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Effects of Fe(II) on N_2O emissions from anammox reactors

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ABSTRACT

In this study, the effects of Fe(II) on N_2O emissions from an anammox reactor were investigated for the first time. The measured N_2O emissions were determined to be 0.06%–0.10% g/g–N removal during this study. Increasing Fe(II) addition from 0.25 mL/L influent to 0.50 mL/L influent, N_2O increased from 0.06% to 0.10% g/g–N removal. The appropriate amount of Fe(II) added should be controlled below 0.25 mL/L (about 0.5 mg/L). The results in this study suggest that Fe(II) coupled with NO_2–N is the cause of N_2O production. Genetic analysis of the 16S rRNA gene indicates that *Nitrosomonas sp. ENI-11* is another probable cause of N_2O production.

Keywords: Fe(II); Anammox; Green-house gas; N_2O

1. Introduction

In wastewater treatment, N_2O is an unwanted gas, which has a global warming potential about 300 times higher than that of CO_2 and is involved in the destruction of the stratospheric ozone layer. In view of this, controlling emission is an important consideration during wastewater treatment. The anammox process, a newly developed biological nitrogen removal process, still produces N_2O. Results from Weissenbacher et al. [1] and Desloover et al. [2] show that N_2O emissions are significant in full-scale plants (up to 6.6% of the total nitrogen (TN) output). Previous studies have generally agreed that emission of N_2O by ammonia oxidizing bacteria (AOB) is the major pathway: nitrifier denitrification, and NH_2OH oxidation. Kampschreur et al. [3], however, reported high N_2O emissions (0.6% of TN output, almost six times higher than that reported by Okabe et al. [4]) from an anammox reactor in a full-scale two-reactor nitritation-anammox process. Okabe et al. [4] indicated that denitrification by putative heterotrophic denitrifiers present in the inner part of the granule was considered the most probable cause of N_2O emissions from anammox reactors. A similar result was also reported by Meng [5], and the average emission of N_2O was only 0.07% nitrogen output using artificial wastewater. Thus, nitrous oxide emissions from an anammox reactor will also have to be considered for their potentially negative impact on the carbon footprint of the technology [6]. Fe(II) is present in the influent due to the dosage of $FeSO_4$, which is widely used as a flocculant. Kampschreur et al. [7] suggest that Fe(II) oxidation can be a significant cause of N_2O formation. To date, there are few reports describing its effects on N_2O emission in an anammox reactor [8]. Emissions of N_2O due to Fe(II) hamper implementation of the anammox process and should therefore be avoided.

In this study, an anammox reactor was used to study the effect of Fe(II) on the N_2O emissions. In addition, genetic analysis using the 16S rRNA gene was employed to characterize the microbial population of the anammox granules.

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2. Materials and methods

2.1. Anammox reactor and substrate

The reactor had an inner diameter of 20 cm with a total liquid volume of 50 L including a reaction zone of 40 L and a recycling vessel of 10 L. The reactor was made of acrylic resin and had a water jacket for temperature control. The influent was introduced to the recycling vessel by the influent pump, and mixed with part of the effluent, which was collected in the recycling vessel (with mixer and heater) for use as recycling water (Fig. 1). The influent and recycling water was introduced into the bottom of the reactor by the input pump. The pH was adjusted by an online pH controller (TPH/T-10, Tengine, China) using 0.5 mol/L H_2SO_4 in the recycling vessel. The reactor was enclosed in a black-vinyl sheet to inhibit growth of photosynthetic bacteria and algae. The anammox reactor was operated as described by Wenjie et al. [9–12]. The composition of substrate was shown in Table 1.

The anammox seed sludge used in the reactor was taken from a pilot-scale anammox reactor (unpublished). The seed sludge was granular activated carbon (GAC)-based granules with settling velocity over 150 m/h (Fig. 2). The initial seeding concentration (mass of mixed liquor suspended solids (MLSS) per liter) was set at 4 g MLSS/L.

2.2. Analytical methods

NO_2–N and NH_4–N were measured by the colorimetric method according to Standard Methods [13]. TN was determined by the persulfate method using the UV spectrophotometric screening method for quantification of TN as NO_3–N (the oxidization product of the persulfate digestion). NO_3–N (of the original sample) was determined by calculation of the difference of TN and the sum of NO_2–N and NH_4–N. The pH was measured by using a pH meter (9010, Jenco, USA), and dissolved oxygen (DO) was measured by using a DO meter (6010, Jenco, USA).

2.3. Gas collection and analysis

Gas was collected through the gas–solid separator and the volume was measured using an inverted cylinder containing tap water with the pH lowered to 3 using 1–N H_2SO_4. Gas analyses were performed by using a GC-112A gas chromatograph (INESA INSTRUMENT, China). The dissolved N_2O gas concentration in the liquid phase was measured by using the headspace method [4]. N_2O emission was calculated relative to the nitrogen removal rate.

2.4. Scanning electron microscopy (SEM)

Samples were first washed in a 0.1-M phosphate buffer solution (pH 7.4) for 5 min. Then samples were hardened for 90 min in a 2.5% glutaraldehyde solution prepared with the buffer solution. Next, samples were washed in the buffer solution three times for 10 min each and then fixed for 90 min in a 1.0% OsO_4 solution prepared with the buffer solution. After washing samples three times for 10 min each in the buffer solution, they were dewatered for 10 min each in serially graded solutions of ethanol at concentrations of 10%, 30%, 50%, 70%, 90%, and 95%. SEM observations were conducted by using a scanning electron microscope (JSM-6380LV, JEOL, Tokyo).

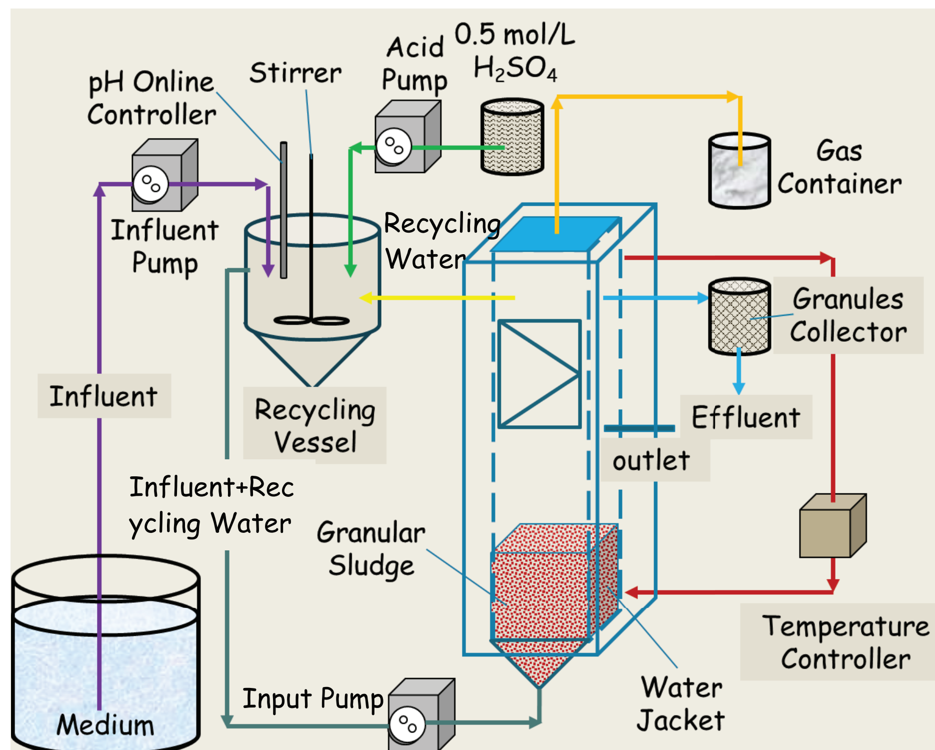


Fig. 1. Schematic view of the anammox reactor system.

Table 1
Substrate composition

Composition	Concentration (mg/L)
$(NH_4)_2SO_4$, $NaNO_2$ (as mg N/L)	200–1,000
$KHCO_3$	1,000
KH_2PO_4	50
$CaCl_2·2H_2O$	100
$MgSO_4·7H_2O$	200
$Na_2S_2O_3$	24.81
Trace element solution 1 (g/L): $FeSO_4·7H_2O$ 10, $C_{10}H_{14}N_2Na_2O_3$ 5.6	Day 1550–1580, 0 mL/L; day 1581–1610, 0.25 mL/L; day 1611–1640, 1.0 mL/L
Trace element solution 2 (g/L): $MnCl_2·4H_2O$ 0.352, $CoCl_2.6H_2O$ 0.096, $NiCl_2·6H_2O$ 0.08, $CuSO_4.5H_2O$ 0.1, $ZnSO_4·7H_2O$ 0.172, $NaSeO_4.10H_2O$ 0.105, $NaMoO_4·2H_2O$ 0.11, $C_{10}H_{14}N_2Na_2O_3$ 5.0	1 mL/L

Fig. 2. Appearance of the granular activated carbon.

2.5. DNA extraction and PCR amplification

Meta-genomic DNA was extracted using an ISOIL kit (Wako, Osaka, Japan) according to the manufacturer's instructions. Amplification of the 16S rRNA gene was performed with Phusion High-Fidelity DNA polymerase (FINNZYMES, Finland) using conserved eubacterial primers 6F (forward primer: 5′-GGAGAGTTAGATCTTGGCTCAG-3′) and 1492r (reverse primer: 5′-GGTTACCTTGTTACGACT-3′). PCR was carried out according to the following thermocycling parameters: 30 s initial denaturation at 98°C, 25 cycles of 10 s at 98°C, 30 s at 51°C, 20 s at 72°C and 5 min final elongation at 72°C. The amplified products were electrophoresed on a 1% agarose gel and extracted fragments were purified using Wizard SV Gel and PCR Clean-Up System (Promega, USA).

2.6. Cloning and sequencing of the 16S rRNA gene

The purified fragments were ligated into the EcoRV site of pBluescript II KS+ (Stratagene, USA) and *Escherichia coli* DH10B was transformed using the constructed plasmids. White colonies including the insert were randomly chosen and the plasmids were extracted by the alkaline method. The nucleotide sequences were determined with a 3130xl genetic analyzer and a BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). The sequences determined in this study were compared with the sequences in the nr database using the basic local alignment search tool program (BLAST) on the NCBI website.

2.7. Denaturing gradient gel electrophoresis (DGGE)

Partial 16S rRNA gene was amplified by PCR with a eubacterial primer set, 1055F-1392R [14]. The extracted meta-genomic DNA and cloned plasmids were used as templates for the sample and markers in the DGGE, respectively. The amplified fragments were purified and combined with the GC-clamp (5′-CGCCCGCCGCGCCCCGCG CCCGTCCCGCCGCCCCCGCCCG-3′) at the 5′ termini by a second PCR using a primer set, 357F with GC-clamp and 534R. The products were resolved by DGGE for 14 h at 90 V at 60°C using the DCode system (Bio-Rad, Hercules, CA, U.S.A.). An 8% polyacrylamide gel with a 30%–65% denaturing gradient was used, where 100% denaturant was defined as 7 Murea and 40% formamide. The gel was stained with SYBR-Gold (Invitrogen, USA) and visualized using the FLA-2000 system (Fuji Photo Film, Tokyo, Japan).

3. Results

3.1. Reactor performance

A nitrogen-loading rate (NLR) of 1.0 kg–N/m^3/d was used at the startup of the anammox reactor, followed by gradual increases in the loading as the anammox population was enriched. The anammox reactor had been in operation for more than 3 years prior to the study (Fig. 3). Throughout the study, the temperature in the reactor was maintained at 33°C ± 1°C, and the DO concentration was held below 0.5 mg/L.

The anammox reactor was operated at a NLR of 2.74 kg–N/m^3/d. The influent NH_4–N and NO_2–N concentrations were set to 210 mg/L and 270 mg/L, respectively (Fig. 3(A)). During the study, effluent NH_4–N, NO_2–N and NO_3–N remained almost unchanged. A TN removal efficiency of 85% was achieved.

Fig. 3(B) shows the ratios of effluent NO_2–N removal to NH_4–N removal, and effluent NO_3–N production to NH_4–N removal. Because the anammox reactor had been in operation for more than 3 years prior to the study, the effluent NO_2–N removal to NH_4–N removal ratio, and the effluent NO_3–N production to NH_4–N removal ratio were an average of 1.25 and 0.2, respectively, which is lower than previously reported values [15]. Nitrifier denitrification [16] and chemical nitrite reduction [7] caused by Fe(II) addition were considered to be the main reasons for additional NO_2–N and NO_3–N removal.

During the study, the anammox granules were sampled to identify the microbial population. Sequence analysis of the major DGGE bands showed that *Kuenenia stuttgartiensis*, *Planctomycete KSU-1* and *Uncultured bacterium clone KIST-JJY001* became dominant (Table 2), which indicates that the anammox reaction prevailed in the reactor.

3.2. Effects of Fe(II) on N_2O emissions

As shown in Table 1, addition of Fe(II) was divided into three phases: 0, 0.25, and 1.0 mL/L influent. During standard operation over the 3 years preceding this study, 0.50 mL/L influent was added according to the suggested value [17]. Therefore, N_2O emissions with 0.50 mL/L influent were analyzed using operational data. The gas products of the anammox reactor were mainly composed of N_2, CO_2 and N_2O. The N_2O emissions increased immediately with an increase of Fe(II) addition from 0.25 mL/L influent to 0.5 mL/L influent (Fig. 4). When the Fe(II) addition was further increased to 1.0 mL/L influent, N_2O emissions remained at 0.10% g/g–N removal (Fig. 4). A total increase of N_2O emissions was 40% from the beginning to the end of the study. Additionally, N_2O emissions remained steady when the Fe(II) addition was decreased from 0.25 mL/L influent to 0 mL/L influent.

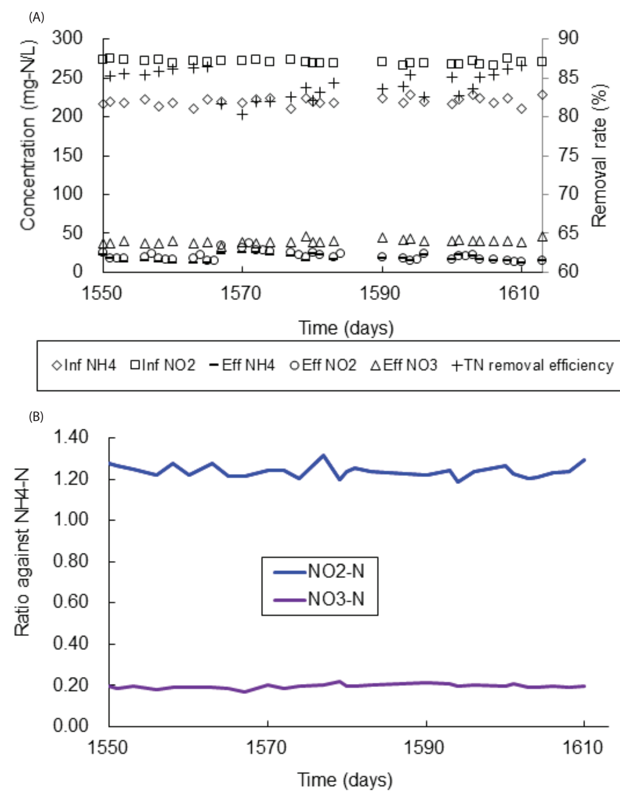


Fig. 3. Performance of the anammox reactor: (A) changes in nitrogen concentrations (nitrogen loading rate, 2.74 kg–N/m³/d) during the study period; (B) ratios of effluent NO_2–N removal/NH_4–N removal, and effluent NO_3–N production/NH_4–N removal.
Note: Inf – Influent; Eff – Effluent.

3.3. Bacteria community analysis

Sequence analyses of the major DGGE bands are summarized in Table 2. Sludge samples were collected from the anammox reactor at the end of each stage. A minimum of three samples were sampled and analyzed three times each to ensure data accuracy. *Kuenenia stuttgartiensis* and *Planctomycete KSU-1*, which are known to be anammox strains, prevailed as the majority of the clones (43%). From the 16S rRNA analysis, *uncultured bacterium clone KIST-JJY030*, *uncultured bacterium clone Dok04* and *uncultured bacterium clone Dok53*, which have often been detected as being commonly co-existing with anammox bacteria, were also identified in this study. The functions of co-existent anammox bacteria in anammox processors are still largely unknown. *Nitrosomonas* sp. ENI-11 and *Nitrobacter winogradskyi strain R1.30*, which are known as AOB and nitrite oxidizing bacteria (NOB), were also detected in the anammox reactor. However, samples from the same reactor with different amounts of Fe(II) addition showed high similarity in community structure.

4. Discussion

Fe(II) shows a positive effect on the bioactivity of anammox bacteria [9,17]. Therefore, Fe(II) is usually added to the feed stock of the anammox bacteria. In this study, increasing Fe(II) addition showed a clear relation to N_2O emissions (Fig. 2). The results of this study indicate that increasing the addition of Fe(II) induced N_2O emissions, which can be explained by the chemical conversions given in Eqs. (1) and (2).

Table 2
Homology search results for 16S rRNA gene sequences of the main bacterial members in the community

Taxon	Identity (%)	Number of clones			
		0.5 mL/L	0 mL/L	0.25 mL/L	1 mL/L
Nitrobacter winogradskyi strain R1.30	96–97	2	2	3	2
Nitrosomonas sp. ENI-11	98–100	2	2	3	2
Uncultured bacterium clone KIST-JJY030	98	2	3	2	2
Uncultured bacterium clone 80	88	1	0	1	0
Kuenenia stuttgartiensis	96–100	10	11	9	10
Planctomycete KSU-1	99	3	4	5	2
Uncultured bacterium clone 37	95	1	1	0	1
Uncultured bacterium clone Dok04	96	1	0	1	0
Uncultured Chloroflexi bacterium clone ST01-SN2H	93	1	1	1	0
Uncultured bacterium clone AA102	88	1	0	0	1
Uncultured bacterium clone Dok53	99	1	0	1	0

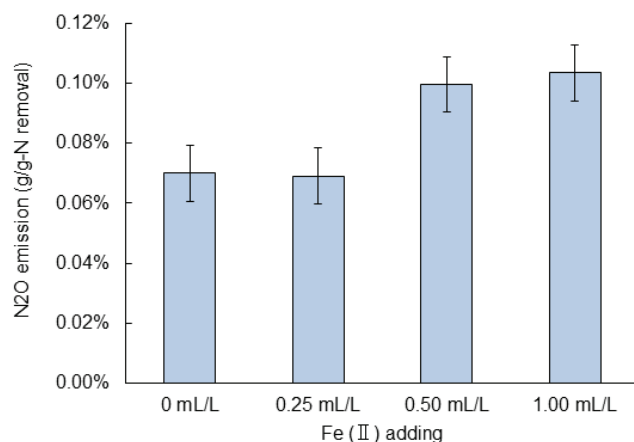


Fig. 4. Effects of Fe(II) addition on N_2O emissions.

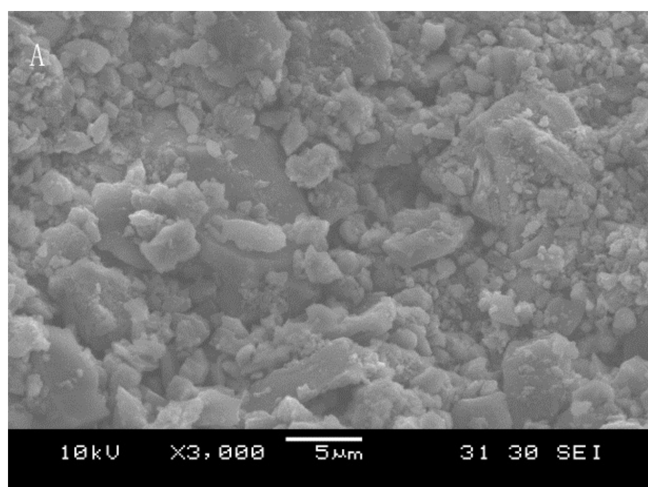
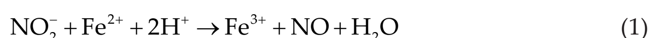


Fig. 5. SEM microphotograph of granules (day 1640).



$$NO_2^- + Fe^{2+} + 2H^+ \rightarrow Fe^{3+} + NO + H_2O \quad (1)$$

$$NO + Fe^{2+} + 1H^+ \rightarrow Fe^{3+} + 0.5N_2O + 0.5H_2O \quad (2)$$

$$Fe^{2+} + 2OH^- \rightarrow Fe(OH)_2 \quad (3)$$

There was an increase from 0.06% to 0.1% in N_2O emissions when the Fe(II) input increased from 0.25 to 0.50 mL/L, but N_2O emissions remained stable when the Fe(II) addition was further increased to 1.0 mL/L. The pH of the anammox reactor rises while treating wastewater [12], and thereafter, a part of the Fe(II) will precipitate as $Fe(OH)_2$ and be removed through sedimentation (Equation 3). The additional Fe(II) will therefore no longer induce N_2O emissions, and thus a further increase in Fe(II) from 0.5 to 1.0 mL/L had no effect on N_2O emissions in this study.

SEM micrographs (Fig. 5) indicate that relatively large numbers of precipitates were attached to the anammox granules. Kampschreur et al. [7] has indicated that the Fe(III) precipitates pulled the above reaction (Eq. (2)). The results of this study are consistent with previous findings that Fe(II) coupled with NO_2–N causes N_2O production [7].

N_2O emissions no longer increased when the Fe(II) addition decreased to zero. This means that at least 0.06% g/g–N was removed by N_2O emissions produced from other sources, in addition to chemical nitrite reduction by Fe (II). These results differ from those of Kampschreur et al. [7]. It is supposed that N_2O is produced as an intermediate of incomplete heterotrophic denitrification due to a low COD/N ratio [4]. However, no denitrification bacteria were detected in this study. Thus, it is difficult to explain the increasing N_2O emissions during this study. Furthermore, *Nitrosomonas* sp. ENI-11 and *Nitrobacter winogradskyi strain R1.30* were detected in all of the samples. Hynes and Knowles [18] indicate that the presence of the oxidizer *Nitrobacter winogradskyi* has no effect on the formation of N_2O. *Nitrosomonas* is a denitrifier which in a state of oxygen stress will use nitrite as a terminal electron acceptor and produces nitrous oxide [18]. Therefore, *Nitrosomonas* sp. ENI-11 might be the primary cause of N_2O emissions in this study, which differs from the results of Okabe et al. [4]. The results of this study are consistent with the literature indicating that AOB is considered the most probable cause of N_2O production (0.6% of the nitrogen load) in a full-scale anammox reactor treating sludge reject water [3].

Reducing N_2O emissions is still a concern for anammox applications [19]. Kampschreur et al. [3] found high N_2O concentrations (0.6%) in one full-scale anammox reactor. Okabe et al. [4] also reported that N_2O emissions of 0.23% were detected with a nitrogen removal rate of 7.5–15 kg–N/m^3/d. In this study, N_2O emissions were found to be lower than the reported values with controlled Fe(II) addition. Fe(II) is widespread in natural aquatic systems and is used as a flocculant. As a possible risk factor for increasing N_2O emission, Fe(II) concentrations in the wastewater should be investigated before application of the anammox process.

5. Conclusions

An anammox reactor was used to investigate the effects of Fe(II) concentrations on N_2O emissions. Results indicate that N_2O emissions, which increased 40% from the beginning to the end of the study, have a positive correlation with influent Fe(II) concentrations. In addition, at least 60% of the N_2O emissions were produced from other sources in addition to chemical nitrite reduction by Fe(II).

Acknowledgements

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