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Long-term effect of temperature on N_2O emission from the denitrifying activated sludge

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The long-term effect of various temperature (4°C, 12°C, 20°C, 25°C and 34°C) on nitrous oxide (N_2O) emission from lab-scale denitrifying activated sludge was studied in terms of activation energy, abundance of functional gene *nosZ* and its transcription. Results showed that temperature had a positive effect on N_2O emissions as well as the maximum biomass-specific reduction rates of N_2O and NO_3^-, ranging from 0.006% to 0.681% of ($N_2O + N_2$), 17.3–116.2 and 5.2–66.2 mg N g^{-1} VSS h^{-1}, respectively. The activation energies (*Ea*) for N_2O and NO_3^- reduction of 44.1 kJ mol^{-1} and 54.9 kJ mol^{-1}, shed light on differences in denitrifying rate variation. The maximum NO_3^- reduction rates were more sensitive to temperature variation than the corresponding N_2O reduction rates under long-term acclimation. As a result, the ratio between N_2O and NO_3^- reduction rates declined to 1.87 at 34°C from 3.31 at 4°C, suggesting great potential capacity for N_2O losses at high temperature. The copy numbers of denitrifiers as *nosZ* gene ($\times 10^8$ copies mL^{-1}) and total bacteria as 16S rRNA gene ($\times 10^{10}$ copies mL^{-1}) did not show obvious relationship with temperature, having relative abundance of 0.42% on average. The transcriptional regulation of *nosZ* gene, in the range of 10^8–10^5 copies mL^{-1}, was affected by reductase activity, substrate concentration as well as its duration. The active *nosZ* gene expression was accompanied with low reductase capacity, high dissolved N_2O and the duration of N_2O accumulation. These results provide insights into activation energy and gene expression responsible for N_2O emission.

[**Key words:** Nitrous oxide emission; Denitrification; *nosZ* gene transcription; Temperature response; Maximum reduction rate; Activation energy]

N_2O is not only present as a potent greenhouse gas, with a 120-year global warming potentials of 320 times that of carbon dioxide, but is also the dominant ozone-depleting compounds (1). Both nitrification and denitrification processes in the wastewater treatment plants (WWTPs), involving nitrogen compounds reduction, are the predominant sources of N_2O (2,3). The global N_2O emission from WWTPs accounted for 3.2% of the anthropogenic source and 1.3% of the total estimated N_2O emission (4), which was estimated at 0.22 Tg N yr^{-1} (5). Therefore, it is essential to explore the mechanisms and optimize the involved processes for the sake of mitigating N_2O emission from WWTPs (6,7).

Denitrification is a sequential reduction of NO_3^- via NO_2^- to NO, N_2O and finally to N_2, catalyzed by nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase, respectively. The inhibition of N_2O reductase, encoded by *nosZ* gene, is the underlying reason for excessive N_2O emission from denitrifying community. Several factors affecting N_2O emission from wastewater have been reported in the literature, such as inappropriate dissolved oxygen, high nitrite concentration and low C/N ratio (8–11). These suboptimal environment factors should be responsible for influencing the functionality at both expression of *nosZ* gene and the activity of N_2O reductase either in pure culture or complex community.

Among these factors, one important controller is temperature, which markedly affects rates of enzyme-catalyzed reactions. In experiments addressing the product ratio (N_2O/N_2), it was found that N_2O represented the larger fraction of the ratio with decreasing temperature (12,13), since N_2O reductase is thought to be affected to a greater extent than the other reductases (NO_3^-, NO_2^- and NO reductase) at low temperature (14). However, several studies made contrary discoveries (15,16), with higher N_2O emission mostly occurring in warm climates. N_2O emission studies with respect to soil have been extensively carried out, whereas seldom focused on long-term effect of temperature, especially in denitrifying activated sludge. To further explore the N_2O emission patterns of denitrifying community, an in-depth investigation of N_2O emission from acclimated activated sludge affected by temperature over the relatively broad range of 4–34°C were performed. Moreover, for the sake of understanding the discrepancy of N_2O emission, the activation energies of NO_3^- and N_2O were also estimated. Total bacteria and denitrifying populations targeting the denitrifying *nosZ* gene using quantitative PCR (q-PCR) as well as the

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transcription of *nosZ* gene during one operational cycle using reverse transcription quantitative PCR (RT-qPCR) were monitored to verify the temperature effects of the denitrifying bacteria population and gene expression under long-term acclimation. This study will be beneficial for understanding the N_2O emission during denitrification process in the activated sludge either from activation energy or functional gene expression scales.

MATERIALS AND METHODS

Reactors Long-term operation of fifteen 1 L sequencing batch reactors (SBRs, three parallel bioreactors of each set) at five selected temperature (4°C, 12°C, 20°C, 25°C and 34°C) were carried out. The SBRs were seeded with sludge from the sludge concentration tank of the municipal wastewater treatment plant in Jimei, Xiamen, China. The sludge was acclimated under anoxic conditions for six months to achieve a steady state with a volume of 0.5 L synthetic wastewater fed in to maintain the hydraulic retention time (HRT) at 2 days. The influent contained (per liter): 1060 mg $CH_3COONa\cdot 3H_2O$, 576 mg KNO_3, 18 mg KH_2PO_4, 10 mg $MgSO_4\cdot 7H_2O$, 10 mg $FeSO_4\cdot 7H_2O$, 10 mg $CaCl_2\cdot 2H_2O$ and 1 mL of trace element. The trace element solution consisted of 0.5 g $CoCl_2\cdot 6H_2O$, 0.5 g $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ per liter. At the beginning of each cycle, the constituents in the reactor included 250 mg L^{-1} of COD (sodium acetate as carbon source) and 40 mg L^{-1} of NO_3^-–N. The dissolved oxygen (DO) concentration of influent was below 0.2 mg L^{-1} by flushing the influent with argon and pH was adjusted at about 7.0 with HCl solution. Each cycle of SBR consisted of 10 min feeding, 23 h operation, 40 min settling and 10 min decanting. The solid retention time (SRT) was maintained at about 20 days and the mixed liquor suspended solid (MLSS) was approximately 1500 mg L^{-1}.

Gas sampling under anoxic incubations The incubation process was conducted in a robotized incubation system similar to that described by Molstad et al. (17), but with improvements developed by the same group (UMB Nitrogen group, Norwegian University of Life Sciences). The system consisted of a gas chromatography (GC, Agilent 7890A, Agilent, Palo Alto, CA, USA) coupled with an auto-sampler, a peristaltic pump and a thermostatic water bath. An electron capture detector (ECD) and a thermal conductivity detector (TCD) were equipped for N_2O and N_2 analysis, respectively. The oven temperature and detector temperature were 50°C and 200°C for N_2 with high purity helium (99.999%) as carrier gas. As for N_2O, 50°C and 340°C were set, with mixture gas of 90% argon and 10% methane as carrier gas. 50 mL of activated sludge from 1 L-SBR was transferred to 120 mL-flasks in triplicate as soon as being fed with the synthetic wastewater. Flasks were then capped with butyl-rubber stoppers and aluminum caps with three cycles of evacuation and filling with helium. A final over-pressure in the flask was released by piercing a needle filling with 0.5 cm water through the rubber stopper. Afterwards, the flasks were transferred to the water bath at 4°C, 12°C, 20°C, 25°C and 34°C, respectively, N_2O and N_2 were then monitored periodically using GC in 24 h-incubation procedure. The gas sample was withdrawn by a peristaltic pump and an equal volume of helium was injected to keep constant atmospheric pressure in the flask. The dilution effect and blank control was taken into account when calculating the accumulation of N_2O and N_2 of denitrification for each time increment. The negative control filled with helium at the end of flush was pretreated as mentioned above. No N_2O signal in the control was detected. Simultaneously, the measured N_2 signal was two orders of magnitude lower than that in the air, indicating that the applied method could effectively eliminate the interference from ambient atmosphere.

Effects of temperature on NO_3^- and N_2O reduction To determine the maximum biomass-specific NO_3^- and N_2O rates accurately, the concentration levels of electron acceptors and donors should be chosen on the basis of results from pre-experiments, to ensure ample supply throughout the whole incubation. Batch tests were conducted at 4°C, 12°C, 20°C, 25°C and 34°C. At the beginning, the reactors were supplied with KNO_3 and sodium acetate, giving a final concentration of 80 mg L^{-1} of NO_3^-–N and 350 mg L^{-1} of COD at 4°C, while 150 mg L^{-1} of NO_3^-–N and 600 mg L^{-1} of COD at other temperature levels. Liquid samples were drawn out and analyzed for NO_3^- concentration every 20 min for 5 h. The specific nitrate reduction rates could be determined by linear regression of concentration–time profile. Regarding N_2O, the absence of NO_3^- was required in measuring N_2O reduction activity, so appropriate anoxic incubation coupled with periodically monitoring was necessary to ensure depletion of NO_3^-. To achieve a high dose of above 50 mg L^{-1}, the N_2O solution was prepared by purging with pure N_2O for 5 min. The supernatant was decanted to one quarter of the reactor volume after settling and N_2O solution as well as adequate sodium acetate was injected as complements. Then the flask with 50 mL pretreated activated sludge was immediately transferred to the water bath with a magnetic stirrer. After about 1 min of equilibrium between liquid and gas phases, the over-pressure was released as mentioned above. Gas was sampled from the headspace of the flask with an interval of 7.5 min until the depletion of N_2O.

Activation energies for NO_3^- and N_2O reduction V_{max} for NO_3^- and N_2O reduction without limitation of carbon source could be estimated from the data by nonlinear regression, using the Michaelis–Menten function (Eq. 1):

$$R = \frac{V_{max}\cdot[S]}{K_m + [S]}X \quad (1)$$

where R is the reduction rate (mg $L^{-1}h^{-1}$), V_{max} is the maximum reduction rate (mg g^{-1} VSS h^{-1}), K_m is the affinity constant for NO_3^- or N_2O (mg L^{-1}), [S] is the average substrate concentration (mg L^{-1}), and X is the biomass concentration (g VSS L^{-1}).

The N_2O concentration in liquid phase (C_{N_2O} in mg N L^{-1}) was calculated based on Eq. 2 (18):

$$C_{N_2O} = \frac{[(kRT\times S_{N_2O}\times V_l)+(S_{N_2O}\times V_g)]}{V_l} \quad (2)$$

where k is the solubility coefficient, R is the perfect gas constant (0.082 L atm mol^{-1} K^{-1}), T is the absolute temperature (K), S_{N_2O} is the concentration of N_2O in the headspace (mg N L^{-1}), V_l is the liquid volume of the flask (L) and V_g is the gas volume of the flask (L).

The NO_3^- and N_2O reduction were fitted to the Arrhenius function of temperature (Eq. 3):

$$V_{max} = A\cdot e^{-Ea/RT} \quad (3)$$

where Ea (kJ mol^{-1}) is apparent activation energy, A is the apparent pre-exponential factor, R is the perfect gas constant (8.314 J mol^{-1} K^{-1}), and T is the absolute temperature (K). Taking the logarithm of the equation, Ea and A can be derived from the slope and intercept values of the plot of ln (V_{max}) vs. 1/T (Eq. 4).

$$\ln(V_{max}) = -\frac{Ea}{R}\cdot\frac{1}{T} + \ln A \quad (4)$$

Extraction of nucleic acids The activated sludge was sampled in the tube and centrifuged at 3000 ×*g* for 5 min, then the supernatant was poured off. The DNA was extracted from 0.5 g activated sludge (wet weight after centrifugation) with the Soil DNA Kit (Omega, Norcross, GA, USA) according to the manufacturer's protocols. For RNA analysis, the samples should be processed immediately without storage since RNA owns weak stability, which is not always fixed. Total nucleic acids were firstly isolated by using the method described by Griffiths et al. (19) with minor modifications. The RNeasy mini kit (Qiagen, Germany) was used to purify the extracted nucleic acids, which had been digested first using the RNase-free DNase I (Fermentas, Canada) at 37°C. The image of large/small subunit Ribosomal RNA by agarose gel electrophoresis was used to assessed RNA integrity. The concentration and purity of DNA and RNA were determined by UV–Vis spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA). After crude extraction and purification, nucleic acids with fine quality were obtained. The DNA and RNA samples were stored at −20°C and −80°C for further analysis, respectively.

Reverse transcription A total of 1 μL of RNA from each sample was performed to create first-strand cDNA with random hexamer primers (50 pmol) and Oligo dT Primers (25 pmol) using the PrimeScript RT reagent Kit (Takara, Japan) following the manufacturer's instructions. PCR procedure was performed at 37°C for 15 min and 85°C for 5 s for reverse transcriptase deactivation.

Real-time PCR analysis Copy numbers of *nosZ*, 16S rRNA gene and *nosZ* transcripts were quantified by an ABI PRISM 7500 Real-Time PCR System (Applied Biosystem, Warrington, UK). The sludge samples for DNA analysis were taken in triplicate at each batch experiment, while the samples analyzed for transcription of functional genes were taken periodically during 24 h-incubation. *nosZ* and 16S rDNA were amplified with the primer pairs *nosZ*-F: *nosZ* 1622R (20) and 338F: 519R (21), respectively. The 20-μL reaction mixture contained 10 μL of SYBR Premix Ex Taq II (Takara), 0.4 μM of each primer, 0.4 μL of 50× ROX II and 2 μL of template DNA. Thermal cycling conditions for *nosZ* and 16S rDNA were as follows: 95°C for 30 s followed by 40 cycles at 95°C for 5 s, 60°C for 60 s and fluorescence was read during each cycle at 82°C for 32 s, followed by melting curve analysis.

During each qPCR run, the standard curves for the quantitative PCR were established using *nosZ* and 16S rDNA gene fragments cloned into plasmid, which were extracted using Plasmid Mini Kit I (Omega) and diluted in series. Plasmid concentration was determined by UV–Vis spectrophotometer. All PCR runs including unknown samples, standard curves and no template control (NTC) were performed in triplicates. The amounts of investigated gene were analyzed using the sequence detection system (SDS 2.3 software, Applied Biosystem). Standard curve for quantitative PCR was linear for *nosZ* and 16S rRNA gene (r^2=0.992–0.999) with a PCR efficiency between 91% and 118%.

RESULTS

N_2O and N_2 production The specific denitrification rates and transient N_2O accumulation as an obligate intermediate of NO_3^- reduction under different temperatures were shown in Fig. 1. N_2 sustained at the concentration level in the range of 62.8–67.3 μmol per flask with 50 mL activated sludge at the end