Article

Ann Rep Osaka City Inst Publ Hlth Environ Sci 72, 41～48 (2010)

# Sensitivity of MPN Method for Enumerating Denitrifying Bacteria by Using Gas Chromatography

Takayuki NISHIO, Taro YOSHIKURA, and Yoshiaki MORI

**Abstract**

Time course studies of most-probable-number (MPN) for denitrifying bacteria were monitored by detecting nitrous oxide generation in vials by using gas chromatography. Results were compared to those of conventional MPN method that counting gas accumulation in an inverted Durham tube. The MPNs obtained by using gas chromatography were of 1-2 orders higher magnitude and required a shorter incubation period to reach the plateau MPN than the conventional MPN method using a Durham tube. Concentrations of organic substances in the media were varied to examine oligotrophic denitrifiers. When samples from an unpolluted site were examined, little difference was found between MPNs with diluted and rich medium. However, the MPNs for samples from a solid waste disposal site increased only gradually in diluted medium and the plateau MPNs were lower than those with richer medium.

**Key words:** denitrification, acetylene inhibition, Durham tube MPN, nitrous oxide, nitrite, nitrate, sea-based solid waste disposal site

## I INTRODUCTION

Most dissimilatory denitrifying bacteria are heterotrophic and facultative anaerobic bacteria, which acquire energy in reducing nitrite and/or nitrate into gaseous nitrogen compounds such as nitrogen ($N_2$) and nitrous oxide ($N_2O$), which are stripped from the liquid media. Denitrifying bacteria play an important role in reducing nitrogen concentrations in biological nitrogen treatment process of wastewater. In addition to monitoring concentrations of nitrogenous compounds, monitoring activity and population of denitrifying bacteria will help maintain active cells in the wastewater treatment system. Rapid and sensitive method to enumerate denitrifying bacteria is required because retention time in denitrifying tank is shorter than the incubation time required for enumeration by conventional cultivation method, e.g., the retention time is 2–4 hours in municipal wastewater treatment plants whereas more than 2 weeks incutation is recommended for MPN measurements.

The denitrifier enzyme activity (DEA) introduced by Tiedje [1, 2] is a good measure of denitrifying activity, however, it does not provide information on population [3, 4]. Probing certain DNA sequences of the gene involved in denitrification may provide a more accurate number of denitrifiers [5, 6], but, a positive test for a gene does not always represent active cells. Monitoring potentially active populations of denitrifiers is necessary for some studies; the most-probable-number (MPN) is still widely applied in enumerating denitrifying bacteria, despite the inherent low accuracy of the method.

The MPN method of enumerating denitrifying bacteria is still widely used; gas bubbles accumulated in inverted Durham tubes placed in 3–5 replicate test tubes of 10-fold serially diluted samples are counted [7]. The accumulation of gas generated through denitrification requires an incubation of 3–4 weeks to reach a plateau MPN and underestimation of denitrifying bacteria is suspected because of the low sensitivity of bubble counting. The accuracy of positive scores for denitrification has been improved by introducing acetylene inhibition technique to confirm gas production as $N_2O$ in combination with the detection of substrate consumption in the media [1, 2]. Acetylene inhibition is a sensitive method of measuring denitrifying activity [8, 9] and its application to MPN enumeration has improved the efficiency of counting denitrifying bacteria. Reduction

Osaka City Institute of Public Health and Environmental Sciences
8-34 Tojo-cho, Tennoji-ku, Osaka 543-0026, Japan

of the incubation period is also expected, however, a two-week incubation is still recommended [2].

In the present study, we examined the time course of MPN of denitrifiers by monitoring N2O generation in every vial by gas chromatography instead of just confirming $N_2O$ production in vials in which the substrate for denitrification is consumed. Concentrations of nutrient and substrate for denitrification were varied to examine the conditions to reduce the incubation period and improve the sensitivity of gas-chromatography-aided MPN (GC-MPN). The time course in Durham tube MPN (DT-MPN) was also monitored and compared with that of GC-MPN.

## Ⅱ MATERIALS AND METHODS

### 1) Organism

*Pseudomonas stutzeri* IAM12668 was obtained from the culture collection of the Institute of Molecular and Cellular Biosciences at The University of Tokyo.

### 2) Medium

BP medium containing 0.5 g of Lab Lemco meat extract (Oxoid, Unipath Ltd., UK) and 0.5 g of Trypticase peptone (BBL; Becton Dickinson Microbiology Systems, USA), 1 mM sodium citrate, 2 mM sodium aspartate, 250 mM NaCl, 25 mM $MgSO_4 \cdot 7H_2O$, 25 mM $MgCl_2 \cdot 6H_2O$, 10 mM KCl in Synthetic basal medium (SBM) was used. The SBM contained 1 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM $CaCl_2 \cdot 2H_2O$, 0.05 mM $K_2HPO_4$, 0.05 mM $NaH_2PO_4 \cdot 2H_2O$, 5 μM Fe-EDTA, 0.2 μM of $ZnSO_4 \cdot 7H_2O$, 0.1 μM $MnCl_2 \cdot 4H_2O$, 0.08 μM of $CuSO_4 \cdot 5H_2O$, 0.04 μM $Na_2MoO_4 \cdot 2H_2O$, and 0.01 μM $CoCl_2 \cdot 6H_2O$ in 1 liter of distilled water (pH 7.8). Nitrite ($NO_2^-$) or nitrate ($NO_3^-$) was added as the substrate at 5 mM unless otherwise noted.

### 3) Enumeration of bacteria

Denitrifying bacteria were enumerated by detecting the denitrifying activity in vials in the GC-MPN method or counting positive tubes which accumulated gas bubble in the inverted Durham tubes in the DT-MPN method. The MPNs were estimated from statistical tables [10–12]. When 95% confidence limits of the two MPNs overlapped, differences were considered insignificant in this study.

Denitrifying activity was examined by means of acetylene inhibition [8, 9]. One milliliter portions of samples that were serially diluted 10-fold with the test medium were dispensed into five 1.6 mL replicate vials and then sealed with a butyl rubber septum and an aluminum cap. In some experiments, samples were diluted 2-fold and dispensed into eight replicate vials. Head space gas was replaced with nitrogen unless otherwise noted and acetylene was injected at about 10 kPa at the beginning of the experiments. Samples were incubated at 30ºC and the accumulation of $N_2O$ in the head space was monitored using a gas chromatograph (GC7A; Shimadzu Corp., Jpn) equipped with an electron capture detector (ECD), on a 3 mm × 3 m column packed with Porapack Q (80–100 mesh; Millipore Corp., USA) operated isothermally at 120ºC.

In DT-MPN measurements, samples were serially diluted 10-fold with the test medium; 1 mL portions were dispensed into five replicate test tubes containing 10 mL of test media with inverted Durham tubes. Gas bubbles accumulated in the Durham tubes through denitrification were scored positive.

Plate counts were made by spreading appropriately diluted samples on BP agar plates. When bacterial numbers in the vials for GC-MPN were monitored, cultures were subsampled with a sterile syringe through the septum of the vials and spread on agar plates after they were diluted appropriately.

### 4) Study sites

Water samples were collected from St. a, a facultative pretreatment pond and St. b, an aerated pond in a sea-based solid waste disposal site (34º 39' N, 135º 25' E), located in Osaka Bay about 4 km from the mouth of the Shin-Yodo river [13], and from St. c, 100 m outside St. a. Chemical oxygen demand (COD) at St. a, St. b, and St.c were, 29.6–57.3, 24.5–45.1, and 2.8–6.0 mg $L^{-1}$, respectively during the study period. Biochemical oxygen demand (BOD) values at Sts. a, b, and c were, 2.5-31.0, 3.5-16.5, and, 0.3–6.6 mg $L^{-1}$, respectively. Total nitrogen at Sts. a, b, and c were, 8.9–31.8, 7.5–26.8, and, 0.78–1.50 mg $L^{-1}$, respectively [14].

## Ⅲ RESULTS

### 1) Sensitivity of MPN measurement with a denitrifying bacterium *P. stutzeri*

Time course studies of MPN of *P. stutzeri*, a typical respiratory denitrifying bacterium, were performed to examine the sensitivity of GC-MPN method and

compared with those obtained using the DT-MPN method. GC-MPN reached a plateau MPNs within 1–2 days and the estimated bacterial numbers in the inocula were 85 to 150% of those obtained by the plate count. When a culture of $3.2 \times 10^9$ (standard deviation: $s$=$4.0 \times 10^8$) cfu $mL^{-1}$ was used as the inoculum, the final GC-MPN was $3.5 \times 10^9$ MPN $mL^{-1}$ after 2 days of incubation (**Fig. 1**). The increase in DT-MPN almost paralleled that of GC-MPN with about a 24 hour delay and reached to a plateau of $3.5 \times 10^9$ MPN $mL^{-1}$ after 3 days of incubation.

In our GC system, $N_2O$ was detected when it accumulated in the head space of the vial at about 20 μL $L^{-1}$. When $N_2O$ become detectable in the headspace of a vial for MPN, the bacterial number in the vial was counted. The cell densities ranged from $2.2 \times 10^5$ ($s$=$2.5 \times 10^3$) to $8.8 \times 10^5$ ($s$=$1.0 \times 10^5$) cfu $mL^{-1}$. On the other hand in DT-MPN, cell densities in the culture ranged from $1.6 \times 10^8$ ($s$=$9.5 \times 10^7$) to $3.8 \times 10^8$ ($s$=$3.0 \times 10^7$) cfu $mL^{-1}$ when bubbles became visible in a Durham tube.

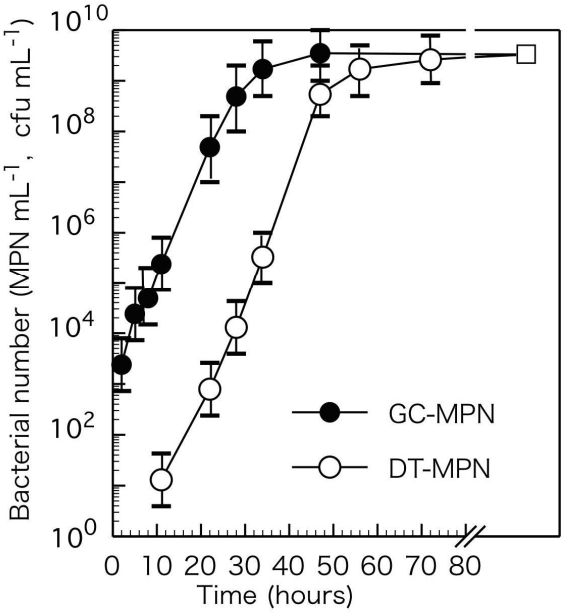


**Fig. 1.** Time course studies of GC-MPN (●) and DT-MPN (○) of *P. stutzeri*. The symbol □ indicates plate count of the inoculum. Bars represent 95% confidence limits.

### 2) Comparison of sensitivity in GC-MPN and DT-MPN using samples from the natural environment

Time course studies of GC-MPN were also performed using samples from natural environments and compared with those of DT-MPN. A typical time course of MPNs of denitrifying bacteria from natural environment is shown in **Fig. 2**. The increases in MPNs were slower than those observed in the experiments with *P. stutzeri*. $N_2O$ accumulation in the head space became detectable after 12 hour of incubation in the vials at low dilutions, while samples at the same dilution level required more than 2 days to be determined positive when using DT-MPN (**Fig. 2**). Many (30 out of 40 samples) required more than 2 weeks to reach a plateau in DT-MPN method, whereas the same samples reached the maximum after a 3–5 day incubation in

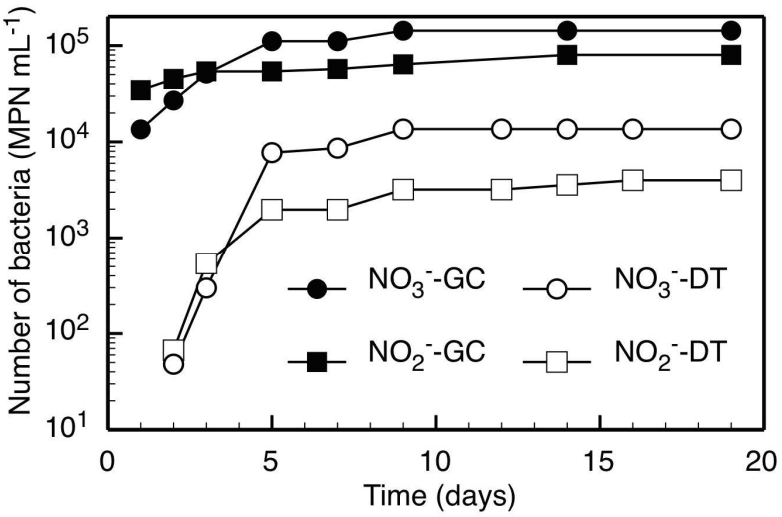


Fig. 2. Time course study of GC-MPN and DT-MPNs of water samples. Open and closed symbols represent DT-MPNs and GC-MPNs, respectively. Samples were taken from St. a (●, ○) and St. b (■, □).

GC-MPN method with some exceptions, which required more than 2 weeks to reach a plateau (9 out of 35 samples). The final MPNs obtained by GC-MPN method were significantly higher than those by DT-MPN method (**Fig. 2**).

### 3) Effects of organic substance concentration on MPN counts

The effect of organic concentration of test media on enumeration of denitrifiers was examined by using GC-MPN method with diluted BP medium and compared with the samples inside (St. a) and outside (St. c) a sea-based solid waste disposal site. In a 10-fold dilution of BP medium (0.1 BP), the MPNs of denitrifiers were 10–100 times lower during the early stage of incubation and a longer incubation was always required to reach the maximal MPNs than in BP medium. BP medium tended to yield significantly higher MPNs of denitrifying bacteria than 0.1 BP medium at St. a and St. b, which were highly polluted areas in the sea-based solid waste disposal site, whereas the differences were insignificant in many samples from St. c, a less polluted area (**Fig. 3**).

The difference between BP and 0.1 BP media was more obvious (probability of occurrence; $P$=0.05) in MPNs of heterotrophic bacteria, which were given by turbidity in media and carbon dioxide generation in head space of vials (**Figs. 3 St. a and St. c**). These observations showed the dominance of eutrophic bacteria in the polluted area and the eutrophic nature of denitrifying bacteria.

## Ⅳ DISCUSSION

### 1) Sensitivity and MPN estimation

Improved sensitivity of GC-MPN method resulted in higher MPNs and shorter incubation time than those by DT-MPN. For model experiments using pure culture, MPNs by both methods reached the cell density of the inoculum within 3 days of incubation, however, GC-MPN was about 400 times more sensitive than DT-MPN method (**Fig.1**).

Only 2.1 nmol of $NO_2^-$ has to be converted to $N_2O$, assuming that $N_2O$ accumulation at 20 μL $L^{-1}$ is positive in our GC-MPN system, although more than 890 nmol must be converted to gaseous compounds, assuming that 10 μL of gas bubbles are counted as positive in DT-MPN method. This estimation is comparative to or slightly lower than the difference in the cell densities at which accumulation of $N_2O$ or $N_2$ gas became detectable, i.e., 2.2–8.8×$10^5$ cells $mL^{-1}$ and 1.6–3.8×$10^8$ cells $mL^{-1}$, respectively in GC-MPN and DT-MPN method. Assuming that single cells were ideally inoculated in the

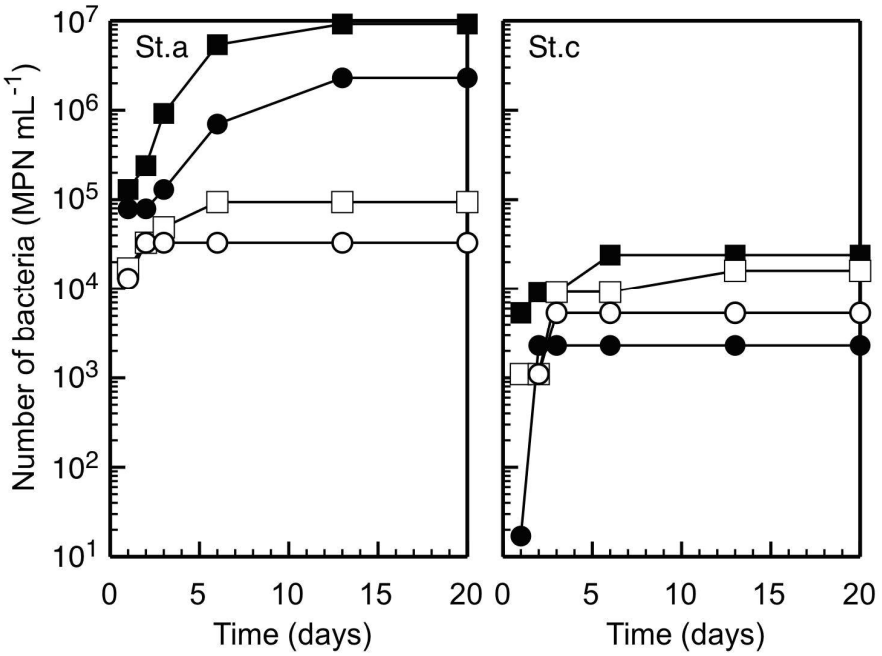


**Fig. 3.** Effects of the organic concentrations of medium on MPNs of heterotrophic bacteria (■, □) and denitrifying bacteria (●, ○) in seawater inside (St. a) and outside (St. c) the solid waste disposal site. BP (■, ●) and 0.1 BP medium (□, ○) containing nitrite at 1 mM were used.

vials at the dilution level of extinction (dilution level between the first not-entirely-positive to the last not-entirely-negative), 18 and 28 times of cell divisions are needed, respectively, to be detectable in GC-MPN and DT-MPN method. Because the generation time of *P. stutzeri* examined by spread plate method was 0.77 hr in the BP medium, the time lag between the two methods corresponding to 10 times cell divisions (18 and 28 times) is expected to be 7.7 hr.

However, the time lag observed between the growth curve in the mid-log growth phase of MPNs obtained by GC-MPN and DT-MPN methods was almost 24 hr; GC-MPN actually gave 20000–60000 times greater MPN counts than DT-MPN did. This discrepancy probably occurs because the diluted sample was diluted further to one-eleventh when a 1-mL portion was inoculated to 10 mL of test medium and because additional time to invade the inverted Durham tube was required in the DT-MPN method.

The increase in GC-MPNs of denitrifiers in the first three days of incubation was on the order of $10^3$; prolonged incubation resulted in additional one order at the most (**Figs. 2** and **3**). Most vials that became positive in 3 days of incubation accumulated $N_2O$ comparative to the criteria for dissimilatory denitrifiers given by Tiedje and co-workers: (i) the specific activity of denitrification monitored by acetylene inhibition must be more than 10 nmol $N_2O$-N $min^{-1}$ $mg^{-1}$ protein [15] and (ii) more than 20% of the $NO_3^-$ added to the media for MPN counting must be converted to gaseous compounds [2, 15]. These criteria can be expressed alternatively as (i) the growth of the single cell to $10^5$ cells in 2 days with $N_2O$ production at 150 μL $L^{-1}$ and (ii) accumulation of $N_2O$ at more than 14000 μL $L^{-1}$ from 10 mM nitrite in 1 mL of BP medium in 1.6-mL vial in our GC-MPN system. Those criteria, however, must be applied carefully to the samples from natural environment as suggested by the large discrepancies found between MPN and enzyme activity found in soil denitrifiers indicate that MPN can recover only a small portion of the denitrifiers in soil [3, 4].

Because freshly prepared preculture of the laboratory stock was used as the inoculum in the model experiment, almost all cells were able to grow to give positive scores and the ultimate MPNs were the same in either of the MPN methods. In contrast, in enumerating samples from natural environment, the discrepancies in MPNs that occurred by either method did not degenerate even after prolonged incubation (**Fig. 2**). The differences in estimated MPNs between GC-MPN and DT-MPN measurements indicated the presence of bacteria, which were active enough to give a positive score for GC-MPN but not for DT-MPN measurements. Heterogeneous bacteria in various physiological states would exhibit various growth rates and lag periods, and not all bacteria will grow to give a positive score because many are supposed to be dormant and resuscitation is limited by the selectivity of the media used.

Most heterotrophic bacteria in the natural environment are supposed to be in ″viable but non-culturable″ state [16]. Some of them usually start to grow exponentially upon confinement [17–19], however, resuscitation occurs only occasionally. Because denitrifying bacteria comprise only several percent of heterotrophic bacteria [19, 20], competition for substrate with the other heterotrophic bacteria, including non-denitrifying nitrate reducers, might limit the substrate and growth of denitrifying bacteria in batch cultures of MPN measurement. Competition for the substrate among heterotrophic bacteria may also reduce the efficiency in enumerating denitrifying bacteria. Reproduction of $N_2O$ was confirmed by transferring the culture in a vial that produced only a small amount of $N_2O$ to a fresh medium. Although such poor generation of $N_2O$ should be assigned to non-denitrifying nitrate reducers [22], it can be said that removal of nitrogenous compounds occurred through biological processes.

### 2) Efficiency in MPN counting with diluted media

Oligotrophic bacteria cannot grow on rich media and neither form colonies on agar plates nor give appreciable turbidity in liquid media [23–25]. Rich nutrient media have been used in MPNs for denitrifying bacteria, probably to exhaust dissolved oxygen in the vials or in inverted Durham tubes. However, such media may not be appropriate to detect denitrifying bacteria in less polluted natural environments. By introducing the sensitive detection procedure to MPN counting of heterotrophs, i.e., detection of $^{14}CO_2$ as the positive test for heterotrophs using $^{14}C$-labeled substrates and diluted media [26], oligotrophic bacteria became detectable and the dominance of oligotrophic

bacteria in a less polluted environment was revealed [27, 28]. Analogous to the $^{14}C$-MPN method, GC-MPNs in diluted nutrient media are expected to yield improved efficiency in enumerating oligotrophic denitrifying bacteria, however, GC-MPNs in 10-fold or more diluted BP medium did not exceed those in BP medium for the samples from the less polluted area. This implies that denitrifying bacteria are virtually eutrophic.

## V CONCLUSIONS

With GC-MPN, the incubation period of denitrifying bacteria can be reduced to 5–7 days from 3–4 weeks. Moreover, the sensitivity can be increased 100-fold relative to DT-MPN method.

Possible underestimation by elimination of oligotrophic denitrifying bacteria was negligible; the BP medium was preferable to 10-fold dilution of BP medium for enumerating denitrifying bacteria.

**Acknowledgements** We thank Dr. Y. Tsujimoto for helpful discussion and K. Kigo, Y. Ogoshi, and Y. Hatta for technical assistance.

## REFERENCES

1) Tiedje J M. Denitrification, p.1011-1026. In Page A. L., Miller R. H., and Keeney D. R. (ed.), Methods of soil analysis, part 2, Am Soc Agron, 1982 WI.
2) Tiedje J M. Denitrification, p. 245-267. In Weaver R. W., Angle J. S., and Bottomley P. S. (ed.), Methods of soil analysis, part 2, Soil Science Society of America, Inc., 1994 Wisconsin.
3) Davidson E A, Strand M K, Galloway L F. Evaluation of the most probable number method for enumerating denitrifying bacteria. Soil Sci Soc Am J. 1984: 49: 642-645.
4) Martin K, Parsons L L, Murray R E, Smith S. Dynamics of soil denitrifier populations: relationships between enzyme activity, most-probable-number counts, and actual N gas loss. Appl Environ Microbiol 1988; 54: 2711-2716.
5) Smith G B, Tiedje J M. Isolation and characterization of a $NO_2^-$ reductase gene and its use as a probe for denitrifying bacteria. Appl Environ Microbiol 1992; 58: 376-384.
6) Ye R W, Fies M R, Bezborodnikov S G, Averill B A, Tiedje J M. Characterization of the structural gene encoding a copper-containing $NO_2^-$ reductase and homology of this gene to DNA from other denitrifiers. Appl Environ Microbiol 1993; 59: 250-254.
7) Alexander M. Denitrifying bacteria. In C. A. Black, et al. (ed.) Methods of soil analysis, part 2. Agronomy 9, 1484-1486. Am. Soc. Agron., 1965 Madison WI.
8) Balderston W L, Sherr B, Payne W. J. Blockage by acetylene of nitrousoxide reduction in *Pseudomonas perfectomarinus*. Appl. Environ. Microbiol. 1976; 31: 504-508.
9) Yoshinari T and Knowles R. Acetylene inhibition of nitrous oxide reduction by denitrifying bacteria. Biochem. Biophys. Res. Commun. 1976; 69: 705-710.
10) Cochran W G. Estimation of bacterial densities by means of the "most probable number." Biometrics 1950; 6: 105-116.
11) Woomer P L. Most probable number counts, p. 59-79. *In* Weaver R. W., Angle J. S., and Bottomley P S. (ed.), Methods of soil analysis, part 2, Soil Science Society of America, Inc., 1994 Wisconsin.
12) Rowe R, Todd R, Waide J. Microtechnique for most-probable-number analysis. Appl. Environ. Microbiol. 1977; 33: 675-680.
13) Takamizawa K, Yamamoto O, Fukunaga I, Inoue Z, Honda A. Design philosophy on leachate treatment facilities at a sea-based solid waste disposal site in Osaka. J. Jpn. Soc. Waste Manage. Experts. 1991; 2: 25-32.
14) Nishio T, Yoshikura T, Fujiwara Y, Fukunaga I. Nitrification and Denitrification Activity in the Facultative Pretreatment Pond of a Sea-based Solid Waste Disposal Site Japanese Journal of Water Treatment Biology, 42; 107-117, 2006
15) Mahne I, Tiedje J M. Criteria and methodology for identifying respiratory denitrifiers. Appl Environ Microbiol 1995; 61: 1110- 1115.
16) Roszak D B. andColwell R R. Surival strategies of bacteria in natural environment. Micobiol Rev 1987; 51: 365-379.
17) Ferguson R L, Buckely E N, Palumbo A B. Response of marine bacterioplankton to differential filtration and confinement. Appl Environ Microbiol 1984; 47: 49-55.

18) Kogure K, Shimidu U, Taga N, Colwell R R. Correlation of direct viable counts with heterotrophic activity for marine bacteria. Appl Environ Microbiol 1987; 53: 2322-2337.
19) Nishio T, Kitano M, Morishita H., Yoshikura T. The effects of confinement of river water on the culturability and heterotrophic activity of the bacterial assemblages. Ann Rep Osaka City Inst Publ Hlth Environ Sci 1994; 56: 53-57.
20) Focht D D and Joseph H. An improved method for the enumeration of denitrifying bacteria. Soil Sci Soc Am Proc 1973; 37: 698-699.
21) Tiedje J M. Ecology of denitrification and dissimilatory nitrate reduction to ammonium, p. 179-244. In Zehnder A J B (ed.), Biology of anaerobic microorganisms, John Wiley and Sons, 1988 New York.
22) Smith M S and Zimmerman K. Nitrous oxide production by nondenitrifying soil nitrate reducers. Soil Sci Soc Am J 1981; 45: 865-871.
23) Ishida Y and Kadota H. Growth patterns and substrate requirements of naturally occurring obligate oligotrophs. Microbiol Ecol 1981; 7: 123-130.
24) Ohta H and Hattori T. Oligotrophic bacteria on organic debris and plant roots in a paddy field soil. Soil Biol Biochem 1983; 15: 1-8.
25) Ohta H and Taniguchi S. Growth characteristics of the soil oligotrophic bacterium: *Agromonas oligotrophica* JCM1494 on diluted nutrient broth. J Gen Appl Microbiol 1988; 34: 349-353.
26) Ishida Y and Kadota H. A new method for enumeration of oligotrophic bacteria in lake water. Arch Hydrobiol Beih Ergebn Limnol 1979; 12: 77-85.
27) Ishida Y, Shibahara K, Uchida H, and Kadota H. Distribution of obligately oligotrophic bacteria in lake Biwa. Bull Jpn Soc Sci Fish 1980; 46: 1151-1158.
28) Eguchi M and Ishida Y. Oligotrophic properties of heterotrophic bacteria and in situ heterotrophic activity in pelagic seawaters. FEMS Microbiol Ecol 1990; 73: 23-30.

大阪市立環科研報告　平成21年度　第72集,41～48 (2010)

# ガスクロマトグラフィーを用いた最確数法による脱窒細菌係数法の感度

西尾孝之、芳倉太郎、森　義明

**要旨**

脱窒細菌の最確数が経時的に変化する様子を、ガスクロマトグラフィーによる亜酸化窒素を検出することにより追跡し、倒置したDurham管底に集積する気泡を計数する従来法と比較した。ガスクロマトグラフィーによる最確数は従来法に比べて1-2桁高い計数値を与え、値が収束するのに要する培養時間も短くなった。貧栄養な脱窒細菌の存在を確かめるために、培地の有機物濃度を変えて検討した。有機汚染が少ない地点の試料は，希釈培地と高濃度培地で最確数にほとんど差が認められなかったが、廃棄物処分場の試料の最確数は希釈培地では極緩慢にしか増加せず、最終的な最確数は高濃度培地よりも低かった。

**キーワード**: 脱窒，アセチレン阻害法，ダラム管最確数法，亜酸化窒素，亜硝酸，硝酸，
海面埋立廃棄物処分場