Environ. Eng. Res. Vol. 9, No. 3, pp. 130~142, 2004
Korean Society of Environmental Engineers

ESTIMATION OF ACTIVE *NITROSOMONAS* AND *NITROBACTER* CONCENTRATIONS IN ACTIVATED SLUDGE USING NITROGENOUS OXYGEN UPTAKE RATE

Sun-Jip Kim †

Mirae G&C Co., Ltd, Junhyun Bldg. 4th FL, 492-16 Sinwol 2-dong, Yangcheon-gu, Seoul, 158-838, Korea

(received April 2004, accepted July 2004)

Abstract : The respirometric mass estimation technique (RMET) classified as an internal standardization method with dominant nitrifier (*Nitrosomonas* and *Nitrobacter*) measuring nitrogenous oxygen uptake rate was evaluated. The coefficients of variation of initial respirometric nitrogenous oxygen uptake rates were 2.6 % and 1.5 % for nitrifier and *Nitrobacter*. Ammonium-nitrogen and nitrite-nitrogen removals equivalent to oxygen uptake were 0.21 mg/mg and 0.61 mg/mg, respectively. The initial substrate to biomass ratio affects the measuring of nitrogenous oxygen uptake rate and requires to be maintained above 11.3 g/g. It is necessary to eliminate the carbon dioxide uptake by nitrifier causing under estimation of nitrifier mass. Washing the sample before initiation of mass estimation is also necessary to minimize soluble organics which causes an interaction between heterotrophs and autotrophs. The RMET as a reliable, rapid and convenient tool can be replaced the conventional water quality analysis method. The severe fluctuations of *Nitrosomonas* and *Nitrobacter* concentrations were observed according to the oxidation and reduction states in the chambers of the Bardenpho 4 stage system fed with grain distillery wastewater having high concentrations of organics and nitrogen. The *Nitrosomonas* concentrations were in the range of 45-193 mg/L through all chambers of the system while the *Nitrobacter* concentrations were 8-129 mg/L.

Key Words : respirometric mass estimation technique, nitrogenous oxygen uptake rate, nitrifier, nitrification, respirometry

INTRODUCTION

Nitrosomonas and *Nitrobacter* concentrations in mixed liquor are usually measured for evaluation of biological nitrogen removal system. Some researchers have studied on enumeration and mass estimation techniques for nitrifier (*Nitrosomonas* and *Nitrobacter*) in sample of activated sludge for several years. The enumeration and mass estimation techniques for nitrifier, which have been developed, are most probable number method, antibody method[1], INT (2-[p-iodophenyl]-5-phenyl tetrazolium chloride) dehydrogenase method[2], external standardization method with dominant nitrifier[3], and internal standardization method with dominant nitrifier.[4,5]

The "Most probable number method" takes about 30 to 40 days due to the long culture period of nitrifying bacteria. For the rapid and easy assay of nitrifier, the "Antibody method," one of the immunological methods, was developed by Yasuda *et al.*[1]. Using this method,

† Corresponding author
E-mail: sunjipkim@yahoo.co.kr
Tel: +82-2-2607-9244, Fax: +82-2-697-4177

they can count the nitrifying bacteria with latex particles sensitized by the antibody of nitrifying bacteria in 3-4 hours. The number of bacteria, possible to be detected, is $5.5x10^5$ cells/mL for the *Nitrosomonas* and $1.8x10^5$ cells/mL for the *Nitrobacter*. Although the antibody method can save time, it needs high cost for analysis. Using "INT method," nitrifier can be classified as active or inactive according to their ability to reduce 2-(p-iodophenyl)-5-phenyl tetrazolium chloride under specified conditions. Although, it takes 3-5 hours for analysis, the assay procedure is complicated. Srinath *et al.*[3] proposed a "Dominant culture equivalent method" for determining the mass of active nitrifier in sample of activated sludge. This method is carried out by simple comparison of the substrate-utilization rates of dominant nitrifier and the nitrifier contained in activated sludge. However, the growth rates between the dominant nitrifier and the nitrifier in sample of activated sludge would be different because of the difference of growth environments. Hall and Murphy[4] developed the mass estimation technique (MET) to measure the active nitrifier mass with consideration of the native environment of sample. They added a known amount of nitrifier dominant culture in the sample of activated sludge, and compared the resulting substrate utilization rates before and after addition. This method will be, however, accomplished by the assumption of that the difference of substrate-utilization rate depends thoroughly on dominant nitrifier added. Interactions between the dominant nitrifier and the nitrifier inherently existed in activated sludge would be occurred and affected the substrate utilization rate with only one addition of dominant nitrifier. To overcome this problem, Copp and Murphy[5] proposed an "*In situ* Mass Estimation Technique (*in situ* MET)". The *in situ* MET technique is the upgraded version of MET technique developed by Hall and Murphy.[4] This proposed technique allows the determination of the *Nitrosomonas* and *Nitrobacter* masses while maintaining the integrity of the native activated sludge environment. The integrity of the native environment can be maintained by dosing of different amount of dominant nitrifier at several reaction vessels in which the same amount of sludge sample is taken. Using this technique, Copp and Murphy[5] measured the nitrifier concentration of the sludge samples taken from Milton Wastewater Treatment Plant in Ontario, Canada. As the results, the active nitrifier concentrations of the sludge samples ranged from 11.5 mg/L to 33.1 mg/L, and estimated errors were 6-14% with 95% confidence limits. Although *in situ* MET technique has several advantages, it is hard to apply when a lot of sludge samples are to be measured because this technique consumes a lot of time and works for water quality analyses. Accordingly, respirometric mass estimation technique (RMET) that measures nitrogenous oxygen uptake rate was evaluated as a reliable, rapid, and simple measurement method.

THEORY

Among the mass estimation techniques developed by several researchers, *in situ* MET technique developed by Copp and Murphy[5] ensures the integrity of the native environment. The basic theory for RMET is derived from the *in situ* MET technique that spikes dominant nitrifier (*Nitrosomonas* and *Nitrobacter*) with different volume in several activated sludge samples and measures nitrification rates. The RMET technique also spikes dominant nitrifier in activated sludge samples and obtained nitrification rates by measuring oxygen uptake rates from nitrogen oxidation. When dominant nitrifier is spiked in the sludge sample containing a certain portion of native nitrifier, total nitrification rate by dominant nitrifier and native nitrifier can be described as follows:

$$-\frac{dN_a}{dt} = K_{PNF} \cdot X_{PNF} + K_{SNF} \cdot X_{SNF} \quad (1)$$

where

N_a : Ammonium-nitrogen concentration, mg/L

K_{PNF} : Specific nitrification rate for dominant nitrifier, g NH_4-N $_{removed}$/g VSS/day

X_{PNF} : Active nitrifier concentration in dominant culture, mg VSS/L

K_{SNF} : Specific nitrification rate for native nitrifier in sludge sample, g NH_4-N $_{removed}$/g VSS/day

X_{SNF} : Active nitrifier concentration in sludge sample, mg VSS/L

The native nitrifier concentration in activated sludge can be obtained by dividing the nitrification rate with the specific nitrification rate for native nitrifier in activated sludge when dominant nitrifier is not dosed (at X_{PNF}=0).

$$X_{SNF} = \frac{-\frac{dN_a}{dt}}{K_{SNF}} \tag{2}$$

When dominant nitrifier is spiked in samples of activated sludge, K_{PNF} is close to K_{SNF} in the native environment. The native nitrifier concentration in sample can be expressed as follows:

$$X_{SNF} = \frac{-\frac{dN_a}{dt}}{K_{PNF}} \tag{3}$$

When dominant *Nitrobacter* is dosed in the samples of activated sludge, active *Nitrobacter* concentration in sludge sample (X_{SNB}) also can be derived as follows:

$$X_{SNB} = \frac{-\frac{dN_n}{dt}}{K_{PNB}} \tag{4}$$

where

N_n : Nitrite-nitrogen concentration, mg/L

K_{PNB} : Specific nitrification rate for dominant *Nitrobacter*, g NO_2-N $_{removed}$/g VSS/day

On the other hand, nitrification rate of ammonium-nitrogen can be obtained by multiplying the coefficient of ammonium-nitrogen removal equivalent to oxygen uptake (Cf_a^{Nx}) in the measured nitrogenous oxygen uptake rate.

$$\frac{dN_a}{dt} = Cf_a^{Nx}\frac{dO_2}{dt} \tag{5}$$

If Eq. (5) is applied to Eq. (3), the native nitrifier concentration in the sample of activated sludge is:

$$X_{SNF} = \frac{-Cf_a^{Nx}\frac{dO_2}{dt}}{K_{PNF}} \tag{6}$$

Nitrobacter concentration also can be derived with the same procedure for nitrifier:

$$X_{SNB} = \frac{-Cf_n^{Nx}\frac{dO_2}{dt}}{K_{PNB}} \tag{7}$$

where

Cf_n^{Nx} : Nitrite-nitrogen removal equivalent to oxygen uptake, mg NO_2-N/L/mL O_2

The schematic diagram of the expected results from RMET is shown in Figure 1.

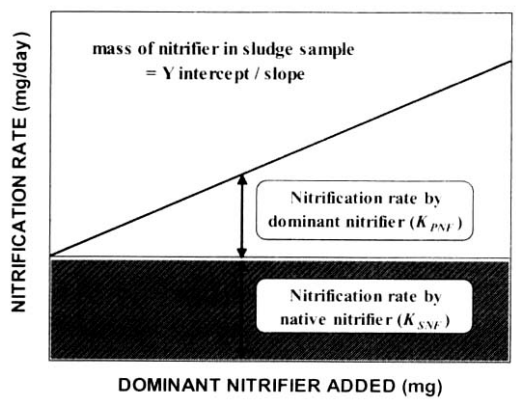

Figure 1. Schematic diagram of the expected results from respirometric mass estimation technique (RMET).

MATERIALS AND METHODS

Cultivation of Dominant Nitrifier

The chemostats for dominant nitrifier and *Nitrobacter*, which were seeded with the effluent of the laboratory-scale biological nitrogen removal system acclimated to grain distillery wastewater, were cultivated in a semi-batch mode. Two glass erlenmeyer flasks (effective volume of 3 liters), which had magnetic stirrer bars and ceramic diffusers with fine pores were installed. Air was saturated with moisture before it was supplied to minimize the evaporation of culture mixtures. Dissolved oxygen concentration was maintained at a near saturation level at both cultures. Temperature was controlled within 1°C of the set point, 20°C, in an incubator. As summarized in Table 1, the growth media of Hall and Murphy[4] was introduced to the cultures with the flowrate of 150 mL/day. Before withdrawing of the mixed liquor the evaporated portion of mixed liquor was made up with distilled water. By hydraulic control, the sludge age of the dominant cultures was maintained at 20 days. The substrate concentration fed to the cultures was chosen such that sufficient nitrifier and *Nitrobacter* biomass for mass estimation could be wasted daily while maintaining the standing crop in the cultures. The ammonium-nitrogen concentration of nitrifier growth media was 1,026 mg/L, and the nitrite-nitrogen concentration of *Nitrobacter* growth media was 3,620 mg/L. DO and pH in the mixed liquor were measured daily. Effluent NH_4-N, NO_2-N and NO_3-N for nitrifier culture and NO_2-N and NO_3-N for *Nitrobacter* culture were analyzed, intermittently. The mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), and specific nitrification rate were also analyzed at both cultures.

Table 1. Constituents of the growth media for nitrifier and *Nitrobacter*[4]

	Nitrifier[(1)]	*Nitrobacter*[(1)]
K_2HPO_4	3.28 g	5.0 g
1 M $MgSO_4$	0.6 mL	0.09 mL
1 M $CaCl_2$	0.16 mL	0.09 mL
30 mM $FeSO_4$/50 mM EDTA	0.26 mL	0.33 mL
10 mM $CuSO_4$	0.04 mL	0.003 mL
KH_2PO_4	54.48 g	0.196 g
$NaHPO_4$	6 g	-
4.1 mM $MnSO_4$	-	0.1 mL
2.4 mM $ZnSO_4$	-	0.1 mL
0.003 mM $(NH_4)_6Mo_7O_{24}$	-	0.1 mL
NaCl	-	3 g
$(NH_4)_2SO_4$	4.72 g	-
$NaNO_2$	-	19.71 g

(1) Make up 1L with distilled water, and control pH to 8.0

Installation and Operation of Biological Nitrogen Removal System

The sludge sample containing nitrifier is required for evaluation of RMET. The plexiglass Bardenpho 4 stage system [pre-anoxic → (first and second) pre-oxic → post-anoxic → post-oxic → settle] having 24 L of effective volume was operated. The HRT of the pre-anoxic, post-anoxic, and post-oxic chambers were 1 day, while the HRT was 2 days and 1 day in the first and second pre-oxic chambers. Diffuser and bubbler were installed at the oxic chambers. Paddle-type mixers were installed at the anoxic chambers. The floated effluent of the anaerobic process having high concentration of organics and nitrogen in the wastewater treatment plant of P distillery factory was selected as a feed. Although the grain distillery wastewater has the typical characteristics of high concentration of organics, nitrogen, and phosphorus, it is tinged with dark brown (true color) and contains a number of solids. The feed with the flowrate of 3,784 mL/day was supplied continuously. As an external carbon source, the raw distillery wastewater, the centrifugal decanter effluent (CDE), was dosed with the flowrate of 216 mL/day in the pre-anoxic chamber. The impure alcohol produced during the alcohol distillation process was selected as another external carbon source for the post-anoxic chamber. The impure alcohol was dosed once a day by manual because of low dosing rate of 1.0 mL/day. The sludge-recycle

Table 2. Characteristics of the feed and the external carbon sources

Parameters	Feed[1]	CDE[2]	Impure alcohol
pH	7.0-8.3	4.4	11.9
Alkalinity, mg $_{CaCO_3}$/L	1,880-3,400 (2,610)	-	2,340
BOD_5, mg/L	390-1,980 (976)	17,500	630,000
TCOD, mg/L	1,947-3,832 (2,940)	31,488	122,900
SCOD, mg/L	720-2,778 (1,831)	18,423	117,800
SS, mg/L	700-2,167 (1,110)	6,280	235
NH_4-N, mg/L	165-796 (504)	62.5	11.9
TKN, mg/L	378-1,319 (689)	559.9	115.9

[1] The floated effluent of the anaerobic process fed with the grain distillery wastewater

[2] After screening with 850 μm sieve.

and mixed-liquor-recirculation rates were 200% and 300% of the influent flowrate. The feed sampled semimonthly, CDE, and impure alcohol were stored in a refrigerator maintained at 4°C. The typical characteristics of the feed and external carbon sources were listed in Table 2. The feed concentration was severely fluctuated according to the raw materials used for alcohol production.

Analytical Methods

The nitrification rate for estimation of nitrifier concentration was measured by respirometry. The respirometer manufactured by Challenge Environmental System, Inc. (AER-200) was used. The specifications of the respirometer were as follows: minimum flowrate detection <0.08 mg, maximum flow capacity 600 mg/hr, calibration precision <1% coefficient of variation (Cv), sensitivity <0.08 mg, and measurement precision <3% Cv. To increase the measuring precision, the flow cells and reaction vessels were maintained at a constant temperature of 20°C in an incubator. Highly purified oxygen (about 99.9%) was supplied for respiration of nitrifier. The water quality analyses followed the procedures in the Standard Methods for the Examination of Water and Wastewater.[6]

RESULTS AND DISCUSSION

Performances of Dominant Nitrifier and *Nitrobacter* Cultures

Dominant nitrifier and *Nitrobacter* (typical nitrifying organisms oxidizing nitrite to nitrate assumed in this study) cultures were cultivated for establishment of respirometric mass estimation technique (RMET) for nitrifier and *Nitrobacter*. At a steady state of the nitrifier culture, the MLSS and MLVSS were 95-99 mg/L and 91-93 mg/L, respectively while pH was maintained at 7.5-7.7. The effluent NH4-N and NO_3-N concentration were 1.4-6.4 mg/L (average 2.6 mg/L) and 1,009-1,026 mg/L (average 1,021 mg/L) while NO_2-N could not be detected during the operation. The nitrification efficiency was 99.4-99.8% (average 99.8%). The specific nitrification rate measured by respirometry is 2.62 g NH_4-$N_{removed}$/g nitrifier/day on the average, smaller than 4.2 g/g/day at 20°C obtained by Copp and Murphy.[5] However, the rate is higher when compared with 1.7 g/g/day at 25°C reported by Hanaki *et al.*.[7] At a steady state of the *Nitrobacter* culture, the MLSS and MLVSS were 62-75 mg/L and 59-66 mg/L, respectively while pH was maintained at 7.8-8.1. The effluent NO_3-N concentration was in the range of 3,476-3,620 mg/L (average 3,572 mg/L). The effluent NO_2-N could not be detected during the operation. The complete nitrification was occurred and the specific nitrification rate measured by respirometry was 3.69 g NO_2-$N_{removed}$/g *Nitrobacter*/day on the average.

Respirometric Nitrification Rate

The reliable, rapid, and convenient respirometric nitrification rate measurement technique is required to estimate the nitrifier mass in biological nitrogen removal system. The nitrification rate measurement technique using nitrogenous oxygen uptake rate by respirometry was evaluated in this study. The reproducibilities of the initial oxygen uptake rate by nitrifier and *Nitrobacter* are shown in Table 3. The same volume of dominant nitrifier, ammonium-nitrogen, and nitrifier growth media were introduced in three reaction vessels. Accumulated oxygen volume consumed during the nitrification reaction was measured according to the elapsed time by respirometry. The initial oxygen uptake rate by nitrifier is 0.397 mL/hr on an average, and the coefficient of variation is 2.6%. When a similar procedure is conducted using dominant *Nitrobacter*, nitrite-nitrogen and *Nitrobacter* growth media, the initial oxygen uptake rate by *Nitrobacter* is 0.147 mL/hr on an average, and the coefficient of variation is 1.5%.

Table 3. Reproducibility of initial oxygen uptake rate

	Nitrifier	*Nitrobacter*
dO_2/dt (mL/hr)	0.397[(1)]	0.147[(1)]
Coefficient of variation (%)	2.6	1.5

-Respirometry test at 20℃
(1) average value

When nitrification rate is measured by respirometry, the amount of oxygen uptake is needed to be converted to the value of ammonium- or nitrite-nitrogen removed. Ammonium-nitrogen was dosed with different volume in six vessels in which the same amount of dominant nitrifier was seeded. Maximum oxygen uptake volume was measured by respirometry, when DO, pH, and reaction temperature were not rate-limiting factors. The reciprocal of the slopes from Figure 2 is the ammonium-nitrogen removal by nitrifier equivalent to oxygen uptake (Cf_a^{Nx}), 0.27 mg NH_4-$N_{removed}$/mL $O_{2\ uptake}$ (0.21 mg NH_4-$N_{removed}$/mg $O_{2\ uptake}$) similar with the theoretical value of 0.22 mg NH_4-$N_{removed}$/mg $O_{2\ uptake}$. The nitrite-nitrogen removal by *Nitrobacter* equivalent to oxygen uptake (Cf_n^{Nx}), the reciprocal of the slopes is 0.81 mg NO_2-$N_{removed}$/mL $O_{2\ uptake}$ (0.61 mg NO_2-$N_{removed}$/mg $O_{2\ uptake}$). This value is lower than the theoretical value of 0.88 mg NO_2-$N_{removed}$/mg $O_{2\ uptake}$.

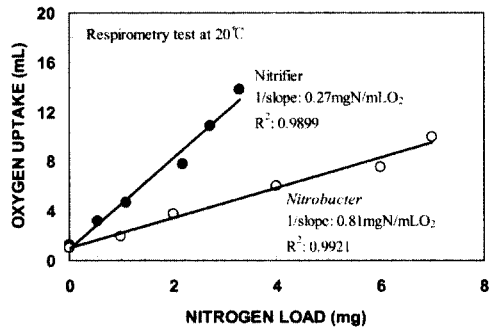

Figure 2. Oxygen uptake equivalent to nitrogen removal.

Initial substrate to biomass ratio (S_0/X_0) in a batch test will affect the values of the kinetic parameters.[8,9] If the S_0/X_0 ratio is high enough, the biomass will adjust it's internal constituents to the level of optimum for unrestricted growth, thereby making the measured kinetic parameters independent of the growth history of the biomass before testing is necessary. Kinetics determined under this condition are termed intrinsic because they represent the inherent maximal activity of the biomass at a given temperature.[10] The initial ammonium-nitrogen or nitrite-nitrogen concentration is required to be maintained above limiting concentration when sample is prepared for measuring of nitrification rate. Figure 3 shows the variation of nitrification rates according to the increasing of initial ammonium-nitrogen/nitrifier ratio and nitrite-nitrogen/ *Nitrobacter* ratio. As the results, initial S_0/X_0 ratios should be above 11.3 g/g for measuring of nitrification rate by nitrifiers and *Nitrobacter*.

Autotrophs use inorganic carbon (carbon dioxide) for cell synthesis rather than organic carbon. When nitrification rate is measured by respirometry the amount of oxygen consumed

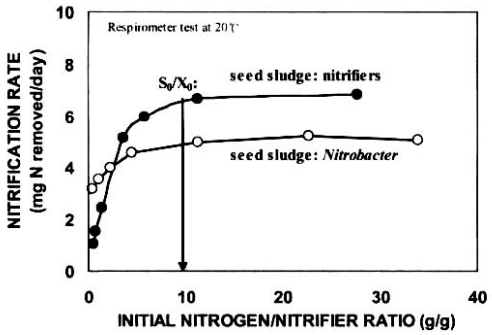

Figure 3. Variation of nitrification rate according to initial NH_4-N/nitrifier ratio and NO_2-N /*Nitrobacter* ratio.

for nitrogen oxidation includes carbon dioxide uptake for cell synthesis. Therefore, oxygen uptake rate should be measured in the absence of carbon dioxide. When 1.0 mol of ammonium and nitrite are oxidized 0.081 mol and 0.031 mol of carbon dioxide are consumed for cell synthesis by nitrifier and *Nitrobacter*, theoretically. If the carbon dioxide presents in the headspace of vessel, nitrifier will uptake not only oxygen but also carbon dioxide causing error for measuring of nitrification rate. For correction of this problem, carbon dioxide, which presents in the headspace of vessel, should be removed immediately with carbon dioxide absorbent. A tube contained the tissue wet with 1.0 mL of 50% KOH as a carbon dioxide absorbent was placed in the headspace of the vessel as shown in Figure 4. Nitrification rates of nitrifier and *Nitrobacter* with and without KOH tube were compared. The initial oxygen uptake rate of 1.52 mL/hr by nitrifier measured without KOH tube was higher than the rate of 1.23 mL/hr measured with KOH tube. The rates by *Nitrobacter* were 0.42 mL/hr and 0.34 mL/hr when carbon dioxide was not absorbed and absorbed, respectively. As the result of this study, the KOH tube should be placed in the headspace of the reaction vessel when nitrification rate of nitrifier is measured.

In general, hetertrophs, autotrophs, particulate organic carbon, particulate organic nitrogen, inert solids, ammonium, nitrite, nitrate, soluble organic nitrogen, soluble organic carbon, etc. are in-

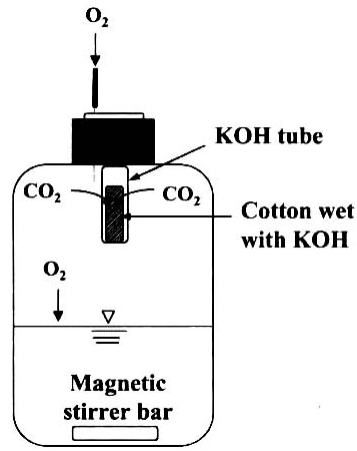

Figure 4. Reaction vessel with KOH tube.

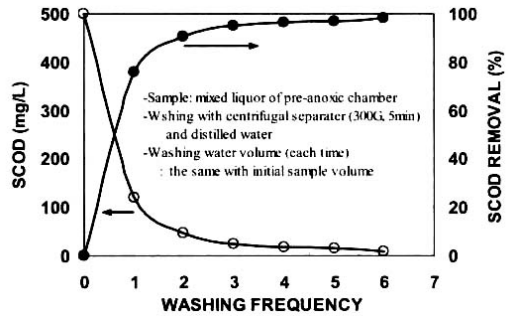

Figure 5. Variation of soluble organic concentration and removal efficiency in sample sludge according to the increasing the washing frequency.

cluded in mixed liquor of biological treatment system. Soluble compounds would be one of the factors affecting nitrification rate. If soluble organic carbon is included in sample sludge, heterotrophs will be activated and will uptake oxygen which cause error in respirometric nitrification rate data. As another possible phenomenon, activating of heterotrophs can lower nitrification rate by competition between nitrifier and heterotrophs. To remove soluble compounds, sample sludge was washed with distilled water in this study. Washing of sludge sample was conducted using centrifugal separator operated with 300 G (Gravity acceleration) for 5 minutes. Following Figure 5 shows that the soluble chemical oxygen demand (SCOD) decreases as the washing frequency increases. The sludge sample incorporated in this study was sampled

from the pre-anoxic chamber of the biological organics and nitrogen simultaneous removal system acclimated to distillery wastewater. At the pre-anoxic chamber, SCOD is the highest among the chambers in the system, and a substantial amount of nitrifier is contained. After centrifuging of sample sludge, the supernatant was decanted, and the settled sludge was resuspended with distilled water. After 6 times of washing, the SCOD of mixed liquor was only 10 mg/L compared with 499 mg/L before washing. Therefore, all of the sludge samples for mass estimation of nitrifier were washed 6 times with centrifugal separator.

The temperature difference between the prepared samples and incubator results in increased initial slope (overestimation of nitrification rate). Endogenous respiration during the incubation time may also lead wrong initial slope. When measured the nitrification rate by respirometry, a seed blank is required to be tested for correction of data.

Respirometric Nitrifier Mass Estimation

The proposed procedure for respirometric mass estimation of nitrifier and *Nitrobacter* based on the above study of the respirometric nitrification rate is shown in Figure 6. The same volume of washed sample is taken in the several reaction vessels. Dominant nitrifier or *Nitrobacter* is dosed into the vessels with different volume. After ammonium- or nitrite-nitrogen is dosed in each vessel, make up same volume with the growth media.[4] The dosing rate of ammonium- or nitrite-nitrogen is decided based on S_0/X_0 which is recommended to maintain higher than 11.3 g/g in previous test. The magnetic stirrer bars and KOH tubes are placed in the vessels. After put stoppers, mount the prepared vessels on the magnetic stirring plate installed in the incubator maintaining the set point of temperature. Insert the needle for supplying pure oxygen. Develop slightly negative pressure in the head-space of the reaction vessels with syringe and check whether oxygen bubbles easily pass through the cells (the device

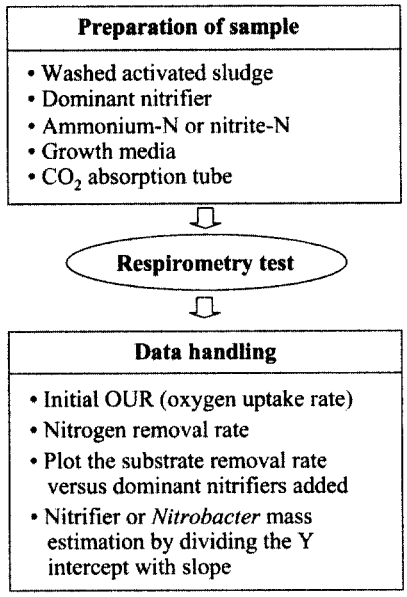

Figure 6. Proposed procedure for nitrifier mass estimation with RMET.

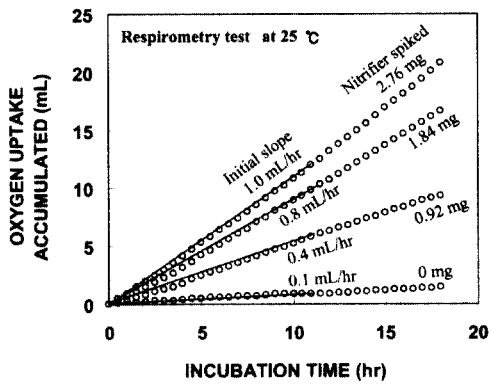

Figure 7. Typical oxygen uptake rate.

for counting of oxygen bubble) to the reaction vessels. Monitor the accumulated volume of oxygen consumed according to the elapsed time. Plot the accumulated volume of oxygen consumed versus elapsed time. Figure 7 shows the typical oxygen uptake rate measured by respirometry. Calculate initial slope (oxygen uptake rate). The initial slope, oxygen uptake rate is converted to substrate removal rate by multiplying of nitrogen removal rate equivalent to oxygen uptake (Cf_a^{Nx} or Cf_n^{Nx}). The initial nitrification rate of each vessel according to the dominant nitrifier dosed is plotted as shown in

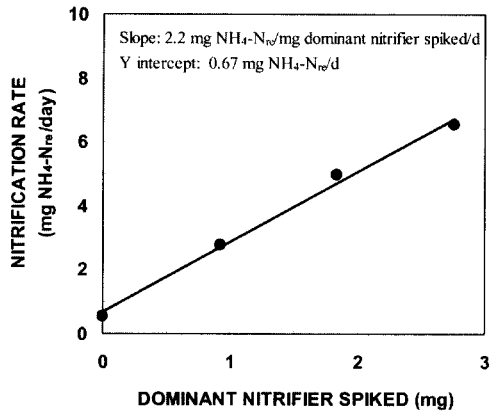

Figure 8. Nitrification rate according to the dominant nitrifier spiked.

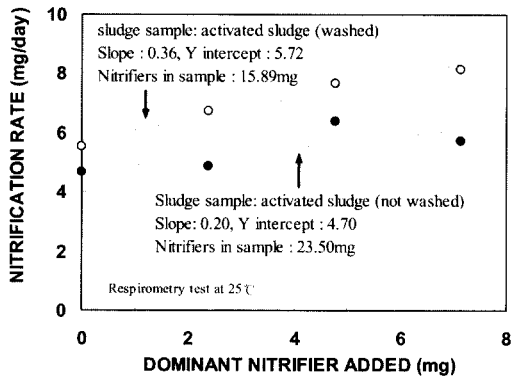

Figure 9. Effect of sludge washing on specific nitrification rate.

Figure 8. A mass of active nitrifier in sludge sample can be calculated by dividing Y-intercept with the slope of the best-fit line.

A test for the effect of sludge washing on the mass estimation of nitrifier was conducted as shown in Figure 9. The same volume of the sludge samples with and without washing was taken in different reaction vessels for the mass estimation. When sludge was washed the mass of nitrifier in the sludge sample was 15.89 mg, while the mass was 23.50 mg when the sludge was not washed. Over estimation of the nitrifier mass when sludge is not washed implies that there are interactions of soluble compounds and/or the competition between heterotrophs and autotrophs. The sludge washing is, therefore, a necessary step in sample preparation for nitrifier mass estimation by respirometry.

Table 4. Comparison of nitrification rates measured by respirometry and direct water quality analysis

	Nitrification rate	
	Respirometry	Direct water quality analysis[1]
Nitrifier (mg NH_4-N removed/hr)	0.43	0.44
Nitrobacter (mg NO_2-N removed/hr)	0.32	0.33

[1] measured by ion chromatography

The nitrification rates measured by respirometry and direct water quality analysis were compared in Table 4. The nitrification rates measured by respirometry using nitrogenous oxygen uptake rate and by direct water quality analysis using ion chromatography were almost same.

Active Nitrifier Concentrations in Biological Nitrogen Removal System

The Bardenpho 4 stage system fed with the grain distillery wastewater (floated effluent of anaerobic pretreatment) having high content of organics and nitrogen was operated with volumetric BOD_5 and COD loading rate of 0.35 g/L/day and 0.80 g/L/day. The water temperature of the system was in the range of 27-29°C. The MLSS ranged 3,978-4,567 mg/L was similar through all chambers of the system. The fraction of MLVSS against MLSS was 0.71-0.74. Relatively long SRT of 21-28 days was maintained. At the oxic and the anoxic chambers, DO concentrations were 2.0-3.5 mg/L and 0.2-0.4 mg/L. The DO concentration in the oxic chamber was maintained above the half saturation concentration of 0.15-2.0 mg/L for nitrification.[11] However, the DO concentration in the anoxic chamber was higher than the half saturation of 0.1 mg/L for denitrification.[11] The pH and alkalinity in the system were in the range of 7.93-8.59 and 1,240-1,580 mg as $CaCO_3$/L at all chambers. The F/M (SCOD/MLVSS) ratio in the pre-oxic chamber (com-

Table 5. Operation performances of the Bardenpho 4 stage system

Item	Influent (mg/L)	Effluent (mg/L)	Removal efficiency (%)
BOD_5	1,471	4.5~31.5 (13)	97.9~99.7 (99.1)
TCOD	5,171	368~594 (489)	88.5~92.9 (90.5)
SCOD	3,890	349~449 (423)	91.3~92.9 (90.5)
NH_4-N	217	3.3~7.9 (5.8)	-
Org-N	329	28.3~59.8 (43.3)	81.8~91.4 (86.8)
NO_2-N	ND	ND	-
NO_3-N	ND	20.5~33.2 (26.1)	-
TKN	546	36.2~63.7 (49.1)	88.3~93.4 (91)
TN	546	56.7~92.6 (75.2)	83.1~89.6 (86.2)

(1) The mixture of the feed and the CDE (dosing ratio was 5.7 % v/v of the feed flowrate)
Note: average values are given in parentheses.

bined first and second pre-oxic chambers) was 0.27-0.28 day^{-1} (average 0.28 day^{-1}).

The operation results of the Bardenpho 4 stage system are summarized in Table 5. The biodegradable organics in the feed is removed with high efficiency. The SCOD/BOD_5 (Biochemical Oxygen Demand) ratio in the effluent is very high as much as 32.5. It implies that the considerable amount of hard-to-biodegradable organics is remained in the effluent. The hard-to-biodegradable COD is associated with color causing pigments. Alkaline pH during processing and high temperatures used for crystallization of sugars lead to the formation of caramels, melanoidins, and a variety of sugar composition products, which in turn, polymerize to form yellowish color.[12] In view of the SCOD/TCOD (Total Chemical Oxygen Demand) ratio of 0.87 in the effluent, the most of hard-to-biodegradable organics is soluble. The nitrogen removal efficiency of the Bardenpho 4 stage system is also high. The nitrification and denitrification efficiencies are 88.3-94.3% (average 92.3%) and 76.1-96% (average 90.1%), respectively. Although high nitrification and denitrification efficiencies are observed, considerable portion of hard-to-biodegradable organic-nitrogen is remained in the effluent. The major species of nitrogen in the grain distillery wastewater are organic-nitrogen and ammonium-nitrogen. A certain portion of organic-nitrogen is difficult to degrade biologically. The volumetric

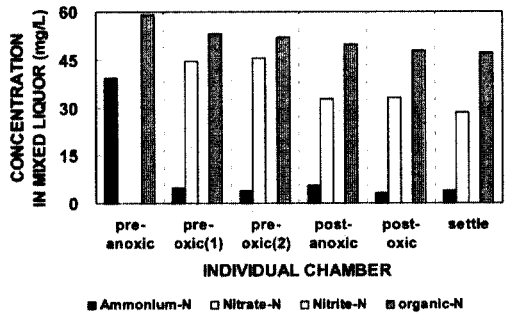

Figure 10. Behavior of nitrogen in the individual chambers of Bardenpho system.

BOD_5 and TCOD removal rates are 235-240 $g/m^3/day$ (average 238 $g/m^3/day$) and 748-785 $g/m^3/day$ (765 $g/m^3/day$) in the system. The volumetric total Kjeldahl nitrogen (TKN) removal rate is in the range of 122-206 $g/m^3/day$ (average 164 $g/m^3/day$). The behavior of nitrogen species in the individual chambers of Bardenpho 4 stage system was presented in Figure 10. The ammonium-nitrogen and organic-nitrogen concentrations from feed are diluted with the nitrogenous mixed liquor recirculated from the second pre-oxic chamber. The mixed liquor having highest ammonium-nitrogen concentration in the pre-anoxic chamber flows to the first pre-oxic chamber. The highest ammonium-nitrogen removal efficiency of 92.6% was observed in the first pre-oxic chamber. The nitrate-nitrogen recirculated from the second pre-oxic chamber was completely denitrified in the pre-anoxic chamber. The nitrate-nitrogen

Table 6. Estimated concentrations of Nitrosomonas, Nitrobacter, and denitrifier in the individual chambers of the Bardenpho 4 stage system

Microorganisms	Pre-anoxic	First pre-oxic	Second pre-oxic	Post-anoxic	Post-oxic
MLVSS (mg/L)	3,500	3,278	3,039	3,533	3,301
Nitrosomonas (mg/L)	128	161	45	138	193
Nitrobacter (mg/L)	86	56	129	64	8
Nitrifier (mg/L)	214	217	174	202	201
Denitrifier (mg/L)	<3,286	<3,061	<2,865	<3,332	<3,101
Nitrifier (%)	6.1	6.6	5.7	5.7	6.1
Denitrifier (%)	93.9	93.4	94.3	94.3	93.9

- Volumetric nitrification rate in oxic chambers (mg TKN removed/L/day): 122-206 (average 164).
- Volumetric denitrification rate in anoxic chambers (mg NOx-N removed/L/day): 180-259 (average 227).

concentration is greatly increased in the first pre-oxic chamber by nitrification. The nitrite-nitrogen was not detected in all chambers. The organic-nitrogen was greatly oxidized in the first pre-oxic chamber and gradually decreased in the following chambers.

An experimental analysis of active nitrifier content provides a rational basis for developing and controlling a desired condition to maximize nitrification process performance. Active nitrifier and *Nitrobacter* concentrations in the Bardenpho 4 stage system were estimated by respirometric mass estimation technique (RMET). The C/N ratio based on the sum of BOD_5 of the feed and the external carbon source dosed was in the range of 2.7-2.9. The active *Nitrosomonas* (typical nitrifying organisms oxidizing ammonium to nitrite assumed in this study) and *Nitrobacter* concentrations in the chambers of the Bardenpho 4 stage systems are shown in Table 6. The active *Nitrosomonas* concentration can be obtained by subtraction of the active *Nitrobacter* concentration from the active nitrifier concentration. The active *Nitrosomonas* and *Nitrobacter* concentrations are severely fluctuated according to the oxidation-reduction states in the chambers. In the first pre-oxic chamber, *Nitrosomonas* concentration is higher than the second pre-oxic chamber where relatively ammonium-nitrogen concentration is lower. By proceeding nitrification reaction, ammonium-nitrogen is oxidized. However, complete nitrification was not achieved in the first pre-oxic chamber. Therefore, considerable nitrite-nitrogen flows to next second pre-oxic chamber. It results that *Nitrobacter* concentration is higher in the second pre-oxic chamber. Considerable concentrations of nitrifier are also observed in the anoxic chambers. Using *in situ* MET technique, Copp and Murphy[5] estimated active nitrifier concentration in the activated sludge samples from the Milton municipal wastewater treatment facility in Milton, Ontario, Canada. The active *Nitrosomonas* and *Nitrobacter* concentrations in activated sludge were 8.3-23.7 mg/L and 3.2-9.4 mg/L, respectively. For all chambers of the Bardenpho 4 stage system, the active nitrifier concentration is in the range of 174-217 mg/L. The active nitrifier content (based on MLVSS) of 5.7-6.6% in the oxic chambers is higher than the range of 2-5% reported by Stensel and Barnard[13] when municipal wastewater is fed and is similar with 8.3% suggested by Metcalf and Eddy Inc.[14] at the C/N ratio of 3.0. Yasuda and Yagishita[1] determined the number of nitrifying bacteria in the aeration basin of sewage treatment plant using "Antibody method". The populations of nitrite bacteria (oxidizing ammonium to nitrite) and nitrate bacteria (oxidizing nitrite to nitrate) were found in the range of 10^7-10^9 cells/mL and 10^5-10^7 cells/mL. Due to the higher content of ammonium-nitrogen in sewage rather than nitrite-

nitrgen, the higher population of nitrite bacteria than nitrate bacteria was observed. It is supported by the result of this study. The *Nitrosomonas* concentration was 2.9 times higher than *Nitrobacter* concentration in the first pre-oxic chamber where highest ammonium- nitrogen removal efficiency was observed. The specific oxygen uptake rates (SOUR) in the first and second pre-oxic chambers were 77.6 g/kg/day and 50.9 g/kg/day based on MLVSS. The SOUR in the oxic chambers were compared with the specific nitrification rates (SNR) based on mass balance in the system and respirometry test at different nitrifier content of mixed liquor as shown in Figure 11. At higher nitrifier content in the first pre-oxic chamber, higher SOUR and SNR could be observed.

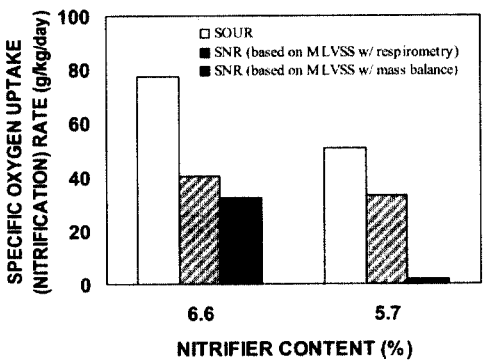

Figure 11. Specific oxygen uptake rate (SOUR) and specific nitrification rate (SNR) at different nitrifier contents in the oxic chambers of Bardenpho system.

There are hterotrophs, autotrophs, and volatile suspended solids in MLVSS. If assumed that all of heterotrophs take part in denitrification reaction and the content of organic suspended solids can be ignored, the relative concentration of denitrifier can be estimated by subtraction of active nitrifier concentration obtained by RMET from total MLVSS. There would be relative relation between the denitrifier concentration estimated in this study and the actual denitrifier concentration. Denitrifier content is similar at the range of 93.4-94.3% of MLVSS through all chambers of the Bardenpho system as shown in Table 6.

Due to installation of the pre-anoxic chamber functioned as a selector, relatively, good settling characteristic of the activated sludge was observed with the sludge volume index (SVI) of 27.3 mL/g measured in the mixed liquor of the post-oxic chamber. Daigger and Nicholson[15] reported that non-bulking activated sludge was produced with anaerobic or anoxic selectors.

CONCLUSIONS

Active *Nitrosomonas* and *Nitrobacter* concentrations in activated sludge were measured by respirometry using nitrogenous oxygen uptake rate. The various affecting factors such as initial substrate to biomass ratio, carbon dioxide uptake by biomass, and soluble organics in sample for measurement of respirometric nitrification rate were investigated. Based on the study on the respirometric nitrification rate measurement technique, the procedure of respirometric mass estimation technique (RMET) for nitrifier was proposed. The RMET was also evaluated using the activated sludge from the Bardenpho 4 stage system fed with the grain distillery wastewater having high concentration of organics and nitrogen.

Relatively high reproducibility of the respirometric nitrogenous oxygen uptake rate was observed. Initial substrate to biomass ratio should be maintained above 11.3 g/g. It is suggested that the carbon dioxide in the headspace of the reaction vessel is to be removed by installation of the carbon dioxide absorption tube (KOH tube) when nitrogenous oxygen uptake is measured by respirometry. Washing the sample before initiation of the biomass estimation is also necessary to minimize soluble organics causing error. As a reliable, rapid, and convenient tool, the respirometric mass estimation technique (RMET) can be replaced the conventional water quality analysis method.

When the RMET is employed, the estimated active *Nitrosomonas* and *Nitrobacter* concentra-

tions are severely fluctuated according to the oxidation-reduction states in the chambers of the Bardenpho 4 stage system acclimated to the grain distillery wastewater. The *Nitrosomonas* concentrations are at the range of 45-193 mg/L through all chambers of the Bardenpho 4 stage system while the *Nitrobacter* concentrations are 8-129 mg/L, respectively.

REFERENCES

1. Yasuda, T. and Yagishita, M., "Development of the determination for nitrifying bacteria with antibody method." *in Poster Symp. 69th Wat. Env. Fed. Tech.*, Dallas, Texas (1996).
2. Okabe, S. O., Hirata, Y. K., and Watanabe, Y., "Relationship between population dynamics of nitrifiers in biofilms and reactor perfirmance at various C:N ratios," *Water Res.*, **30**(7), 1563-1572 (1996).
3. Srinath, E. G., Loehr, R. C., and Prakasam, T. B. S., "Nitrifying organism concentration and activity," *J. Env. Eng. Div. American Soc. Civ. Engr.*, **102**(EE2), 449-463 (1976).
4. Hall, E. R. and MurphyK. L., "Estimation of nitrifying biomass and kinetics in wastewater," *Water Res.*, **14**(4), 297-304 (1980).
5. Copp, J. B. and Murphy, K. L., "Estimation of the active nitrifying biomass in activated sludge," *Water Res.*, **29**(8), 1855-1862 (1995).
6. American Public Health Association, American Water Works Association, and Water Environment Federation, *Standard Methods for the Examination of Water and Watewater*, 18th ed., Greenberg, A. E., Clesceri, L.S. and Eaton, A. D. (Eds), APHA, Washington, D.C. pp. 1.1-10.37 (1992).
7. Hanaki, K., Wantawin, C., and Ohgaki, S., "Effects of the activity of heterotrophs on nitrification in a suspended-growth reactor," *Water Res.*, **24**(3), 289-296 (1990).
8. Buittron, G. and Capdeville, B., "Uptake rate and mineralization of hexadecane and naphthalene by a mixed aerobic culture," *Water Res.*, **27**(5), 847-853 (1993).
9. Chudoba, P., Capdeville, B., and Chudoba, J., "Explanation of biological meaning of the S_0/X_0 ratio in batch cultivation," *Water Sci. Technol.*, **26**(3-4), 743-751 (1992).
10. Grady, C. P. L. Jr., Smets, B. F., and Barbeau, P. S., "Variability in kinetic parameter estimates : a review of possible causes and a proposed terminology," *Water Res.*, **30**(3), 742-748 (1996).
11. U.S. EPA, *Nitrogen Control*, EPA/625/R-93/010, U.S. EPA, Washington, D.C., pp. 87-110 (1993).
12. Rao, A. V. S. P., Karthikeyan, J., and Iyengar, L., "Treatment of distillery wastewater (spent wash) - Indian experience," in *Proc. 44th Ind. Waste Conf.*, Purdue University, West Lafayette, pp. 787-794 (1989).
13. Stensel, H. D. and Barnard, C. W., "Principles of biological nutrient removal," *Design and Retrofit of Wastewater Treatment Plants for Biological Nutrient Removal*, Randall, C. W., Barnard, J. L., and Stensel, H. D. (Eds.), Technic Publishing Co., Inc., Lancaster, Pennsylvania, pp. 25-84 (1992).
14. McCarty, P. L., Beck, L., and St. Amant, P., "Biological denitrification of wastewaters by addition of organic materials," *Proceedings of the 24th Industrial Waste Conference*, Purdue University, Lafayette, Indiana, pp. 1271-1285 (1969).
15. Daigger, G. T., and Nicholson, G. A., "Performance of full-scale nitrifying wastewater treatment plants incorporating selectors," *J. WPCF*, **62**(5), 676-683 (1990).