1 **Partial nitrification in a continuous pre-denitrification submerged membrane**
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6 **bioreactor and its nitrifying bacterial activity and community dynamics**
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Abstract

In this study, nitrogen removal by partial nitrification via nitrite was completely achieved for >40 days in a continuous submerged membrane bioreactor configured with both a pre-denitrification (10 L) and a nitrification tank (20 L). The possible aeration saving achieved with this configuration was 29% compared to that required for nitrogen removal via nitrate. Partial nitrification in the submerged membrane bioreactor was achieved by controlling the influent ammonium concentration (161 mgN/L) and the dissolved oxygen concentration in the nitrification tank (0.6 mg/L). Measurement of oxygen uptake rates during batch experiments confirmed that increasing influent ammonium concentration (161 mgN/L) decreased nitrite oxidizing activity of the biomass to almost zero. Furthermore, real-time PCR assays revealed that increasing influent ammonium concentration led to a decrease not of *Nitrobacter* but *Nitrospira* in the submerged membrane bioreactor. These results suggest that partial nitrification can be achieved by inhibiting the activity and growth of *Nitrospira* spp. with high ammonium concentration.

Keywords:

Activated sludge; Membrane bioreactor (MBR); Nitrifying bacteria; Nitrogen removal; Oxygen uptake rate (OUR); Wastewater treatment

1. Introduction

The necessity for nitrogen (N) removal to protect closed water systems like lakes and inner bays from eutrophication has been a long recognized need in wastewater treatment [1]. N removal from wastewater is generally achieved by biological nitrification and denitrification in combination. Nitrification is an aerobic process where ammonia (NH_3) is oxidized to nitrite (NO_2^-) and nitrate (NO_3^-) [2], and is performed in two sequential steps by two phylogenetically different groups of bacteria; the autotrophic ammonia-oxidizing bacteria (AOB) and the nitrite-oxidizing bacteria (NOB) [3]. It is now clear that ammonia-oxidizing *Archaea* (AOA) may also carry out NH_3 oxidation in wastewater treatment plants [4]. On the other hand, denitrification is an anoxic process where NO_3^- and NO_2^- are reduced to N_2 gas via nitric oxide (NO) and nitrous oxide (N_2O) by anaerobically respiring chemoorganoheterotrophic bacteria, and thus requires an organic carbon source [5].

Several novel processes have been developed to enhance the economic attractiveness of nitrification and denitrification by inhibiting NO_2^- oxidation to NO_3^- which is a technically feasible process [6]. The oxygen requirements during the aerobic nitrification phase involving NO_2^- are theoretically 25% lower than those when NO_3^- is produced, while the carbon requirements for subsequent denitrification are 40% less

[7–10]. Many methods have been reported to inhibit NO_2^- oxidation to NO_3^- where the AOB accumulate and NOB are washed out of the reactor, based on their different activation energies, growth rates, differences in their dissolved oxygen (DO) half-saturation coefficients, and anti-toxic tolerances [11]. To exploit these, reactor temperature [12], pH [13], dissolved oxygen (DO) levels [14], sludge retention times (SRT) [15], substrate concentrations and loads [16], operational and aeration patterns [17] and addition of inhibitors [18] have been manipulated.

Membrane bioreactors (MBR) have been increasingly widely used in wastewater treatment to minimize solid phase –liquid phase separation problems often encountered in conventional activated sludge processes [19]. MBR systems also have the advantages of operating at high mixed liquid suspended solids (MLSS) concentrations, generating a lower excess sludge production, and the treated water can be reused [20]. In addition, biological nutrient removal (BNR) processes with MBR can be attractive [21–23] because the space occupied is greatly reduced because of the absence of settling tanks [19].

So far, nitrification/denitrification via NO_2^- has been examined in several MBR systems such as sidestream MBR [24], sequencing batch SBR [25] to save the aeration costs. However, the examination has not been reported in a widely used MBR

system (i.e. continuous pre-denitrification SMBR system). In this study, N removal via NO_2^- was completely achieved in the continuous pre-denitrification SMBR by controlling the influent ammonium concentration and the dissolved oxygen concentration in the nitrification tank. Thus, the authors report the operational conditions to attain the N removal via NO_2^- and also its aeration saving effect. Furthermore, the authors report the reason why the control of influent ammonium concentration could achieve partial nitrification by analyzing the activity and community of nitrifying bacteria in the SMBR.

2. Materials and Methods

2.1. Experimental set-up

A SMBR reactor was configured with a working volume of a 10 L (radius = 80 mm) pre-denitrification tank and 20 L ($L \times W \times H = 0.4 \text{ m} \times 0.1 \text{ m} \times 0.5 \text{ m}$) nitrification tank (Fig. 1). Those two tanks were connected at the bottom. The membrane module (Mitsubishi Rayon, Tokyo, Japan) consisted of polyethylene (PE) hollow-fiber membranes with an effective filtration area of 0.2 m^2 and pore size of $0.4 \mu\text{m}$. It was fully immersed in the nitrification tank. The air for washing the membrane surface was supplied continuously at 10 L/min from an aeration pipe located directly below the

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3 87 membrane module, while that supplied to the biomass was supplied intermittently from
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6 88 a diffuser located at the bottom of the nitrification tank at 5 L/min. The net volume of
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9 89 supplied air for membrane and biomass was monitored with a flow instrument
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12 90 (CMS0020 / 0050, Yamatake Corporation, Tokyo, Japan). The filtrate was obtained with
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15 91 a roller pump with a suction mode of 12-min-on and 3-min-off. Constant flux (0.234
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18 92 m/d) was maintained by frequent adjustment of the pump rotation rate, and the
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24 94 times that of effluent flow. The trans-membrane pressure (TMP) was monitored to
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27 95 indicate the extent of fouling with a pressure gage (AP-51A, Keyence, Osaka, Japan).
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30 96 The membrane module was washed with 2000 mg/L of sodium hypochlorite for 2 h or
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33 97 changed for a new module when the TMP reached 30 kPa, at which value it was
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36 98 difficult to maintain flux at constant level.
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41 99 Two concentrated synthetic wastewaters (SWW(A) and SWW(B)) were
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44 100 prepared, and each contained the following (g/L) respectively: SWW(A): CH₃COONa,
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47 101 12.7; Bacto Pepton, 9.53; Bacto Yeast Extract, 1.56; CaCl₂·2H₂O, 3.17; and a trace
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50 102 element solution (4.7 ml) prepared as previously reported [26]; SWW(B): NH₄Cl, 7.45;
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53 103 KH₂PO₄, 0.429; Na₂HPO₄·12H₂O, 1.02; MgSO₄·7H₂O, 3.52; MgCl₂·6H₂O, 6.25. Note
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56 104 that concentrations of NH₄Cl and CH₃COONa were varied from 0-10.9 g/L and
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8.00-12.7 g/L respectively to meet the operating requirements as shown below. The concentrated SWWs were made up with milliQ water and sterilized by autoclaving. The SWW(A) and SWW(B) were dosed at constant flow 1.54 L/d and 3.08 L/d respectively into the pre-denitrification tank with a peristaltic pump. A level sensor, connected to a roller pump to provide tap water, was used to maintain a constant water level. The SWW(A) and SWW(B) were diluted with the same tap water.

Inoculum was obtained from an anaerobic-anoxic-aerobic (A₂O) biological nutrient removal process treating municipal wastewater in Yokohama, (Japan). Influent contained 9.7 mg N/L of ammonium (NH₄⁺) and 0.4 mg N/L of NO₃⁻. Treated effluent contained 0.2 mg N/L of NH₄⁺ and 3.5 mg N/L of NO₃⁻. No NO₂⁻ was detected in either the influent or effluent. Sludge retention time (SRT) and hydraulic retention time (HRT) were controlled at 15 d and 6.7 h respectively.

2.2. Operating conditions and analytical methods

The MBR was operated for 194 days in a water bath at 25°C, and the operation divided into four runs. The concentrations of NH₄⁺ (mg N/L) in the SWW(B) diluted with tap water and DO (mg/L) in the nitrification tank were changed for each run as follows: Run 0 (days 0-63), NH₄⁺ 0-249, DO 0.35-0.65; Run 1 (days 64-94), NH₄⁺ 107, DO 2.0; Run 2 (days 95-122), NH₄⁺ 107, DO 0.6; Run 3 (days 123-194), NH₄⁺ 161, DO

0.6. Also the concentration of acetate (CH_3COO^-) (mg C/L) and expected BOD (mg/L) in the SWW(A) diluted with tap water were changed as follows: Run 0 (days 0-46), 152 and 140; Run 0 (days 47-63), 120 and 111; Run 1 (days 64-74), 108 and 100; Runs 1-3 (days 75-194), 96.2 and 88.7 respectively. The BOD concentrations were given by multiplying CH_3COONa concentration by 0.27 (g BOD/g CH_3COONa). The BOD volumetric load ($\text{kg BOD/m}^3/\text{day}$) was 0.111-0.176. Bulk liquid dissolved oxygen (DO) concentrations were measured with a DO probe (DO-24P, DKK-TOA, Tokyo, Japan), and controlled by turning the air from the diffuser on and off. The pH was controlled at 8.0 ± 0.1 using a pH controller (TPX-98 with FX-300H probe, TOKO, Tokyo, Japan). HRT was controlled at 19.2 h, but the SRT was uncontrolled without wasting biomass.

Activated sludge samples were taken from the MBR periodically. Nitrate (NO_3^- -N), nitrite (NO_2^- -N) and acetate (CH_3COO^- -C) were measured by ion chromatograph LC-10A with a Shim-pack IC-A3 column (Shimadzu, Kyoto, Japan). Ammonium (NH_4^+ -N) and mixed-liquor suspended solid (MLSS) were determined according to standard methods [27].

2.3. Oxygen uptake rate measurements by batch experiments

The oxygen uptake rate (OUR) of activated sludge in the SMBR was measured with a batch experimental instrument TS checker (Ogawa, Kamakura, Japan) according

to the supplied instructions. The sample for each batch experiment consisted of 1 L of activated sludge collected from the nitrification tank. It was then diluted with tap water to a biomass level of about 4 g-MLSS. The OUR profile was obtained using the measured DO profile in the liquid phase of the instrument. After measuring the endogenous respiration rate (R_{ir}) of the activated sludge and the mass transfer coefficient ($K_L a$) of the instruments, OUR for exogenous respiration by activated sludge was determined as shown in Eq. (1):

$$OUR = K_L a(DO_s - DO) - R_{ir} - \frac{dDO}{dt} \quad (1)$$

where DO_s is the saturation value of DO. NH_4Cl (20 mg N/L) or $NaNO_2$ (20 mg N/L) were used as a substrate for the endogenous respiration.

2.4. Real-time PCR assay

Quantitative PCR was performed for *amoA* of AOB and 16S rRNA genes of NOB (*Nitrospira* sp. and *Nitrobacter* sp.) on the Thermal Cycler Dice Real Time System (Takara Bio). Amplification reactions were carried out with the SYBR Premix Ex Taq (Takara Bio) in a total volume of 20 μ l using the primer sets amoA1F-amoA2R for AOB [28], NSR1113F-NSR1264R for *Nitrospira* sp. [29] and FGPS872-FGPS1269' for *Nitrobacter* sp. [30], respectively. Each reaction mixture contained 1 μ l (50 ng) of template DNA, 10 μ l of SYBR Premix Ex Taq, and 0.05 μ l (5 pmol) of each primer. The

real-time PCR protocols for the *amoA* and NSR primer sets were as follows: 95°C for 10 s, and 60 cycles at 95°C for 5 s, 60°C for 30 s; and 95°C for 15 s, 60°C for 30 s, 95°C for 15 s; respectively. The real-time PCR protocols for the FGPS primer set was as follows: 95°C for 10 s, and 50 cycles at 94°C for 45 s, 50°C for 45 s, 72°C for 90 s; and 95°C for 15 s, 60°C for 30 s, 95°C for 15 s. All measurements were carried out in triplicate. The copy numbers of the target DNAs were quantified as previously reported [31].

3. Results and discussion

3.1. Reactor performance

In this study, the DO in the nitrification tank and NH_4^+ concentration in influent were varied to achieve partial nitrification leading to production of NO_2^- . The MBR and reactor performances during the operation are given in Fig. 2. DO levels in the nitrification tank (Fig 2a) were controlled at 0.65 mg/L (temporally 0.35 mg/L), and NH_4^+ concentration in the influent was changed from 0 to 249 mg N/L for Run 0 (days 0-63). However, high NH_4^+ concentrations were measured in the nitrification tank (Fig. 2b), suggesting that nitrification was not stable with these operational conditions. Therefore, DO in the nitrification tank and NH_4^+ concentrations in influent were

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3 177 changed to 2.0 mg/L and 107 mg N/L respectively for Run 1 (days 64-94) in attempts to
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6 178 stabilize conventional nitrification through to NO_3^- . As a result, NH_4^+ concentration in
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9 179 the nitrification tank was almost undetectable at 0 mg N/L. Because nitrification activity
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12 180 seemed to be high, DO in the nitrification tank was decreased to 0.6 mg/L for Run 2
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15 181 (days 95-122). Although NO_2^- could be detected at the end of Run 2, its concentration
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18 182 (2.60 mg N/L) was much lower than that of NO_3^- (27.1 mg N/L) (Fig. 2c). Thus, NH_4^+
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22 183 concentration in the influent was increased from 107 mg N/L to 161 mg N/L for Run 3
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25 184 (days 123-194) to attempt to increase the NO_2^- concentration, which gradually increased
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28 185 to 44.7 mg N/L on day 150. Both NH_4^+ concentration and NO_3^- concentration in the
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32 186 nitrification tank were then maintained at almost 0 mg N/L until the end of Run 3 (day
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35 187 194), while the NO_2^- concentration in the nitrification tank remained at around 40 mg
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41 189 Concentrations of CH_3COO^- in both the pre-denitrification and nitrification
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44 190 tanks increased during Run 0 (Fig. 2d), which seemed to result from low oxidative
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47 191 activity from the low NO_x^- and DO levels in the pre-denitrification and nitrification
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51 192 tank respectively. As DO was increased from 0.6 to 2.0 mg/L from Run 1, oxidation of
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54 193 CH_3COO^- in the nitrification tank seemed to increase too, being about 10 mg C/L. On
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57 194 the other hand, CH_3COO^- in the pre-denitrification tank increased about 10-30 mg C/L
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in Run 1, despite its level in the influent being decreased from 120 to 96.2 mg C/L.

While DO levels in the nitrification tank were maintained at 0.6 mg/L for Run 2, CH_3COO^- concentrations in the nitrification tank were kept at 15 mg C/L. The low CH_3COO^- concentrations seemed to result from some active denitrification in the pre-denitrification tank during Run 2 because its concentration in the pre-denitrification tank in Run 2 was less than in Run 1. When the influent NH_4^+ concentration was raised from 107 mg N/L to 161 mg N/L for Run 3, concentrations of CH_3COO^- in both tanks fell to almost zero. Biomass was not withdrawn except for sampling during the operational period. Therefore, the MLSS increased by about 6 g/L to 16 g/L (Fig. 2a).

3.2. Aeration saving by achieving nitrogen removal via nitrite

The total daily aeration volume was measured with a flow instrument during Runs 2 and 3 (Fig. 3). To compare these aeration volumes, each was divided by the amount of NH_4^+ removed, which was the difference between dosed and released NH_4^+ .

The released NH_4^+ was calculated by multiplying the NH_4^+ concentration in the nitrification tank by the treatment volume (40 L) per day. When the average volume (4.14 L/mg N) in Run 2 (days 96-105), where $\text{NO}_2^-/\text{NO}_x^-$ was 0%, was compared to that (2.98 L/mg N) in Run 3 (days 149-194) where $\text{NO}_2^-/\text{NO}_x^-$ was 90-100%, the aeration requirement was reduced in Run 3 by about 29%. This result showed that N removal via

NO_2^- reduced the aeration demand, at a level higher than the theoretical oxygen reduction ratio (25%) [7–10].

To discuss the high aeration reduction ratio (29%), theoretical oxygen demand (ThOD) to oxidize NH_4^+ (ThOD(N) and also CH_3COO^- (ThOD(C)) removed from the nitrification tank was calculated according to the following equations respectively.

$$\text{ThOD(N)} \left[\frac{\text{mg O}}{\text{day}} \right] = \text{removed } \text{NH}_4^+ \times \left(\frac{64.0}{14.0} \times \frac{\text{NO}_3^-}{\text{NO}_x^-} + \frac{48.0}{14.0} \times \frac{\text{NO}_2^-}{\text{NO}_x^-} \right) \quad (2)$$

$$\text{ThOD(C)} \left[\frac{\text{mg O}}{\text{day}} \right] = \text{removed } \text{CH}_3\text{COO}^- \times \left(\frac{64.0}{24.0} \right) \quad (3)$$

where the amount of CH_3COO^- removed was calculated by multiplying the CH_3COO^- concentration difference between the nitrification and pre-denitrification tanks by four times of the treatment volume (40 L×4) per day. The obtained ThOD(N) and total ThOD(ThOD (T)) (i.e. ThOD(N) + ThOD(C)) were divided by the amount of removed NH_4^+ respectively and those values were shown in Fig. 4. If DO was consumed only for oxidation of NH_4^+ , ThOD values per NH_4^+ should be 4.57 or 3.43 according to Eq. (2) when the $\text{NO}_3^-/\text{NO}_x^-$ ratios were 100% or 0% respectively. Those values are shown as dashed lines in Fig. 4. ThOD(N)s per NH_4^+ during Run 2 and Run 3 were 4.57 and around 3.43 respectively, and the average value during Run 3 (3.51) was 77% of that during Run 2 (4.57). On the other hand, ThOD(T)s per NH_4^+ during Run 2 and Run 3 were above 4.57 and around 3.43 respectively, and the average value during

Run 3 (3.52) was 51% of that during Run 3 (6.86). As mentioned above, the aeration requirement was reduced in Run 3 by about 29%, which is higher than the reduction in ThOD(N) (23%) in Run 3 but lower than the reduction in ThOD(T) (49%) in Run 3. Thus, not only NH_4^+ but also CH_3COO^- removal in the nitrification tank seemed to affect the aeration reduction in Run 3. Moreover, one reason why the reduction ratio of aeration was lower than that of ThOD(T) may reflect differences in oxygen transfer efficiency during Run 2 and Run 3. Several studies have indicated that oxygen transfer ratios exponentially decrease with increases in MLSS [32]. As the average MLSS during Run 3 (after 150): 15.4 g/L was higher than that during Run 2 (day 96-105): 13.1 g/L, the oxygen transfer efficiency during Run 2 may reach 1.11-1.22 times greater than that achieved during Run 3 according to several studies [32].

3.3. Nitrification activity of activated sludge

As mentioned in section 2.2, HRT of MBR was controlled at 19.2 h. As the nitrification tank occupied two thirds of the total MBR volume, HRT of the nitrification tank was controlled at 12.8 h. NH_4^+ , NO_2^- and NO_3^- concentrations in nitrification tank shown in Fig. 2b and 2c were measured during the long HRT. Therefore, those data seemed not to show the nitrification activity in detail. Thus, oxidation uptake rate (OUR) was measured by batch experiment after Run 1 to check community NH_4^+ and

247 NO_2^- oxidizing activity (Fig. 5). Fig. 5 shows three OUR profiles: i) OUR with NH_4Cl
 248 (20 mg N/L) (OUR(NH_4Cl)), ii) OUR with NaNO_2 (20 mg N/L) (OUR(NaNO_2)), iii)
 249 the difference between OUR(NH_4Cl) and OUR(NaNO_2) (ΔOUR). OUR(NH_4Cl),
 250 OUR(NaNO_2) and ΔOUR seemed to show OUR by both AOB and NOB, only by NOB,
 251 and only by AOB respectively. The OUR(NaNO_2) was kept at almost 2 mg/h g-MLSS,
 252 whereas that ΔOUR increased from 1.2 mg/h g-MLSS to 8.0 mg/h g-MLSS during Run
 253 1. This increase in OUR (from NH_4^+ oxidation) seems to explain the stable removal of
 254 NH_4^+ in the nitrification tank during Run 1, as shown in Fig. 2b. The NH_4^+
 255 accumulation in the nitrification tank during Run 0 also seemed to be caused by low
 256 NH_4^+ oxidation activity. Although this activity decreased from 15 mg/h g-MLSS to 10
 257 mg/h g-MLSS during Run 2 where DO was held at 0.6 mg/L, no NH_4^+ accumulation in
 258 the nitrification tank could be detected. Since the OUR(NaNO_2) was also at >3.5 mg/h
 259 g-MLSS during Run 2, only small amounts of NO_2^- were detected at the end.
 260 OUR(NaNO_2) decreased until day 169 in Run 3 when influent NH_4^+ concentrations
 261 were increased to 161 mg N/L, while ΔOUR remained at almost 5 mg/h g-MLSS.
 262 Although ΔOUR then increased to around 9 mg/h g-MLSS on days 188 and 194,
 263 OUR(NaNO_2) was almost undetectable until at the end of Run 3. Thus, NO_2^-
 264 accumulation seemed to be achieved as shown in Fig. 2c.

As mentioned above, partial nitrification in this SBR system was achieved by controlling NH_4^+ concentration in the influent together with low DO concentrations in the nitrification tank for >40 days in this study. Influent NH_4^+ concentration seemed to be a critical parameter in establishing partial nitrification, as data shown in Fig. 2a and 2c demonstrate. Anthonisen et al. [33] reported that the relationship between free-ammonia (FA) concentration and pH is as shown below:

$$FA(\text{mg} / \text{L}) = \frac{17}{14} \times \frac{[\text{NH}_4^+ - N(\text{mg} / \text{L})] \times 10^{\text{pH}}}{K_a / K_w + 10^{\text{pH}}} \quad (4)$$

$$\text{Where } K_a / K_w = e^{6344 / (273 + T)} \quad (5)$$

Here, T is temperature ($^{\circ}\text{C}$), K_a and K_w are ionization constants of ammonia and water, respectively.

They also reported that AOB and NOB respectively were inhibited at >10-150 mg/L and >0.1-1.0 mg/L of FA [33]. In our SBR experimental setup, the recycling rate from the nitrification tank to the pre-denitrification tank was kept at three times the effluent flow. Therefore, the influent was diluted by about 25% in the pre-denitrification tank, and the NH_4^+ concentration there was about a quarter of that in the influent. As this is the same as that in the influent to the nitrification tank, FAs in the pre-denitrification tanks at Run 2 and Run 3 were calculated from NH_4^+ concentrations in the pre-denitrification tank (Fig. 2b) according to Eqs. (4) and (5) as 1.21-1.83 mg N/L and 1.94-3.99 mg N/L for

Run 2 (except for day 110) and Run 3 respectively. Such concentration ranges are above the NOB inhibition limit and below the AOB inhibition limit, but that for Run 2 was closer to the lower limit for NOB inhibition. Thus, NO_2^- accumulation levels in the nitrification tank in Run 2 seemed to be low (<3.00 mg N/L).

Sinha and Annachhatre [34] have reviewed and compared the literature values for FA inhibition of nitrite oxidation in wastewater treatment. They drew from the results of Balmelle et al. [35], where a process was operated with changing NH_4^+ concentration under similar conditions (temperature (25°C) and pH (8.1)) to those in this study, except for their DO concentration (2.5 mg/L). When they changed the FA concentration from 1.06 to 6.64 mg N/L, inhibition ratios of NO_2^- oxidation changed by 55-100% [35]. They also showed that having a FA concentration close to the lower limit of the NOB inhibition range was critical in achieving partial nitrification. In our study, OUR by NOB (Fig. 5) decreased markedly from those in Runs 2 to 3. The results also seem to support the view that the increase in FA concentration was responsible for inhibiting NO_2^- oxidation activity by NOB.

As mentioned above, 161 mg N /L of NH_4^+ was required in the influent to inhibit NO_2^- oxidation activity by NOB in this study. Most WWTPs will not experience such high NH_4^+ concentrations in their influent. However, in this study, the recycling

rate from nitrification tank to pre-denitrification tank was kept at three times that of effluent flow. If the recycling rate can be decreased compared to that configured in this study, required NH_4^+ concentration in the influent to inhibit NO_2^- oxidation activity should decrease and the partial nitrification with FA concentration could be more widely applied to WWTPs.

3.4. Quantification of AOB and NOB

Changes in population levels of nitrifying bacteria in the SBR were assessed by quantifying AOB *amoA* and NOB (*Nitrospira* sp. and *Nitrobacter* sp.) 16S rRNA genes. Real-time PCR assays were conducted with samples taken on days 69 (Run 1) to 194 (Run 3) and the results are shown in Fig. 6. The copy numbers of *Nitrospira* sp. and *Nitrobacter* sp. 16S rRNA genes were almost the same until day 118 (the end of Run 2), whereas the copy numbers of AOB *amoA* slightly decreased from $2.64 \times 10^2 \pm 3.85$ copies per ng-DNA to $8.02 \times 10^1 \pm 1.23 \times 10^1$ copies per ng-DNA. The copy number of *Nitrospira* sp. 16S rRNA genes then kept dropping after increasing the influent NH_4^+ concentration on day 123 (sharply on day 169) and finally reached $1.19 \times 10^2 \pm 6.91 \times 10^1$ copies per ng-DNA on day 194. However, the copy number of *Nitrobacter* sp. 16S rRNA genes remained at almost same value ($3\text{--}5 \times 10^3$ copies per ng-DNA) during Run 3. The AOB *amoA* copy number increased after day 123 and reached $7.78 \times 10^2 \pm 9.91$

319 $\times 10^1$ copies per ng-DNA on day 140. However, the copy number then decreased to 4.36

320 $\times 10^1 \pm 4.71$ copies per ng-DNA on day 194.

321 Real-time PCR assay of NOB (Fig. 6) suggested that both *Nitrobacter* sp. and
322 *Nitrospira* sp. were present in the current SMBR. Daims et al. [36] have suggested that
323 *Nitrobacter* could take advantage of the temporarily elevated nitrite concentration and
324 compete successfully with *Nitrospira* in the SBR. Furthermore, Kim and Kim [37]
325 reported that the distribution of *Nitrobacter* and *Nitrospira* depended largely on NO_2^-
326 concentrations. In their study, *Nitrospira* dominated in a continuous biofilm airlift
327 reactor running under NO_2^- limiting conditions, while *Nitrobacter* was dominant in a
328 sequencing batch reactor with high NO_2^- concentrations (250 mgN at the beginning of a
329 cycle). In our study, NO_2^- concentrations in the nitrification tank of the SMBR were low
330 (<3mgN/L) until Run 2, and then increased to 40 mgN/L during Run 3. Therefore, it
331 might be expected that the copy number of *Nitrospira* 16S rRNA genes showed about a
332 hundredfold of those of *Nitrobacter* sp until Run 2 and then fell markedly to 1/10 of
333 those in *Nitrobacter* sp during Run 3. Note that NO_2^- oxidation activity gradually
334 decreased eventually to zero during Run 3 (Fig. 5). Therefore, it is incomprehensible
335 that the copy numbers of 16S rRNA genes in *Nitrobacter* could be maintained under
336 these conditions. Physiological studies with the NOB by Prosser [38] showed that many

Nitrobacter spp. could grow mixotrophically on acetate, butyrate, and propionate. Moreover, Bock et al. [39] reported that *Nitrobacter* utilized pyruvate under anoxic conditions. Thus, *Nitrobacter* species might survive in our SMBR system using organic substrates as electron donors under anoxic conditions by denitrification. If denitrification by *Nitrobacter* was carried out during Run 0-2, population levels of *Nitrobacter* would be maintained by their denitrification activity during the operation period. *Nitrospira* sp. and not *Nitrobacter* sp. seemed to play a role in NO_2^- oxidation in this SMBR system, and the partial nitrification in the SMBR might result from inhibiting the activity and growth of *Nitrospira* sp.

4. Conclusions

N removal via NO_2^- (partial nitrification to NO_2^-) was achieved at 25 °C in a continuous SMBR system configured with both a pre-denitrification and nitrification tank. It was achieved by operating the reactor at low DO concentrations (0.6 mg/L) in the nitrification tank and high influent ammonium concentrations (161 mg N/L). Aeration saving in by partial nitrification was determined by experimental measurement, and the saving ratio shown to be 29% on a removed NH_4^+ N basis compared to N removal via NO_3^- .

When N removal via NO_2^- was achieved, oxygen uptake rates for NO_2^- substrate were reduced to zero mg/h g-MLSS. The decrease in NO_2^- oxidizing activity with high FA concentration in the influent seemed to cause NO_2^- accumulation. Moreover, the copy number of *Nitrospira* 16S rRNA genes gradually decreased during accumulation of NO_2^- . This reduction also seemed to be correspondence to a decrease in NO_2^- oxidizing activity. However, the copy number of *Nitrobacter* 16S rRNA genes remained at a constant level irrespective of any decrease in NO_2^- oxidizing activity. The partial nitrification demonstrated in this study might be attained by inhibiting the activity and growth of *Nitrospira* sp. with high FA concentration.

Acknowledgement

The authors gratefully acknowledge Prof. R. Seviour for his suggestion and proofreading of the manuscript. The authors thank Environmental Planning Bureau of Yokohama City Government for their provision of activated sludge. This research was supported by the Japan Society for the Promotion of Science (JSPS), Grant-in-Aid for Scientific Research (B) 18310054.

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Figure Captions

Fig. 1. Schematic diagram of experimental setup. “D tank” and “N tank” mean denitrification tank and nitrification tank respectively.

Fig. 2. Changes in operational controls in SMBR and results of chemical analysis for
activated sludge suspension through the operational period. Fig. 2a: Control changes in
 NH_4^+ -N concentrations in diluted influent and DO concentrations in nitrification tank,
and variations in MLSS. Fig. 2b: Variations in NH_4^+ -N concentrations in denitrification
and nitrification tanks. “D tank” and “N tank” in explanatory note mean denitrification
tank and nitrification tank respectively. Fig. 2c: Variations in NO_2^- -N and NO_3^- -N
concentrations in nitrification tank. “N tank” in explanatory note means nitrification
tank. Fig. 2d: Control changes in CH_3COO^- -C concentration in diluted influent and
variations in CH_3COO^- -C concentrations in denitrification and nitrification tanks. “Inf”,
“D tank” and “N tank” in explanatory note mean influent, denitrification tank and
nitrification tank, respectively.

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502 Fig. 3. Comparison of provided aeration volume for Run 2 to that for Run 3. Note that
503 the aeration volume was divided by the NH_4^+ concentration removed in the nitrification
504 tank as the NH_4^+ -N concentration in influent for Run 2 was different from that for Run
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3 508 Fig. 4. ThOD(N) and ThOD (T) divided by removed NH_4^+ -N during Run 2 and Run 3.
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6 509 Thick and thin dashed lines shows ThOD(N) divided by removed NH_4^+ -N when the
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9 510 $\text{NO}_3^-/\text{NO}_x^-$ ratios were 100% and 0% respectively.
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Fig. 5. Variations in OURs using NH_4Cl and NaNO_2 by activated sludge biomass collected from the nitrification tank, and the NO_2^- -N/ NO_x^- -N ratios in the nitrification tank. The differences between OURs using NH_4Cl and NaNO_2 were shown as ΔOUR . Measurements were performed in samples taken from Runs 1 and 3.

Fig. 6. Variations of the quantity of 16S rRNA gene copy number of *Nitrobacter* and *Nitrospira* and *amoA* copy number per DNA unit for the 16 samples that were taken on day 69 to day 194. The samples were analyzed in triplicate, and mean values $\pm$ S.D. are shown.

Figure 1

Figure 2a

Figure 2b

Figure 2c

Figure 2d

Figure 3

Figure 4

Figure 5

Figure 6