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Source identification of nitrous oxide on autotrophic partial nitrification in a granular sludge reactor

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ABSTRACT

Emission of nitrous oxide (N_2O) during biological wastewater treatment is of growing concern since N_2O is a major stratospheric ozone-depleting substance and an important greenhouse gas. The emission of N_2O from a lab-scale granular sequencing batch reactor (SBR) for partial nitrification (PN) treating synthetic wastewater without organic carbon was therefore determined in this study, because PN process is known to produce more N_2O than conventional nitrification processes. The average N_2O emission rate from the SBR was 0.32 ± 0.17 mg-N L^{-1} h^{-1}, corresponding to the average emission of N_2O of 0.8 ± 0.4% of the incoming nitrogen load (1.5 ± 0.8% of the converted NH_4^+). Analysis of dynamic concentration profiles during one cycle of the SBR operation demonstrated that N_2O concentration in off-gas was the highest just after starting aeration whereas N_2O concentration in effluent was gradually increased in the initial 40 min of the aeration period and was decreased thereafter. Isotopomer analysis was conducted to identify the main N_2O production pathway in the reactor during one cycle. The hydroxylamine (NH_2OH) oxidation pathway accounted for 65% of the total N_2O production in the initial phase during one cycle, whereas contribution of the NO_2^- reduction pathway to N_2O production was comparable with that of the NH_2OH oxidation pathway in the latter phase. In addition,

spatial distributions of bacteria and their activities in single microbial granules taken from the reactor were determined with microsensors and by in situ hybridization. Partial nitrification occurred mainly in the oxic surface layer of the granules and ammonia-oxidizing bacteria were abundant in this layer. N_2O production was also found mainly in the oxic surface layer. Based on these results, although N_2O was produced mainly via NH_2OH oxidation pathway in the autotrophic partial nitrification reactor, N_2O production mechanisms were complex and could involve multiple N_2O production pathways.

Keywords: Nitrous oxide production pathway; Sequencing batch reactor; Isotopomer analysis; Microsensors; In situ hybridization; Hydroxylamine

1. Introduction

Nitrous oxide (N_2O) emissions draw attention since N_2O is expected to be a major stratospheric ozone-depleting substance in the future (Ravishankara et al., 2009) and is an important greenhouse gas with a global warming potential of about 300 times higher than CO_2 (Desloover et al., 2012; IPCC, 2007). It is generally accepted that nitrogen removal processes in a wastewater treatment system are an anthropogenic source of N_2O (Desloover et al., 2012). Conventionally, biological nitrogen removal is achieved by a combination of nitrification and denitrification processes. In contrast, an alternative and innovative approach is the use of a partial nitrification (PN) process followed by an anaerobic ammonium oxidation (anammox) process (PN-anammox process), which has several advantages, such as no need for external carbon addition, less energy and oxygen

requirement, and less sludge production (van Dongen et al., 2001; Kartal et al., 2010). The PN-anammox process is applicable to reject water (Desloover et al., 2011; Kampschreur et al., 2008; 2009a; Joss et al., 2009; Okabe et al., 2011), landfill leachate (Wang et al., 2010), and wastewater from semiconductor factory (Tokutomi et al., 2011). N_2O emission from PN-anammox processes, especially from the PN process, has been reported (Desloover et al., 2011; Kampschreur et al., 2008; Okabe et al., 2011). Especially, a granular sludge reactor for PN process draws attention because of high specific nitrification rate, efficient biomass retention and excellent settleability.

There are three main microbial pathways involved in N_2O production. During nitrification, it is produced from hydroxylamine (NH_2OH) as a side product of the oxidation of ammonium (NH_4^+) to nitrite (NO_2^-) (Poughon et al., 2001; Hooper and Terry, 1979). During denitrification, N_2O is produced as an intermediate during reduction of nitrate (NO_3^-) to N_2 by heterotrophic denitrifiers (Lu and Chandran, 2010; Schmidt et al., 2004). Some ammonia-oxidizing bacteria (AOB) reduce NO_2^- to N_2O or N_2 through a process called nitrifier denitrification (Tallect et al., 2006; Wrage et al., 2001; Colliver and Stephenson, 2000). Many studies have been conducted to estimate N_2O emission rate of PN processes (Kong et al., 2013a; Kong et al., 2013b; Okabe et al., 2011; Law et al., 2011; Desloover et al., 2011; de Graff et al., 2010; Kampscheur et al., 2008). In contrast, there are few studies on N_2O production pathways. Nitrifier denitrification was the key biological pathway of N_2O production in an intermittently aerated sequencing batch biofilm reactor for PN treating synthetic ammonium-rich wastewater (Kong et al., 2013b) while NH_2OH oxidation pathway was the main source of N_2O in a sequencing batch reactor (SBR) for PN (PN-SBR) (Law et al., 2011; Yang et al., 2009). To determine

which pathway is responsible for N_2O production in a wastewater treatment process is still challenging, because a variety of operational parameters (concentrations and loading rates of nitrogenous compounds, dissolved oxygen (DO) and organic carbon, pH, a ratio of organic carbon and nitrogenous compounds (COD/N) and temperature) influence N_2O production in a PN process (Tallec et al., 2006; Kampschreur et al., 2009b; Desloover et al., 2012; Wunderlin et al., 2012; Law et al., 2011). Furthermore, their temporal changes also affect N_2O production.

Analyses of the intermolecular distributions of ^{15}N in N_2O (isotopomers) are regarded as useful parameters to infer the predominant N_2O production pathway (Wunderlin et al., 2013; Sutka et al., 2006; Toyoda et al., 2005; 2011). Isotopomer ratios (site-specific N isotope ratios in asymmetric molecules of NNO) give us qualitative information on N_2O production and consumption pathways. Toyoda et al. (2011) and Wunderlin et al. (2013) conducted isotopomer analysis and distinguished N_2O produced during NH_2OH oxidation from N_2O produced during NO_2^- reduction in wastewater treatment processes. However, N_2O production pathways in a PN-SBR have not been investigated by isotopomer analysis. In a PN-SBR, temporal changes in the operational parameters (DO, NH_4^+ and NO_2^- concentrations and pH level) are more significant than conventional activated sludge processes, which likely play an important role in N_2O production pathways.

In this study, source of N_2O produced in an autotrophic granular PN-SBR was investigated. A lab-scale PN-SBR was operated and N_2O emission from the reactor was determined with an on-line monitoring system. Dissolved N_2O in the reactor was monitored with a microsensor for N_2O. N_2O, DO, pH, NH_4^+, NO_2^- and NO_3^-

concentrations in the PN-SBR for one cycle were continuously monitored. We measured temporal changes in intermolecular ^{15}N-site preference (SP) in N_2O in the PN-SBR for one cycle. In addition, the spatial distribution of N_2O, DO, pH, NH_4^+, NO_2^--and NO_3^- in the PN granules were determined with the microsensors to estimate net production and consumption rates of N_2O, NH_4^+ and NO_2^- in single granules. The spatial distribution of AOB and other bacteria in the PN granules was determined by FISH. The combination of microsensor measurements and FISH analysis allows us to deduce function of AOB. Finally, these results were compared and we discussed the source of N_2O in the PN-SBR.

2. Materials and methods

2.1 Operation of a lab-scale autotrophic PN-SBR

A lab-scale autotrophic PN-SBR with working volume of 2.0 L was operated. The reactor was inoculated with 0.3 L of PN granules (3-5 mm in diameter), which was obtained from the PN reactor operated in our laboratory (Okabe et al., 2011). One cycle of the reactor operation was 4 h. It consisted of feeding of a synthetic wastewater (3 min), aeration (232 min), settling of the granules (3 min), and discharging of treated wastewater (2 min). The composition of a synthetic wastewater was as follows: $(NH_4)_2SO_4$ (1650 mg L^{-1}), $KHCO_3$ (3300 mg L^{-1}), $CaCl_2 \cdot 2H_2O$ (135 mg L^{-1}), $MgSO_4 \cdot 7H_2O$ (300 mg L^{-1}), and KH_2PO_4 (22 mg L^{-1}). Trace element solutions I and II were prepared and added as described by van de Graaf et al. (1996). The influent pH was adjusted to 7.7 ± 0.1. The hydraulic retention time (HRT) of the PN reactor was fixed at 8 h. The airflow rate was changed according to reactor performance until the PN process became stable. After the PN process became stable, airflow rate was fixed at 0.2 L min^{-1}.

2.2 Water and gas analyses

The PN reactor performance was determined by collecting grab samples of influent and effluent at arbitrary time intervals during the operation. NH_4^+, NO_2^- and NO_3^- concentrations in the influent and effluent were measured by using ion-exchange chromatography (DX-100, DIONEX, CA., USA) with an IonPac CS3 cation column and IonPac AS9 anion column after filtration with a 0.45-μm pore size membrane (ADVANTEC, Tokyo, Japan).

The N_2O concentrations in the off-gas from the reactor were determined with a 1412 Photo acoustic Field Gas-Monitor (INNOVA, Copenhagen, Denmark). Grab samples were taken from 115 min to 125 min during 4-h cycles. For batch tests, the N_2O concentrations in the off-gas were determined once every minute. The dissolved N_2O (D-N_2O) concentration in the effluent of the reactor was measured with a N_2O microsensor (Unisense, Aarhus, Denmark). N_2O emission rate into the headspace of the PN-SBR was calculated by multiplying the N_2O concentration in the off-gas by gas emission rate, and D-N_2O discharge rate into the effluent of the PN-SBR was calculated by multiplying the D-N_2O concentration in the effluent by hydraulic flow rate.

2.3 Isotopomer analysis

Isotopomer ratios (δ) in N_2O in the off-gas from the PN reactor were measured to identify N_2O production pathway. The notations of isotopomer ratios are shown below.

$$\delta^{15}N^{\alpha} = ({}^{15}R^{\alpha}_{sample} - {}^{15}R^{\alpha}_{standard}) / {}^{15}R^{\alpha}_{standard}$$

$$\delta^{15}N^{\beta} = ({}^{15}R^{\beta}_{sample} - {}^{15}R^{\beta}_{standard}) / {}^{15}R^{\beta}_{standard}$$

Where, $^{15}R^{\alpha}$ and $^{15}R^{\beta}$ donates $^{14}N^{15}N^{16}O/^{14}N^{14}N^{16}O$ and $^{15}N^{14}N^{16}O/^{14}N^{14}N^{16}O$, respectively, for samples and standards (atmospheric N_2). Here, we define a certain parameter called ^{15}N-site preference (SP) as an illustrative parameter of intermolecular distribution of ^{15}N that was defined as follows (Toyoda et al., 2005; 2011).

^{15}N-site preference (SP) = $\delta^{15}N^{\alpha} - \delta^{15}N^{\beta}$

The off-gas samples of the PN reactor were collected into evacuated 50 mL glass bottles at arbitrary time intervals in the aeration phase. The isotopomer ratios of the collected gas samples were measured on an isotope-ratio monitoring mass spectrometer (MAT 252; Thermo Fisher Scientific K.K, Yokohama, Japan) using an online analytical system at the Tokyo Institute of Technology, Japan (Toyoda et al., 2005; 2009; 2011). The precision of the isotopomer ratios were typically better than 0.5‰ for $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$.

Characteristic SP values of 33‰ and 0‰ for NH_2OH oxidation and NO_2^- reduction (nitrifier denitrification and heterotrophic bacterial denitrification), respectively, which were estimated in specific pure cultures, were used for estimation of the contribution to each process (Sutka et al., 2006). Approximate contributions of NH_2OH oxidation and NO_2^- reduction to N_2O production were estimated by assuming that each process is linearly proportional to the SP value using the following equation:

Contribution of NH_2OH oxidation (%) = SP/(SP for NH_2OH oxidation - SP for NO_2^- reduction) × 100 = SP/33 × 100

Contribution of NO_2^- reduction (%) = 100 - contribution of NH_2OH oxidation

2.4 Microsensor measurements

The steady-state concentration profiles of DO, N_2O, NH_4^+, NO_2^-, NO_3^- and pH in the PN granules were measured in a synthetic medium for microsensor measurements with microsensors. DO and N_2O microsensors were purchased from Unisense (Aarhus, Denmark). LIX-type NH_4^+, NO_2^-, NO_3^- and pH microsensors were constructed in our laboratory as described by de Beer et al. (1997) and calibrated and used according to a protocol reported by Okabe et al. (1999a). The synthetic medium for the microsensor measurements of DO, N_2O and pH was as follows (mg L^{-1}): NaH_2PO_4 (19), $(NH_4)_2SO_4$ (990), $NaHCO_3$ (2770), $NaNO_2$ (690), $MgSO_4 \cdot 7H_2O$ (300), $CaCl_2 \cdot 2H_2O$ (135) and trace element solution I and II (van der Graaf et al., 1996). Trace element solution I contained EDTA (5 g L^{-1}) and $FeSO_4$ (5 g L^{-1}), and trace element solution I1 contained EDTA (15 g L^{-1}), $ZnSO_4 \cdot 7H_2O$ (0.43 g L^{-1}), $CoC1_2 \cdot 6H_2O$ (0.24 g L^{-1}), $MnC1_2 \cdot 4H_2O$ (0.99 g L^{-1}), $CuSO_4 \cdot 5H_2O$ (0.25 g L^{-1}), $NaMoO_4 \cdot 2H_2O$ (0.22 g L^{-1}), $NiC1_2 \cdot 6H_2O$ (0.19 g L^{-1}), $NaSeO_4 \cdot 10H_2O$ (0.21 g L^{-1}), and H_3BO_4 (0.014 g L^{-1}). pH was adjusted to 7.5. For NH_4^+, NO_2^- and NO_3^- concentration measurements, the concentration of the species to be measured was adjusted to 250 μM, 250μM and 50μM, respectively. The PN granules with diameters of 2 to 3 mm were sampled from the reactor at 120 min after aeration was started and positioned with five insect needles in the flow chamber (2.0 L) that was filled with the synthetic medium. DO concentration in the medium was controlled at the required value by continuous bubbling with N_2 gas (99.9%) and/or atmospheric air, which also provided sufficient mixing of the medium. The granules were acclimated in the medium at least 3 h to ensure that steady-state profiles were obtained. At least five concentration profiles of each species were measured in different granules taken in one cycle. The concentration profiles were determined in five cycles.

Net volumetric production rates of N_2O, NH_4^+ and NO_2^- in the granules were estimated from the averaged steady-state concentration profiles by using Fick's second law of diffusion as previously described by Santegoeds et al. (1999). Diffusion coefficients of 1.38×10^{-5} cm^2 s^{-1}, 1.25×10^{-5} cm^2 s^{-1} and 2.10×10^{-5} cm^2 s^{-1} were used for NH_4^+, NO_2^- and N_2O, respectively, at 25°C for the calculation of net volumetric production rates (Okabe et al., 2011).

2.5 Fluorescence in situ hybridization (FISH)

Ten granules were taken from the reactor in a cycle at 120 min after aeration was started. The sampling was conducted in five cycles from day 100 to day 200. The granule samples were fixed in 4% (w/v) paraformaldehyde solution at 4°C for 24 h, washed three times with phosphate-buffer saline (PBS; 10mM sodium phosphate buffer, 130 mM sodium chloride; pH 7.2), and embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) at -30°C overnight to infiltrate the OCT compound into granules. 20-μm-thick vertical thin sections were prepared by using a cryostat (Reichert-Jung Cryocut 1800, Leica, Bensheim, Germany). FISH was performed as previously described by Okabe et al. (1999b). The 16S rRNA-targeted probes used in our present study were as follows; Mixture of EUB, EUBII, and EUBIII probes (Daims et al., 1999) in an equimolar for all bacteria and Nso1225 probe (Mobarry et al., 1996) for betaproteobacterial ammonia-oxidizing bacteria. Hybridized samples were observed using a model LSM510 confocal laser-scanning microscope (CLSM, Carl Zeiss, Oberkochen, Germany) equipped with an Ar ion laser (458 and 488 nm) and two He-Ne ion lasers (543 and 633 nm).

3. Results and discussion

3.1 Reactor performance

Figure 1 shows concentrations of NH_4^+, NO_2^-, NO_3^- and D-N_2O in the influent and the effluent, N_2O concentrations in the off-gas, and N_2O emission rates into the headspace and D-N_2O discharge rate into the effluent of the PN-SBR. The reactor was operated at an average nitrogen loading rate (NLR) (± standard deviation) of 43 ± 2.7 mg-N L^{-1} h^{-1}. In the initial stage of the reactor operation, airflow rate was adjusted to achieve stable NO_2^- production. The stable PN was achieved at day 30 and the airflow rate was fixed at 0.2 L min^{-1}. The average concentration of NH_4^+ in the influent was 350 ± 21 mg-N (Figure 1A). The average NH_4^+ and NO_2^- concentrations in the effluent were 168 ± 18 mg-N L^{-1} and 182 ± 29 mg-N L^{-1}, respectively. Approximately 50% of the influent NH_4^+ was converted to NO_2^- with the NH_4^+ oxidation rate of 22 ± 2.9 mg-N L^{-1} h^{-1}, indicating that a favorable NH_4^+/NO_2^- ratio for anammox process was achieved. NO_3^- concentration in the effluent was 0.5 ± 0.1 mM.

Figure 2A shows an image of the PN granules. The average diameter and the settling velocity of PN granules were approximately 2 mm and 160 cm min^{-1}, respectively. FISH, using a TRITC-labeled Nso1225 probe and a Cy5-labeled EUB338 mix probe, revealed that the outer layer (ca. 600 μm thick) was dominated by bacteria and AOB were found in the upper 400 μm. The probe specific for anammox bacteria was not applied.

N_2O concentrations in the off-gas and the effluent of the PN reactor were measured (Figure 1B). The N_2O concentration in the off-gas varied from 30 ppm to 230 ppm (89 ± 48 ppm on average). D-N_2O concentration in the effluent varied between 14 μg-N L^{-1} and

420 μg-N L^{-1}. Fluctuation of N_2O concentrations in the off-gas and the liquid phase might be due to fluctuation of airflow and hydraulic flow rates.

The N_2O emission and D-N_2O discharge rates from the PN-SBR are shown in Figure 1C. The N_2O emission rate from the PN reactor was 0.67 ± 0.34 mg-N h^{-1} per reactor and 0.32 ± 0.17 mg-N L^{-1} h^{-1} as specific rate. Fluctuation of the N_2O emission might be due to fluctuation of airflow and hydraulic flow rates, followed by the change in microbial activities of production or consumption of N_2O. A large portion (more than 96%) of N_2O produced in the PN reactor was evolved to the headspace by aeration. The average ratio of N_2O production rate to NLR was 0.8 ± 0.4% (or 1.5 ± 0.8% of the converted NH_4^+ in the PN reactor).

The ratios of N_2O production rate to NLR and the parameters affecting the N_2O production rate in the PN-SBR were compared with those reported in the previous studies (Table 1). The ratio of N_2O production rate to NLR in this study (0.8%) was in the same order (between 0.28% and 0.85%) of the other reactors. The ratios of a lab-scale column biofilm reactor (Okabe et al., 2011) and a full scale floc based sequential PN reactor (Desloover et al., 2011) were higher than the other reactors. The variation in N_2O emission in previous studies (Figure 1) is attributed to a complicated pathway of biological and chemical N_2O production, for example, NH_2OH oxidation by AOB, NO reduction by heterotrophic bacteria and AOB, and chemodenitrification (Poughon et al., 2001; Lu and Chandran, 2010; Wrage et al., 2001; van Cleemput, 1998) and consumption (N_2O reduction by heterotrophic bacteria and AOB (Pan et al., 2012; Schmidt et al., 2004)). Therefore, it was obvious that difference in operational parameters (DO concentration, NH_4^+ and NO_2^- concentration, COD/N ratio, NH_4^+ loading rate, and pH)

of the PN reactors could strongly affect them (Kampschreur et al., 2009a; Law et al., 2011; Burgess et al., 2002).

3.2 Dynamic N_2O emission in one cycle of the PN-SBR operation

The dynamics of N_2O, NH_4^+, NO_2^-, NO_3^- and DO concentrations and pH level in one cycle of the PN-SBR are shown in Figure 3. In the settling period (-7 min to -4 min) both N_2O and D-N_2O concentrations decreased due to gas-liquid equilibrium and dilution of off-gas with air (an insertion panel in Figure 3A). In the discharging (-4 min to -2 min) and feeding (-2 min to 0 min) periods, N_2O concentrations in off-gas further decreased due to dilution with air. In contrast, D-N_2O concentration in the bulk liquid increased in the feeding period. N_2O accumulation in the settling granular sludge bed was experimentally confirmed by a N_2O microsensor measurement (Figure S1). Subsequently, N_2O concentration in off-gas suddenly increased just after starting the aeration due to release of N_2O from the bulk liquid and decreased over the operation. D-N_2O concentration was gradually increased in the initial 40 min of the aeration period and was decreased thereafter. Thus, the net N_2O production rate was higher in the initial phase of aeration period. These trends were reproducible. We measured these concentrations in five cycles and found the same trend of changes in them. However, the level of N_2O was different between each test, which agreed with the result shown in Figure 1C.

Dynamics of N_2O emission from the PN-SBR suggests that the continuous measurement of N_2O in off-gas is necessary for reliable estimation of N_2O emission rate from a PN-SBR. Dynamics of N_2O emission in our reactor (Figure 3A) might be

attributed to perturbation of the operating conditions, such as DO and pH (Figure 3C). N_2O emission rate was also high in the initial phase of aeration period of a lab-scale PN-SBR (Kong et al., 2013a). Difference of N_2O sampling methods (e.g., timing and amount of a sample) among studies reported in Table 1 might result in difference of N_2O emission rates.

3.3 N_2O isotopomer analysis

The $\delta^{15}N^{\alpha}$, $\delta^{18}O$, and calculated SP value for the off-gas samples collected at different stages of the aeration period in the PN-SBR are shown in Figure 4A. The SP values ranged from 23‰ to 16‰. No significant increase in $\delta^{18}O$ in the remaining N_2O indicates that contribution of N_2O reduction to N_2 was not strongly occurred in the reactor (Groenigen et al., 2005; Schmidt et al., 2004). Production of N_2 as estimated based on the N balance calculation was 3.5 ± 4.7%, which might not be strong enough to influence the $\delta^{18}O$ values and the SP value. Therefore, approximate contributions of NH_2OH oxidation and NO_2^- reduction (nitrifier denitrification and heterotrophic bacterial denitrification) to N_2O production were estimated by assuming that each process is linearly proportional to the SP value (Figure 4B). The result indicates that N_2O was produced in the PN-SBR by combination of NH_2OH oxidation and NO_2^- reduction pathways. Within initial 60 min of the aeration phase, about 70% of the totally produced N_2O was produced via the NH_2OH oxidation pathway. After 60 min, the contribution of the NH_2OH oxidation pathway to the total N_2O production gradually decreased. At the end of the aeration phase, the contribution of the NH_2OH oxidation pathway was comparable with that of the NO_2^- reduction pathway. To the best of our knowledge, this

is the first study to distinguish contribution of nitrification and denitrification processes to N_2O production pathways in an autotrophic granular PN-SBR.

Higher contribution of NH_2OH oxidation on N_2O production within the initial 60 min is due to sudden fluctuation in DO and NH_4^+ concentrations and pH level. Wunderlin et al. (2012) reported that N_2O production by NH_2OH oxidation pathway was favored at high NH_4^+ and low NO_2^- concentrations, in contrast, the contribution of nitrifier denitrification increased under the condition of higher NO_2^- and lower NH_4^+ concentrations. In addition, N_2O production was only observed during recovery to aerobic conditions after a period of anoxia in chemostat cultures of model nitrifying bacteria (Yu et al., 2010), and N_2O production rates of the AOB enriched culture were increased with increases in pH and NH_4^+ oxidation rate (Law et al., 2011; 2012). Increase in the contribution of NO_2^- reduction pathway might be due to relative enhancement of denitrification caused by increase in NO_2^- concentration and decrease in NH_4^+ concentration in the reactor (Wunderlin et al., 2012). The contribution of the NH_2OH oxidation pathway to the total N_2O production was about 65% throughout one cycle, indicating that the NH_2OH oxidation pathway was the key pathway of N_2O production in the autotrophic PN-SBR. In the latter phase the N_2O production via the NO_2^- reduction pathway was comparable with that via the NH_2OH oxidation pathway.

In contrast, in the previous studies to investigate N_2O production pathways in NH_4^+ oxidation process in wastewater treatments by isotopomer analysis, NO_2^- reduction contributed to N_2O production greater than NH_2OH oxidation did (Wunderlin et al., 2013; Toyoda et al., 2011). It might be because operational parameters affect N_2O production pathways. In the present study, isotopomer analysis was conducted in only one cycle.

Reproducibility of the trend of shift in N_2O production pathways should be confirmed in the future study. N_2O production pathways have also been investigated with the use of mathematical models (Ni et al., 2013; Law et al., 2012) and by addition of substrates (NH_2OH or NO_2^-) (Wunderlin et al., 2012; Yang et al., 2009). In contrast to this method, isotopomer analysis can reveal the N_2O production pathways directly and quantitatively with high reliability. However, there are some limitations to isotopomer analysis, for example, it cannot distinguish N_2O production by heterotrophic denitrification from that by nitrifier denitrification. For more specific and quantitative identification of N_2O source in a PN-SBR, other analytical methods (e.g., functional gene expression analysis (Philippot and Hallin, 2005)) have to be combined with isotopomer analysis.

3.4 Spatial distributions of bacteria and their activities in single PN granules

The steady-state concentration profiles of DO, pH, NH_4^+, NO_2^-, NO_3^-, and N_2O in the PN granules were measured under the typical operational conditions of the PN-SBR and the spatial distributions of net volumetric production rates of NH_4^+, NO_2^- and N_2O were calculated (Figure 5A). N_2O was detected throughout the granule and the net N_2O production rate was higher in the oxic layer (within 300 μm) of the granules (Figure 5B). NH_4^+ consumption and NO_2^- production were found mainly in the oxic surface layer without a significant production or consumption of NO_3^-, demonstrating that partial nitrification occurred efficiently in the PN granules. Moreover, FISH results revealed that AOB were abundant in the outermost layer of the granules (Figure 2). These results reflect that AOB might be responsible for N_2O production in the PN granules. Unfortunately, based on the microsensor measurements we cannot conclude that the

NH_2OH oxidation by AOB was the main N_2O production pathway rather than NO_2^- reduction by AOB and/or heterotrophic denitrifiers, as could be demonstrated by isotopomer analysis.

Less but detectable N_2O production probably by heterotrophic denitrifiers in the deeper anoxic parts of the granules were found (Figure 5B), which could be expected by isotopomer analysis. Although the PN-SBR operated without an external organic carbon supply, it has been hypothesized that heterotrophic bacteria scavenge organic matter derived from biomass decay and substrate metabolism of nitrifying bacteria (Okabe et al., 2005). In addition, under the limited availability of biodegradable carbon, N_2O can be produced due to the incomplete denitrification process and/or endogenous denitrification (Chung and Chung, 2000; Itokawa et al., 2001). As a result, the microsensor measurements revealed that the N_2O production mechanisms in the PN granules involve multiple N_2O production pathways, because there were steep vertical gradients of physicochemical parameters in the PN granules.

4. Conclusions

A lab-scale sequencing batch reactor for partial nitrification treating synthetic wastewater without organic carbon was operated to identify source of N_2O in an autotrophic partial nitrification reactor.

- The average N_2O emission rate from the reactor was 0.32 ± 0.17 mg-N L^{-1} h^{-1} and the average emission of N_2O was $0.8 \pm 0.4\%$ of the incoming nitrogen load.
- N_2O emission rate and N_2O production pathways were dynamic during one cycle of the sequencing batch reactor operation; N_2O emission rate was high in the initial phase of

the aeration period, where hydroxylamine oxidation pathway accounted for 65% of the total N_2O production.

- The active N_2O production as well as partial nitrification was found in the oxic surface layer of the granule, where ammonia-oxidizing bacteria were abundant.
- Based on all experimental results (including isotopomer analysis, microelectrode and FISH), although N_2O was produced mainly via NH_2OH oxidation pathway in the autotrophic partial nitrification reactor, N_2O production mechanisms were complex and could involve multiple N_2O production pathways.

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Figure captions

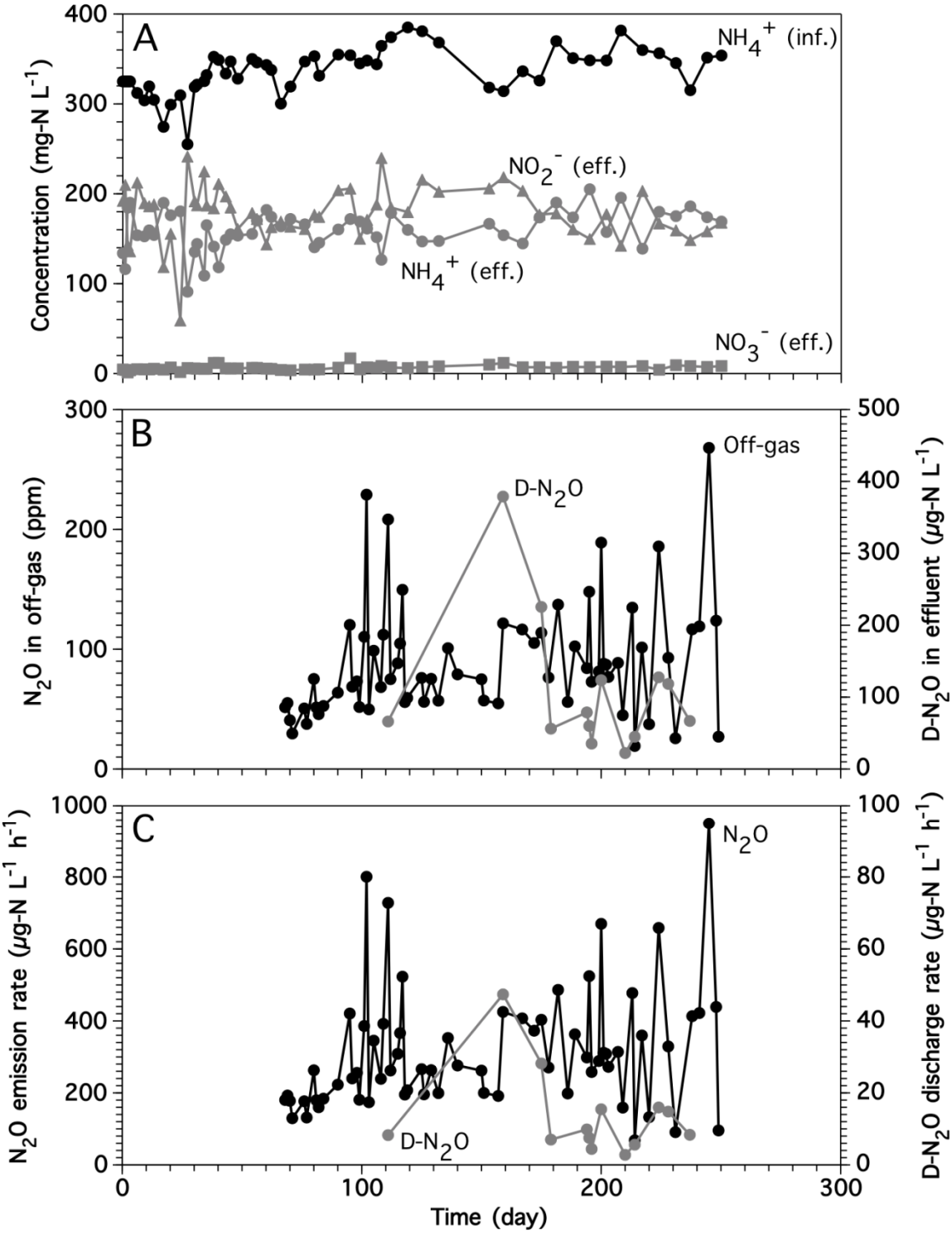

Figure 1. (A) Concentrations of NH_4^+ in the influent, and NH_4^+, NO_2^- and NO_3^- in the

effluent of the PN reactor. (B) N_2O concentration in the off-gas and D-N_2O concentration in the effluent. (C) N_2O emission rates into the headspace and into the effluent.

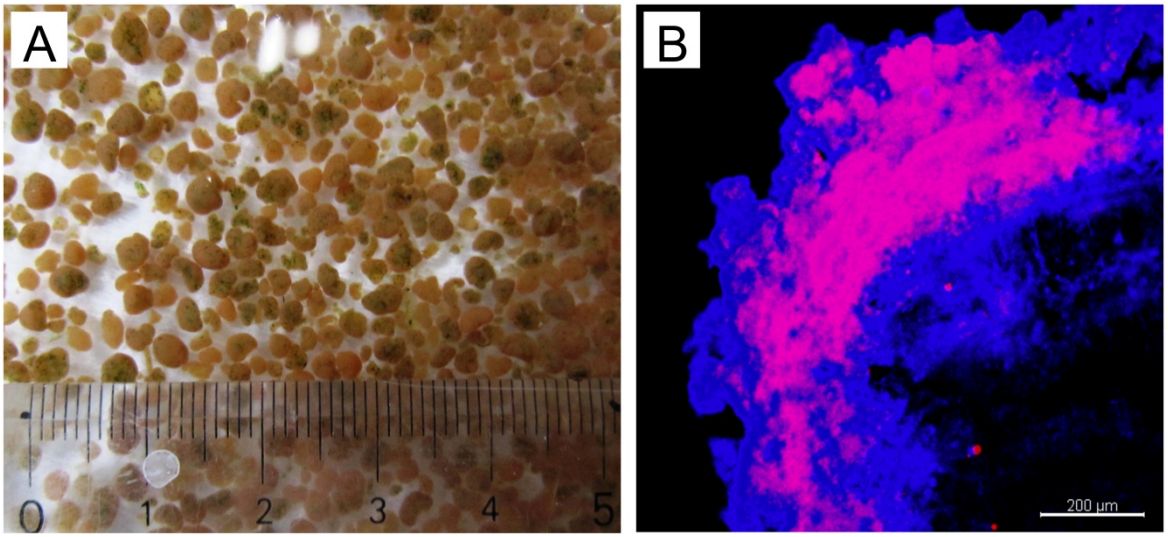

Figure 2. (A) An image of the PN granules. **(B)** Confocal laser scanning microscope images of thin cross-section of the PN granules showing in situ spatial distribution of AOB (magenta) and other bacteria (blue) after fluorescence in situ hybridization with Cy5-labeled EUB338 mix probe and TRITC-labeled Nso1225 probe.

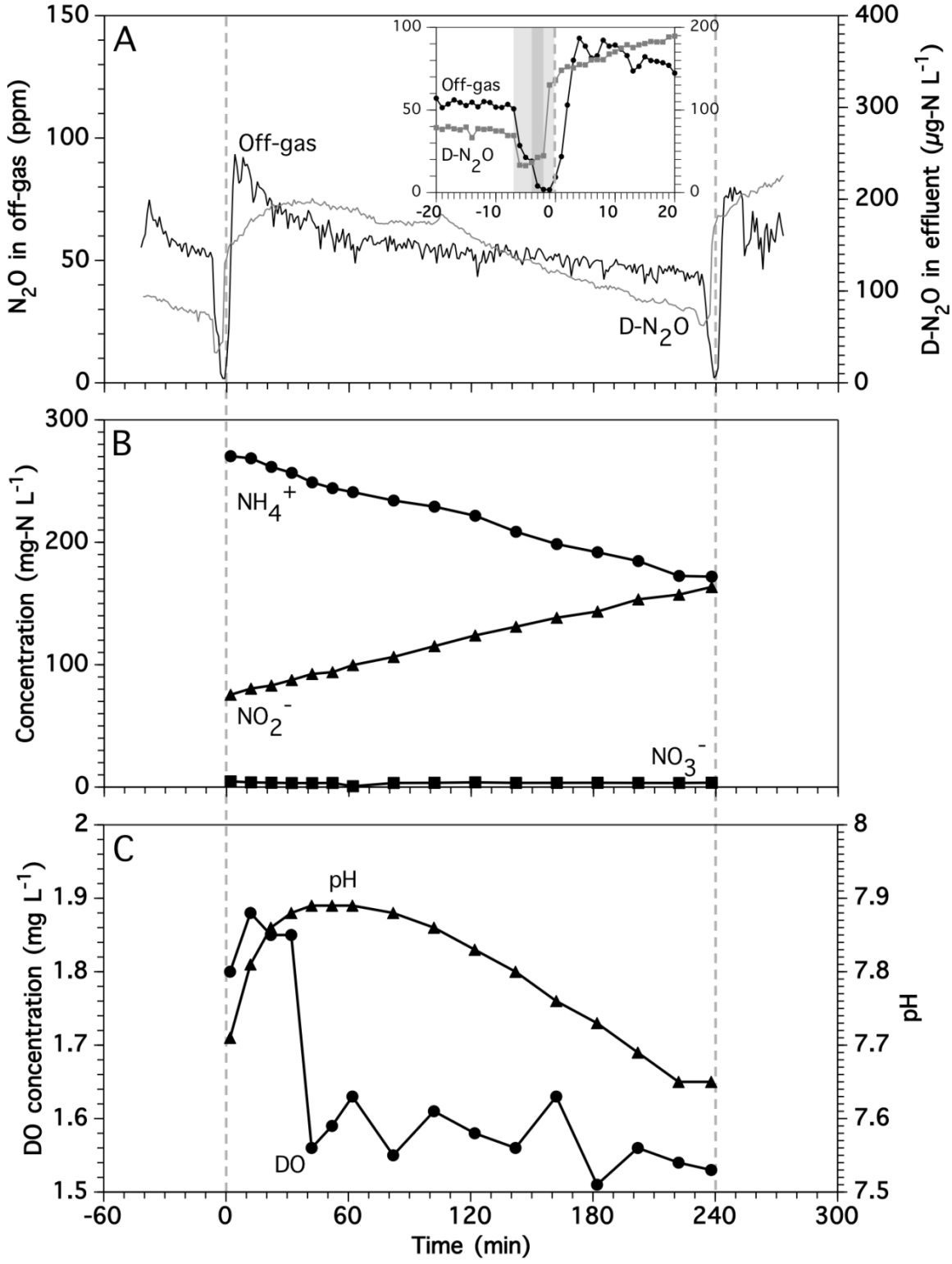

Figure 3. The concentration profiles of (A) N_2O in the off-gas and D-N_2O, (B) NH_4^+, NO_2^- and NO_3^-, and (C) DO and pH during one typical cycle of the sequencing batch reactor operation. Inset of panel A shows the concentration profiles of N_2O in the off-gas

and D-N_2O from -20 min to +20 min. A one cycle of the reactor operation was 4 h and aeration was started at 0 min.

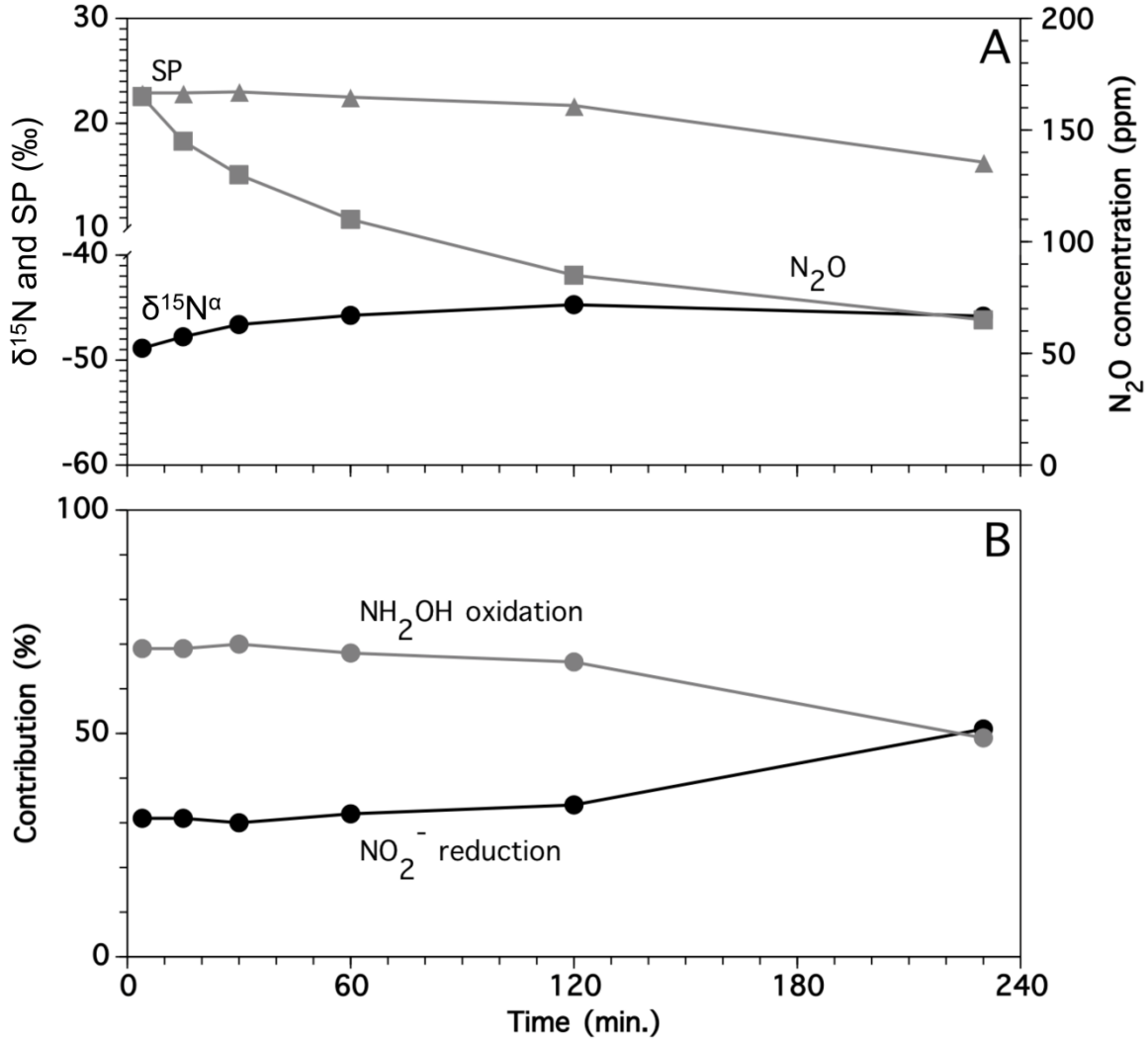

Figure 4. (A) N_2O concentration, isotopomer ratios and SP values in off-gas at each sampling time over one cycle. (B) Contribution of NH_2OH oxidation and NO_2^- reduction pathways to N_2O emission from the PN reactor.

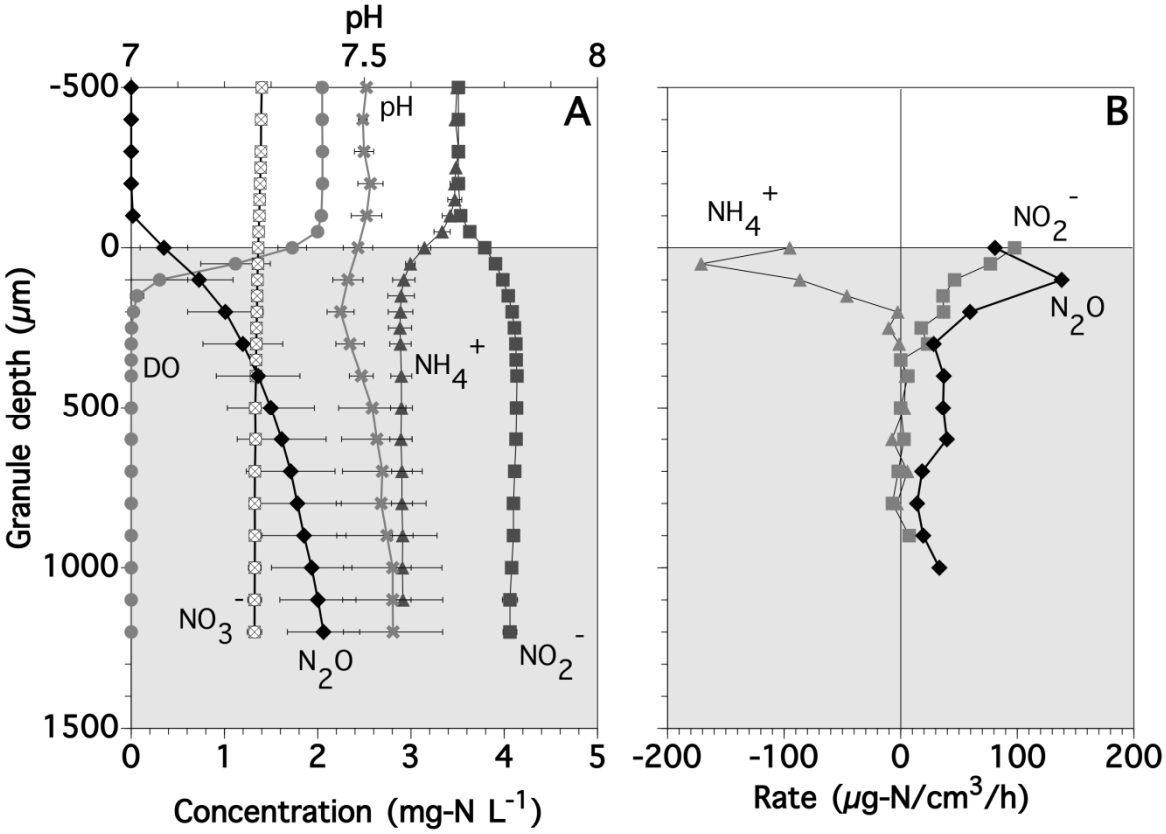

Figure 5. (A) Steady-state concentration profiles of DO, pH, NH_4^+, NO_2^-, NO_3^-, and N_2O in the PN granules. (B) Net volumetric production rates of NH_4^+, NO_2^- and N_2O in the PN granules. Positive and negative values indicate production and consumption rates, respectively.

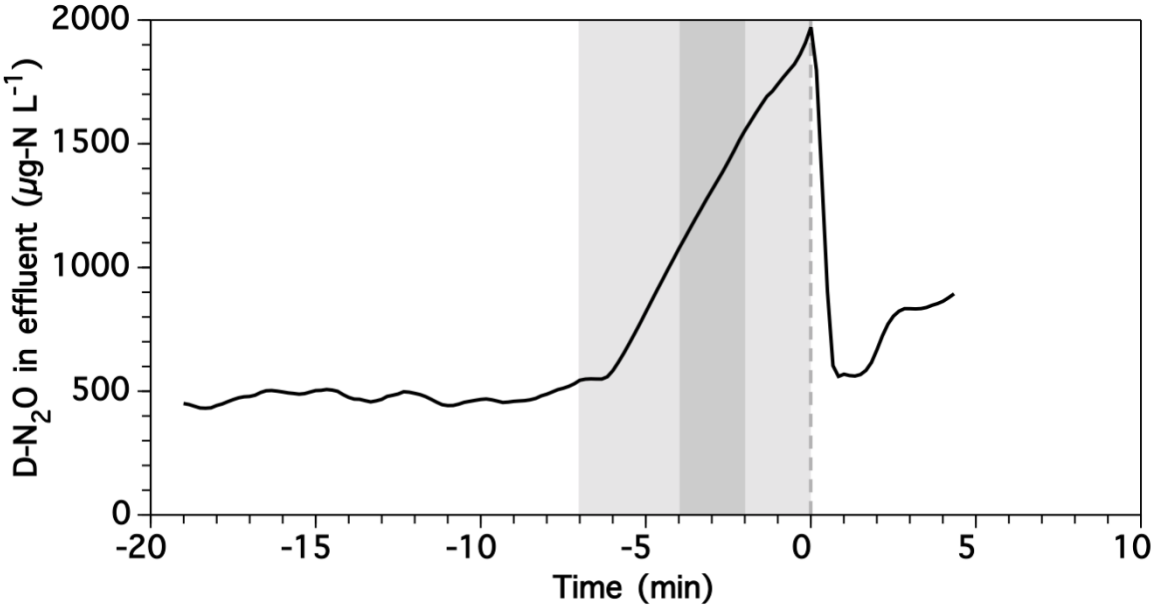

Figure S1. The profile of D-N_2O concentration in the PN reactor during the settling (-7 min to -4 min), discharging (-4 min to -2 min) and feeding (-2 min to 0 min) periods.

Table 1 Summary of the ratios of N_2O production rate to nitrogen loading rate and parameters afecting the N_2O production rate in PN reactors

Type of reactor	NLR[a] (mmol-N/L/d)	The ratio of N_2O production to NLR (%)[b]	DO (mg/L)	pH	Ref.
A lab-scale PN SBR	71 ± 7	0.8 ± 0.4	2.0 ± 0.3	7.4 – 7.8	This study
A full-scale nitritation CFR	46.7	0.85	2.5		Kampscheur et al. 2008
A single-stage PN-anammox reactor	71.4	0.6	5.0		Kampscheur et al. 2009a
A lab-scale PN CFR	37.1	0.3 – 1.3	4.1 ± 0.73	6.8 ± 0.33	de Graff et al. 2010
A lab-scale PN SBR	89.3	0.75	1.3	7.1 – 7.5	Kong et al., 2013b
A lab-scale PN SBR	214	0.4	1.0	6 – 7.5	Kong et al., 2013a
A lab-scale PN SBR	571	0.28	0.5 – 0.8	6.4 ± 0.05	Law et al., 2011
A lab-scale PN CFR	179	2.0 ± 0.8	< 2.0		Okabe et al., 2011
A full-scale PN SBR	14.7	2.55 – 3.3	0.75 ± 0.05	7.5 ± 0.1	Desloover et al., 2011

[a] nitrogen loading rate.

[b] The ratio was calculated to divide N_2O production rate (μmol-N L^{-1} h^{-1}) by NLR.

SBR: Sequencing batch reactor.

CFR: Continuous flow reactor.