stratified eutrophic pond and lakes*

Since denitrifying bacteria are heterotrophic, the ratio of their number to total number of aerobic, heterotrophic bacteria (D/Aeh) represents the dominancy of denitrifying bacteria in a given environment.

In the same manner as their absolute number, D/Aeh ratio is a useful indicator for the ecological importance of denitrifying bacteria. Numbers of heterotrophic bacteria and denitrifying bacteria were determined in the Himon-ya Pond (at 0 and 1 m water depths) from 1970 to 1975, in Lake Kitaura (0, 4 and 6 m), Lake Kasumigaura (0, 3 and 5 m) from 1974 to 1975, and in Lake Suwa (0, 3 and 5 m). Figure 1 shows the relationship between the number of denitrifying bacteria and the D/Aeh ratio in these waters. As the number of denitrifying bacteria increased, the D/Aeh ratio tended to increase. The reason for this is that the number of denitrifying bacteria fluctuated greatly (10^0 – 10^3 cells·ml⁻¹), whereas the aerobic heterotrophic bacterial population was relatively constant (10^4 – 10^5 cells·ml⁻¹). Chemical and physical factors of the environments were measured in order to identify the main factors affecting the distribution of denitrifying bacteria. The relationship between the amount of total nitrogen and the number of denitrifying bacteria is shown in Fig. 2. The level of total nitrogen in the water of the Himon-ya Pond, Lake Kitaura and Lake Kasumigaura was 1.8–3.0, 0.4–1.2 and 0.3–1.1 mg·l⁻¹,

Fig. 1. Relationship between the number of denitrifying bacteria and its ratio to the number of aerobic heterotrophic bacteria (D/Aeh) in non-stratified eutrophic pond and lakes. ○, Pond Himon-ya; ●, Lake Kitaura; ×, Lake Kasumigaura; △, Lake Suwa.

Fig. 2. Relationship between the amount of total nitrogen and number of denitrifying bacteria in non-stratified eutrophic pond and lakes. ○, Pond Himon-ya; ●, Lake Kitaura; ×, Lake Kasumigaura.

respectively. In most cases, there were more denitrifying bacteria in the Himon-ya Pond than in Lake Kasumigaura. As a whole, the denitrifying bacteria tended to increase with increase in total nitrogen; however, this tendency was not clear in each body of water. The coefficient of correlation (r) between them was calculated as 0.73 from this regression plot. There was no correlation between D/Aeh ratio and such environmental factors as dissolved oxygen, nitrate and nitrite, other nitrogenous compounds, pH and temperature.

(2) *Distribution of denitrifying bacteria and its controlling factors in the stratified eutrophic lake*

The bacterial number was measured in the water of 0, 6 and 12 m depths and just above the sediment surface in Lake Nakanuma from Dec. 1974 to Jun. 1975. The denitrifying bacterial population fluctuated greatly (10^0 – 10^3 cells·ml⁻¹), and that of aerobic heterotrophic bacteria varied between 10^3 and 10^5 cells·ml⁻¹. The denitri-

fying bacterial population tended to increase with increase in water depth in each period. The relationship between the number of denitrifying bacteria and D/Aeh ratio is shown in Fig. 3. The more denitrifying bacteria, the higher was the D/Aeh ratio. The D/Aeh ratio fluctuated between 0.0001 and 0.1.

Fig. 3. Relationship between the number of denitrifying bacteria and its ratio to the number of aerobic heterotrophic bacteria (D/Aeh) in Lake Nakanuma.

Several environmental factors were measured to determine the main factors controlling the distribution of denitrifying bacteria. The amount of total nitrogen in Lake Nakanuma was smaller than in the Himon-ya Pond, whereas the D/Aeh level in Lake Nakanuma was frequently higher than in the Himon-ya Pond. There was no direct correlation between the amount of total nitrogen and the number of denitrifying bacteria. These observations suggest that other environmental factors than total nitrogen affected the fluctuation in the number of denitrifying bacteria.

In Lake Nakanuma, anoxic water layer developed during the summer stratification period. Oxygen content in water influences the metabolism of denitrifying bacteria, so it is considered to cause the change in their population. The highest D/Aeh ratio was obtained for water containing no dissolved

Fig. 4. Relationship between dissolved oxygen and the ratio of the number of denitrifying bacteria to that of aerobic heterotrophic bacteria (D/Aeh) in Lake Nakamura. Arrow means the value lower than that.

oxygen. The D/Aeh ratio tended to increase with the decrease in oxygen content (Fig. 4). Other factors, including concentrations of nitrate and nitrite, were not found to have a direct correlation with their population.

(3) *Distribution of denitrifying bacteria and its controlling factors in a polluted river*

Distribution of denitrifying bacteria was investigated at three stations in the midstream of the River Tamagawa from 1975 to 1979. Attached substance was collected from stones within a quadrat. Surveys were carried out on more than 10 successive days after vigorous rainfall. Number of denitrifying bacteria in both water and attached substance on the gravels increased toward downstream. Figure 5 shows the relationship between the number of denitrifying bacteria in water and the attached

Fig. 5. Relationship between BOD and the number of denitrifying bacteria in water and attached substance in the midstream of the River Tamagawa. ○, water; ●, attached substance.

substances and BOD of water. The number tended to increase with BOD. However, there was no good correlation between the D/Aeh ratio and BOD. BOD indicates the amount of organic matter available for heterotrophic bacteria, so its increase is presumed to be generally favorable for denitrifying bacteria as well as aerobic heterotrophic bacteria.

To examine the growth of denitrifying bacteria in attached substance, artificial substrata were used. Plates of polyvinylchloride were immersed in the river at St. 3. Figure 6 shows the increase in the number of denitrifying bacteria in the course of development of a sessile microbial community in August 1978. Particulate organic nitrogen increased for the first 10 days, although the range of the variation among plates increased with time because of the heterogeneity in microbial film on the plates (Fig. 6a). It decreased after 10 days' immersion. The number of denitrifying bacteria increased rapidly for the first 2 days, and thereafter gradually up to 8th day (Fig. 6b). The number decreased after 10 days' immersion, when particulate organic nitrogen was maximum. Variation in the

Fig. 6. Changes in the amount of particulate organic nitrogen (a), and bacterial number (b) on artificial substrata immersed at station 3 in the River Tamagawa. In b; ○, denitrifying bacteria; ●, aerobic heterotrophic bacteria. The mean $\pm$ SD ($n=3$) was indicated.

number of aerobic heterotrophic bacteria showed a similar pattern to that in the number of denitrifying bacteria. Anaerobic heterotrophic bacteria were counted at the same time using a gas-pak anaerobic system (BBL). The mean population of three plates at 2, 4, and 12 days was 1×10^5 , 6×10^5 and 4×10^7 , respectively. This suggests the development of anaerobic conditions in the sessile microbial community in terms of its growth.

If the micro-environment suitable for denitrifying bacteria develops in the sessile microbial community, the D/Aeh ratio probably becomes high. Possible changes in the D/Aeh ratio during the increase in attached substance (as PON) were investigated. The growth of denitrifying bacteria was examined in the microbial community on the underside of the plates within the

Fig. 7. Relationship between particulate organic nitrogen (PON) and the ratio of the number of denitrifying bacteria to that of aerobic heterotrophic bacteria (D/Aeh) in 8 days' immersion in water at St. 3. ○, Mar. 1978 (back surface of the plates); ●, Aug. 1978 (top surface of the plates); ×, May. 1978 (top surface of the plates).

6-day immersion period to minimize the complexity. Only a few algae were observed in the community. In Fig. 7, the relationship between the D/Aeh ratio and the particulate organic nitrogen is given. The results obtained for the microbial community on the upside of the plates within the 8 day immersion period were also shown in this figure. Aerobic heterotrophic bacteria and denitrifying bacteria increased in number with increase in particulate organic nitrogen. The D/Aeh ratio became high as particulate organic nitrogen increased to a certain level.

Figure 8 shows the relationship between the number of denitrifying bacteria and particulate organic nitrogen in the attached substances sampled after various immersion times (2-120 days) from 1975 to 1978. The population varied greatly even at the same level of particulate organic nitrogen. However, the maximum number of denitrifying bacteria became greater as particulate organic nitrogen increased. In almost all

Fig. 8. Relationship between particulate organic nitrogen (PON) and the number of denitrifying bacteria in attached substance at St. 3 in the River Tamagawa.

cases, the D/Aeh ratio was higher than 0.01.

(4) *Taxonomic investigations on denitrifying bacteria in freshwater environments*

Dominant species of denitrifying bacteria were isolated from various freshwater environments and identified at the generic level. The results are summarized in Table 1. All of the isolates were coccoidal rods or rods and not pigmented on the 1/10 strength nutrient-agar plate. *Pseudomonas* was the genus most frequently isolated from water and sediment. Bacteria belonging to *Moraxella*, *Aeromonas* and *Alcaligenes* were sometimes isolated. A gram-positive denitrifying bacterium (*Bacillus*) was isolated only once in the Himon-ya Pond.

5. Discussion

The denitrifying bacterial population fluctuated greatly in both non-stratified and stratified eutrophic lake waters, although the aerobic heterotrophic bacteria varied little (Figs. 1 and 3). TERAJ (1979) also reported that the number of denitrifying

bacteria in Lake Kizaki fluctuated greatly. KONDA and TEZUKA (1979) found that bacterial flora in Lake Motosu varied remarkably even if the number of aerobic heterotrophic bacteria did not. Change in bacterial flora is probably more clear than in bacterial number in a lake. The number of denitrifying bacteria in this study was lower than the range of 10^2 - 10^4 cells·ml⁻¹ which was reported by several investigators for eutrophic lakes in the Soviet Union (summarized by KUZNETSOV, 1970). However, the number of denitrifying bacteria differs frequently according to the counting method. Since the counting method was not described in his book, the values obtained in the present investigation are difficult to compare with his ones.

In non-stratified eutrophic lakes, the number of denitrifying bacteria tended to increase as the amount of total nitrogen in water increased (Fig. 2). The amount of total nitrogen indicates the degree of lake eutrophication. KUZNETSOV (1970) showed, based on the data from several authors, that the number of denitrifying bacteria increased as eutrophication progressed. The D/Aeh ratio in freshwater is usually in the order of 0.01 or less. Exceptionally high D/Aeh values were shown in a fish-culturing pond and an aquarium, which contained much organic matter (SUGIYAMA and KAWAI, 1978; KAWAI and SUGIYAMA, 1979). According to SUGIYAMA and KAWAI (1978), denitrification proceeded more rapidly in water with much organic matter rather than in water with a lower dissolved oxygen level. JANNASCH (1960) found that denitrification of a denitrifying bacterium occurs in the presence of particulate organic matter even under aerobic conditions. In such anaerobic microsphere within particulate organic matter, the growth of denitrifying bacteria probably increases. However, denitrifying bacteria are facultative anaerobes and their growth is not always based on denitrifying activity. Anaerobic microspheres and/or available organic matter for denitrifying bacteria may increase as eutrophication proceeds.

Table 1. Genera of denitrifying bacteria isolated from freshwater environments.

Location	Sampling date	Water depth (m)	Genus
P. Himonya	Sep. 1975	0	<i>Aeromonas</i> (1)
		1	<i>Bacillus</i> (1)
	Mar. 1976	mud	<i>Pseudomonas</i> (1)
	May 1976	0	<i>Pseudomonas</i> (1)
		mud	<i>Pseudomonas</i> (4)
L. Takahoko (Aomori Pref.)	Aug. 1974	3	<i>Pseudomonas</i> (2)
		6	<i>Pseudomonas</i> (1)
L. Nakanuma	June 1976	0	<i>Moraxella</i> (1)
		6	<i>Pseudomonas</i> (9), <i>Alcaligenes</i> (2)
			<i>Aeromonas</i> (1)
		12	<i>Pseudomonas</i> (1)
	Nov. 1976	mud	<i>Aeromonas</i> (1)
		6	<i>Pseudomonas</i> (3), <i>Moraxella</i> (2)
			<i>Alcaligenes</i> (1)
		12	<i>Pseudomonas</i> (1)
R. Tamagawa	St. 1	Sep. 1975	A
		Jan. 1976	W
			A
			<i>Pseudomonas</i> (1)
	St. 2	Sep. 1975	A
		Jan. 1976	W
			A
			<i>Pseudomonas</i> (3), <i>Alcaligenes</i> (1)
	St. 3	Sep. 1975	W
			A

(), number of isolates; A, attached substance; W, water; NI, not identified (Gram negative).

In Lake Nakanuma, a stratified eutrophic lake, the D/Aeh ratio tended to increase with decrease in the concentration of dissolved oxygen (Fig. 4). Denitrification in anaerobic microsphere was probably not appreciable in Lake Nakanuma, because denitrifying activity was not observed even in the water with low oxygen concentration. NAKAJIMA and OCHIAI (1979) investigated the influence of oxygen on the bacterial number in Lake Nakanuma. They reported that the number of denitrifying bacteria under aerobic conditions decreased to one or two orders of magnitude lower than that of denitrifying bacteria under semi-anaerobic conditions, whereas numbers of

aerobic heterotrophic bacteria remained relatively constant under both conditions. It seems unlikely that oxygen would directly suppress their growth. NAGATSUKA and FURUSAKA (1977) showed that oxygen tension influenced bacterial flora in paddy soil. Also in Lake Nakanuma, bacterial flora probably was altered with changes in oxygen tension, and the number of bacteria which possess denitrifying ability might increase with decrease in dissolved oxygen. Further investigations must be carried out to clarify the effect of oxygen on the number of denitrifying bacteria in Lake Nakanuma.

A large number of denitrifying bacteria

was observed in the River Tamagawa (Figs. 5 and 6). The value of 10^3 cells·ml⁻¹ enumerated at a scarcely polluted site was in the same order of their maximum number in eutrophic lakes. The D/Aeh ratio in both water and attached substances was relatively higher than in water of eutrophic lakes (cf. Figs. 1 and 2). Nitrate or nitrite and organic matter were rich especially in polluted water in the River Tamagawa (TEZUKA *et al.*, 1974; AIZAKI, 1978, 1979). Since anaerobic conditions develop in the inner part of microbial film when the thickness of the film increases to a certain level (ECKENFELDER, 1961; WUHRMAN, 1963; SANDERS, 1966), development of sessile microbial film on gravel offers favorable conditions for denitrification. NAKAJIMA (1979) reported that sessile microbial community of the River Tamagawa showed denitrifying activity even in oxic river water. Occurrence of denitrification probably was caused by high D/Aeh ratio in this river.

In some cases of sessile microbial community in early stages in development, the D/Aeh ratio tended to increase slightly as particulate organic nitrogen increased to a certain level (Fig. 7). Anaerobic zones must have been formed as particulate organic nitrogen increased. However, the tendency is not clear when all results, including those of long-term immersion, were plotted. Periphyton community varied quantitatively and qualitatively in the River Tamagawa (AIZAKI, 1978, 1979, 1980). Exfoliation of the attached substances and the difference in organisms constituting sessile microbial community must be some of the factors causing the scattered plots as shown in Figs. 7, 8 and 9.

In the present investigation, most of the isolates were classified as *Pseudomonas* (Table 1). There are reports that the dominant species of denitrifying bacteria isolated from natural environments were *Pseudomonas* (SREENIVASAN and VENKATARAMAN, 1952; KEENEY *et al.*, 1971; GAMBLE *et al.*, 1977; TERA, 1979). Although taxonomic investigations on dominant denitri-

fying bacteria are few, *Pseudomonas* seems to be, in general, the most dominant genus in freshwater environments.

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摘 要

陸水における脱窒細菌の分布と、それらの分布に影響を及ぼしている環境要因を調べた。

脱窒細菌の計数は、ペプトン・イーストエキス・硝酸塩培地を用い、MPN 法によった。

富栄養化した湖沼の脱窒細菌数は、 $10^0 \sim 10^3$ cells·ml⁻¹ であって、その変動は、好氣的従属栄養細菌数の場合より顕著であった。脱窒細菌数の従属栄養細菌数に対する比 (D/Aeh) は、0.000~0.1 であった。夏季成層しない富栄養湖沼における水中の脱窒細菌数は、全窒素量が増加するにしたがい増加する傾向にあった。成層する富栄養湖では、D/Aeh 比が、溶存酸素の減少に伴って増加する傾向があった。有機汚濁の進んだ河川での脱窒細菌は、水中および河床付着物のいずれにおいても BOD の増加にしたがい多く計数され、D/Aeh 比は 0.01 以上であった。碑文谷池・中沼および多摩川より分離された脱窒細菌のほとんどがグラム陰性菌であった。どの水域でも *Pseudomonas* 属が多く分離された。

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- (著者 : 中島拓男, 東京都立大学理学部, 東京都世田谷区深沢; Takuo NAKAJIMA, Department of Biology, Faculty of Science, Tokyo Metropolitan University, Setagaya-ku, Tokyo 158)

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