Journal of Bioscience and Bioengineering
VOL. 118 No. 3, 289–297, 2014

www.elsevier.com/locate/jbiosc

Abundance, transcription levels and phylogeny of bacteria capable of nitrous oxide reduction in a municipal wastewater treatment plant

Kang Song,[1] Toshikazu Suenaga,[1] Aki Hamamoto,[2] Kouichi Satou,[2] Shohei Riya,[1] Masaaki Hosomi,[1] and Akihiko Terada[1,*]

Department of Chemical Engineering, Tokyo University of Agriculture & Technology, Tokyo 184-8588, Japan[1] and Tokyo Metropolitan Govt., Bur Sewerage, Tokyo 163-8001, Japan[2]

Received 28 October 2013; accepted 27 February 2014
Available online 13 April 2014

Nitrous oxide (N_2O) production and expression of genes capable of its reduction were investigated in two full-scale parallel plug-flow activated sludge systems. These two systems continuously received wastewater with the same constituents, but operated under distinct nitrification efficiencies due to mixed liquor suspended solid (MLSS) concentration and the different hydraulic retention times (HRTs). A shorter HRT in system 2 resulted in a lower nitrification efficiency (40–60%) in conjunction with a high N_2O emission (50.6 mg-N/L/day), whereas there was a higher nitrification efficiency (>99%) in system 1 with low N_2O emission (22.6 mg-N/L/day). The DNA abundance of functional genes responsible for nitrification and denitrification were comparable in both systems, but transcription of *nosZ* mRNA in the lower N_2O emission system (system 1) was one order of magnitude higher than that in the higher N_2O emission system (system 2). The diversity and evenness of the *nosZ* gene were nearly identical; however, the predominant N_2O reducing bacteria were phylogenetically distinct. Phylogenetic analysis indicated that N_2O-reducing strains only retrieved in system 1 were close to the genera *Rhodobacter*, *Oligotropha* and *Shinella*, whereas they were close to the genera *Mesorhizobium* only in system 2. The distinct predominant N_2O reducers may directly or indirectly influence N_2O emissions.

[**Key words:** Nitrous oxide reduction; Wastewater treatment plants; N_2O reductase gene (*nosZ*); Abundance of DNA and mRNA; Gene transcription; N_2O reduction bacteria]

Nitrous oxide (N_2O) has a greenhouse gas effect about 300 times greater than that of carbon oxide (CO_2) (1); therefore, it has attracted a great deal of attention from policy makers, practitioners and researchers (2). N_2O emissions have increased annually by 0.2–0.3% from the preindustrial period to 1998, and they currently account for up to 7.9% of the global anthropogenic greenhouse gas (GHG) emissions as CO_2 equivalents (3). In addition to its high greenhouse potential, N_2O is reportedly the dominant ozone-depleting substance emitted in the 21st century, and has a long lifetime of 114 years (1,4).

Global N_2O emissions from municipal wastewater treatment plants (WWTPs) were reportedly 0.22 Tg-N/year in 1990, which was equivalent to 3.2% of the total anthropogenic N_2O emissions (1). The United States Environmental Protection Agency reported that N_2O from the wastewater sector accounted for about 3% of N_2O emissions from all sources, making it the sixth largest contributor (5). N_2O emissions from wastewater handling has been estimated to contribute 26% of the total greenhouse gas emissions (CO_2, Methane (CH_4), N_2O) of the water chain, including drinking water production, water transport, wastewater and sludge treatment and discharge (6). However, N_2O emissions from WWTPs have been underestimated because of the assumption that they do not contain facilities for nitrogen removal, which inevitably produce N_2O via biotic conversion of nitrogen. In addition, innovative nitrogen removal technologies designed to enable cost-effective methods of biological nitrogen removal such as nitrification/denitrification via nitrite and nitritation/anammox may increase N_2O production by WWTPs (7,8). Accordingly, it is important to develop novel techniques for mitigation of N_2O emissions from WWTPs.

N_2O is biologically produced via nitrification and denitrification pathways, as well as through abiotic formation via hydroxylamine (9). While there are three pathways to the production of N_2O, oxidation of hydroxylamine (10,11), nitrifier denitrification (12–14) and incomplete heterotrophic denitrification (15–17), there is only one biological pathway to the conversion of N_2O into nitrogen gas (N_2) by heterotrophic denitrifying microorganisms. In WWTPs, this reduction occurs in the final step of denitrification, which is mainly conducted by denitrifying bacteria that produce N_2O reductase (NOS) encoded by the *nosZ* gene (2,18,19). Hence, harnessing bacteria harboring NOS has the potential to mitigate N_2O emissions from WWTPs. Despite its crucial role in mitigating N_2O emissions, knowledge regarding the phylogeny, abundance and physiology on *nosZ* in WWTPs remains limited (20). Accordingly, it is important to combine information regarding these processes to elucidate a rationale regarding N_2O dynamics and ultimately develop an operational strategy to mitigate N_2O emissions in WWTPs.

This study was conducted to investigate the abundance, gene expression and phylogeny of bacteria capable of N_2O reduction in

* Corresponding author. Tel.: +81 42 388 7069; fax: +81 42 388 7731.
E-mail address: akte@cc.tuat.ac.jp (A. Terada).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2014.02.028

two different trains of activated sludge systems for a full-scale municipal WWTP showing distinct nitrogen removal performance. These data were compared with N_2O emissions from these systems, underpinning the significance of monitoring bacteria responsible for N_2O consumption.

MATERIALS AND METHODS

Sampling site In this study, activated sludge samples were collected from a full-scale municipal WWTP in Tokyo with a population equivalent to 1.57 million. This municipal WWTP employs conventional activated sludge as the main biological system. The plant has four trains in each activated sludge tank, employing a conventional plug-flow system. Wastewater after the primary sedimentation tank flows into any of the four trains. Municipal wastewater after the primary sedimentation tank and return activated sludge enters one end of the tank and flows to the other end (Fig. 1). In the present study, two trains (systems 1 and 2) with different nitrification efficiencies were investigated. To accomplish this, four sampling points were set along the distance of each train from one end to the other of the tank (denoted A to D from upstream to downstream). Samples were collected from each sample point at 9:30, 11:30, 13:30 and 15:30 on November 14, 2012. The operational conditions of each system are summarized in Table 1.

Analytical methods Each point consisted of a duct 1 m above the water surface with a pump in the middle that conveyed exhausted gas to the end of a line, where it was collected and directed into Multigas analyzer (VA-3000; Horiba, Tokyo, Japan) to measure N_2O gas online. Concentrations of nitrogen compounds (ammonium (NH_4^+), nitrate (NO_3^-), and nitrite (NO_2^-)) were measured by ion chromatography with cations (ICS-1600, Dionex, Osaka, Japan) and anions (ICS-2100, Dionex, Osaka, Japan). Dissolved oxygen (DO) concentration was measured by a DO meter (YSI-58, YSI, Yellow Springs, Ohio). Gaseous N_2O concentration was measured using a Horiba VS-3000 (Tokyo, Japan). COD_{Mn} and mixed liquor suspended solids (MLSS) were assayed according to the Standard Methods for the Examination of Water and Wastewater (21).

DNA/RNA extraction and quantification Biomass samples were taken by a stainless bucket tied with a rope, after which there were transported to the laboratory on ice, where DNA was extracted upon arrival using a Fast DNA spin kit (FastDNA Spin Kit for Soil, Bio101, Qbiogene Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The purities and concentrations of the DNA extracts were then evaluated based on the absorbance at 260 nm and 280 nm (NanoDrop 2000c, Thermo Scientific, Wilmington, DE, USA). Activated sludge samples for RNA extraction were submerged in RNA*later* RNA Stabilization Reagent (Qiagen, Venlo, The Netherlands) to prevent RNA degradation during transportation. Subsequently, RNA was extracted using an RNA PowerSoil Total Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions and eluted in nuclease-free water. DNA removal and reverse transcription from the extracted RNA was then conducted using a QuantiTect Reverse Transcriptase kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocols.

Ammonia oxidation (Bacterial and Archaeal *amoA*), *Nitrobacter* nitrite oxidation (*nxrA*), nitrite reduction of heterotrophic bacteria (*nirK* and *nirS*) and nitrous oxide reduction (*nosZ*) by all bacteria were quantified by real-time polymerase chain reaction (qPCR) based on functional gene targeted primer sets (Table 2) using a CFX96 Touch Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA, USA). The reaction conditions were as follows: initiation by pre-heating at 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, annealing at the specified temperature (dependent on primers) for 30 s, extension at 72°C for 1 min, and plate reading. The annealing temperatures for bacterial *amoA*, archaeal *amoA*, *nxrA*, *nirK*, *nirS*, and *nosZ* were 58°C, 56°C, 55°C, 63°C, 58°C and 67°C, respectively. All samples including control reactions without template DNAs were measured in triplicate. Additionally, plasmid DNA (positive control) was diluted to attain standard solutions containing 1.0×10^8–1.0×10^1 copies per 5 μL. There were high correlations between logarithmic gene copy numbers and PCR cycle number required to reach a threshold of fluorescence ($r^2 > 0.99$), and the detection limits were around 1.0×10^2 copies per 5 μL for all primer sets. Reverse transcription quantitative PCR (RT-qPCR) based on messenger RNA (mRNA) was performed to confirm gene expression dynamics of *nosZ* responsible for N_2O reduction to nitrogen gas in the WWTPs. The absence of primer dimers and artifacts in each primer set was confirmed by melting curve analysis for each round of qPCR and RT-qPCR (data not shown).

TABLE 1. Relevant parameters of each system.

Parameter	System 1		System 2	
Influent volume [m^3/h]	1470		1860	
HRT [h]	9:30	4.65	9:30	4.05
	11:30	4.69	11:30	3.90
	13:30	4.71	13:30	3.66
	15:30	4.71	15:30	3.84
SRT [d]	4.8		5.0	
Average temperature [°C]	21.5		21.6	
Average DO [mg/L]	Point A	0.4	Point A	0.4
	Point B	3.1	Point B	1.1
	Point C	0.7	Point C	1.2
	Point D	1.6	Point D	1.8
Average MLSS [mg/L]	1500		1380	
Average influent COD_{Mn} [mg/L]	54.0		53.8	

Pyrosequencing The extracted DNA was amplified using V4 region forward primer 563F with different "barcode" sequences attached to the 5′ end and a cocktail of four equally mixed reverse primers, R1, R2, R3 and R4 at the 3′-end of the V4 region (*Escherichia coli* positions 785–802) (28). The thermal cycling conditions for PCR were as follows: an initial cycle of 98°C for 5 min followed by 40 cycles of 98°C for 45 s, 55.5°C for 45 s, and 72°C for 1 min and then 1 cycle at 72°C for 5 min. PCR amplicons were purified using a quick-spin kit (iNtRON, Seoul, Korea), after which their concentrations were measured by spectrometry (NanoDrop-1000, Thermo Scientific, Wilmington, DE, USA). Amplicons from different sludge samples were then mixed to achieve equal mass concentrations in the final mixture, which was sent to Takara Bio Company (Mie, Japan) for pyrosequencing by GS FLX+ (Roche Applied Science, Penzberg, Germany).

Identification of bacteria capable of nitrous oxide reduction The extracted DNA samples were subjected to PCR using primers specific for the *nosZ* gene. The thermal conditions for PCR were as follows: initial denaturation at 95°C for 15 min, followed by 6 cycles of 95°C for 15 s, 67°C for 30 s with a touchdown of −1°C per cycle, 72°C for 30 s and then 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s, after which the samples were kept at 4°C. Following PCR, the product was confirmed by agarose gel electrophoresis, purified using a wizard PCR clean-up system (Promega, Madison, WI) and ligated into a pGEM-T Easy plasmid vector according to the manufacturer's instructions (Promega, Madison, WI, USA). The vector was then

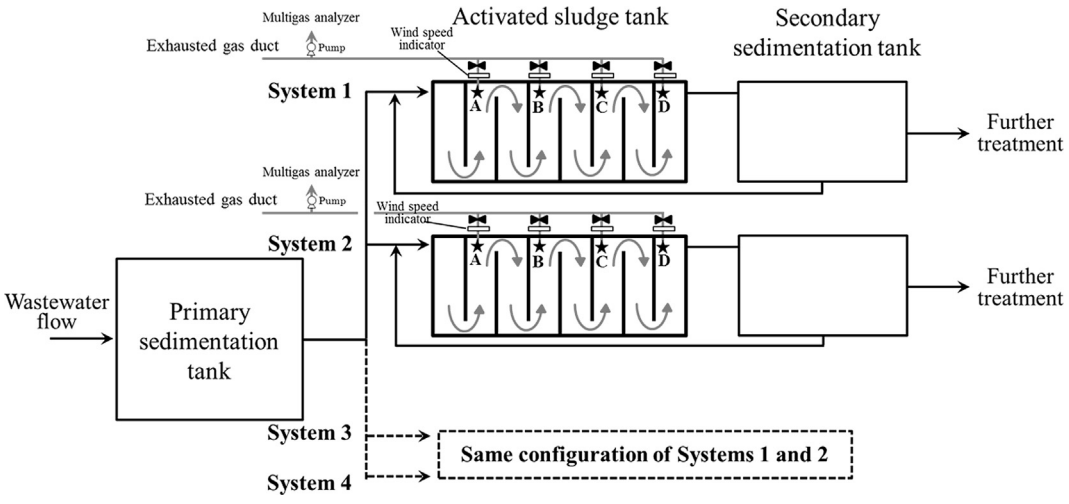

FIG. 1. Schematic of wastewater treatment systems. The stars (A to D) represent sampling points for gas and liquid measurements.