Journal of Bioscience and Bioengineering
VOL. 118 No. 3, 277–283, 2014

www.elsevier.com/locate/jbiosc

Effect of ammonium nitrogen concentration on the ammonia-oxidizing bacteria community in a membrane bioreactor for the treatment of anaerobically digested swine wastewater

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Received 6 November 2013; accepted 14 February 2014
Available online 26 March 2014

A membrane bioreactor (MBR) was developed for the treatment of anaerobically digested swine wastewater and to investigate the effect of ammonium nitrogen concentration on biological nitrogen removal and ammonia-oxidizing bacteria (AOB) community structures. The MBR achieved a high NH_4^+-N removal efficiency of 0.08 $kgNMLSS^{-1}d^{-1}$ and removed 95% of the influent NH_4^+-N. The TN removal rate was highest of 82.62% at COD/TN and BOD_5/TN ratios of 8.76 ± 0.30 and 3.02 ± 0.09, respectively. With the decrease in ammonium nitrogen concentrations, the diversity of the AOB community declined and showed a simple pattern of DGGE. However, the AOB population size remained high, with abundance of 10^7–10^9 copies mL^{-1}. With the decrease of ammonium nitrogen concentrations, *Nitrosomonas eutropha* gradually disappeared, whereas *Nitrosomonas* sp. OZK11 showed constant adaptability to survive during each treatment stage. The selective effect of ammonium concentration on AOB species could be due to the affinity for NH_4^+-N. In this study, the changes of ammonium nitrogen concentrations in digested swine wastewater were found to have selective effects on the composition of AOB community, and biological nitrogen removal was improved by optimising the influencing parameters.

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[**Key words:** Nitrification; Ammonia-oxidizing bacteria; Anaerobically digested wastewater; Membrane bioreactor; Biological nitrogen removal]

Livestock production farms produce large amounts of highly polluted wastewater, which causes groundwater eutrophication when discharged without proper treatment. Biogas technology is a widely used approach for decreasing organic matter and retrieving renewable energy; however, this technique also produces large amounts of digested liquid, which contains high concentration of ammonium nitrogen. The digested liquid is generally applied to farmlands as fertilizer. Because of large scale of animal breeding in some regions and the restriction on excessive nitrogen returned to the field, many countries had paid attention to the treatment of the digested liquid (1–3). Therefore, it is necessary to explore a feasible treatment process for removing the nitrogen from anaerobically digested swine wastewater.

Anaerobically digested swine wastewater has high concentration of ammonium nitrogen and low C/N ratio (1,2,4). Biological nitrogen removal (BNR) process was adopted to remove nitrogen pollutants using intermittent aeration reactors (IARs), sequencing batch reactors (SBRs) and anoxic/oxic-membrane bioreactors (A/O-MBRs) which were widely adopted to treat digested liquid (3,5,6). Biological nitrogen removal involves the transformation of NH_4^+-N to NO_2^--N by ammonia-oxidizing bacteria (AOB) and further oxidation of NO_2^--N to NO_3^--N by nitrite-oxidizing bacteria (NOB). Subsequently, denitrifying bacteria produce N_2 from NO_2^--N or NO_3^--N. Nitrification of the anaerobically digested liquid at ammonium nitrogen concentrations of 1000–3000 mg/L (1,2) requires high aeration rates, addition of alkalinity and long hydraulic retention time (HRT) (1,7). This process is not economical (8), and high concentrations of ammonium nitrogen will likely inhibit ammonium oxidation (9,10). A COD/TN ratio of 6–8 is required for denitrification, and thus, the low C/N ratio (COD/TN ratio of 1–3) of the digested liquid is insufficient for TN removal (11). Therefore, to biologically treat anaerobically digested swine wastewater, it is necessary to decrease the ammonium nitrogen concentration and increase the C/N ratio.

The ammonia oxidation is the rate-limiting step of BNR (12). To further understand the BNR process, the changes of the composition of AOB community structure in response to variations of environmental factors should be studied. The ammonium nitrogen concentration (13), C/N (carbon-nitrogen) ratio, pH, DO (dissolved oxygen) (14), organic carbon concentration (15) and inorganic carbon concentration are all known to influence the AOB community structure. Most of the previous studies on AOB used synthetic feed water. There have been few studies on AOB communities in biological treatment of anaerobically digested swine wastewater. Besides, for the treatment of digested swine wastewater the effect of ammonium nitrogen concentrations on the shifts of AOB community structures has not been investigated yet.

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1389-1723/$ – see front matter © 2014, The Society for Biotechnology, Japan. All rights reserved.
http://dx.doi.org/10.1016/j.jbiosc.2014.02.017

In this study, digested swine wastewater with different ammonium nitrogen concentrations was treated using a membrane bioreactor to enhance the ammonium oxidation and TN removal efficiencies. The effects of ammonium nitrogen concentration on the shift of AOB community were investigated to explore the ammonium oxidation mechanisms involved. With a proper regulation of parameters, the AOB community could have high-efficiency on ammonia oxidation.

MATERIALS AND METHODS

Experimental design and MBR operation strategy The reactor was made of Plexiglas and had a working volume of 40 L (W 28 × L 46 × H 31 cm). The reactor was divided by a baffle into two parts: the biofilm zone (B zone) and the membrane zone (M zone) with volumes of 26.7 L and 13.3 L, respectively. A recycle ratio of 400% was maintained between the B and M zones. The B zone was filled with polyethylene carriers (diameter of 25 mm, height of 9 mm, porosity of 95%, specific surface area 500 of m^2 m^{-3} and filling rate of 50%). The membrane module (Mitsubishi Rayon, Japan) was installed in the M zone. The PVDF membrane had a pore size of 0.1 μm and an effective area of 0.14 m^2.

The inoculated sludge was collected from the aeration tank used to treat the anaerobically digested liquid from a swine farm. The inoculated sludge volume was 40 L with a concentration of 3000 mgSS/L. The reactor was allowed to acclimate for over 3 months. Because of the recycle flux, there were not only biofilm but also free sludge contained in B zone. The MLSS was 3500 mgSS/L before the experiment started. The water temperature in the reactor was maintained at 25°C. Two continuously aerating perforated pipes were installed at the bottom of the membrane module to provide oxygen and flush the membrane surface. The aeration rate was determined by compared with the aeration for microbial consumption and the aeration intensity recommend by membrane manufacturer (100–150 m^3 m^{-2} h^{-1}), and chose the bigger one. On the condition of the membrane was not fouled, the aeration rate in the M zone was adjusted according to the influent organic matter and ammonium nitrogen concentrations. The B zone was not aerated. The pH in the reactor was not controlled and remained consistently higher than 7.0. The effluent was sucked by a peristaltic pump (YZ100, Qili, China) in constant-flow mode with filtration/idle ratio of 8 min/2 min. The membrane module was physically cleaned with tap water and then submerged in 2000 mg $NaClO_3$/L solution for 24 h for recovery of the membrane flux, when the suction pressure reached −35 kPa. The SRT was on average of 90 d by discharging 450 mL of MLSS from M zone every day.

The anaerobically digested wastewater used in this study was collected from the effluent of an anaerobic digester (effective volume of 500 m^3) treating the manure and flushing water of the swine farm mentioned above. The operational period was divided into four stages, as shown in Table 1. Between each two stages there was a transition period of about 20 d, during which the influent ammonium nitrogen concentration was decreased stepwise. Table 1 presents the data at stable status. Because the water quality of the influent varied over time, the organic loading rate of the MBR was maintained at the identical level of 0.5 kg COD m^{-3} d^{-1}. The feed water was the raw digested swine wastewater in stage I. To test the effect of the ammonium nitrogen concentration and C/N ratio on the BNR and the AOB community, a portion of the ammonia was stripped according to the method of Guštin et al. (16). The ammonia was stripped prior to the MBR in stages II, III and IV to achieve the desired ammonium nitrogen concentrations and C/N ratios. From stage I to IV, the ammonium nitrogen concentration decreased, and meanwhile the C/N ratio increased from 1.78 ± 0.06 to 8.76 ± 0.30.

During the four stages, microbial samples were collected at eight time points (sample 1–sample 8), and each sample was consisted of mixed liquor samples from B zone and M zone. The samples were collected twice for every stage, i.e., at the beginning and the end. The samples were preserved by freezing at −70°C.

TABLE 1. Operation parameters of the MBR in four stages.

Parameter	Stage I	Stage II	Stage III	Stage IV
Duration (d)	30	32	18	15
HRT (d)	8	8	5	3
Organic loading rate (kg COD $m^{-3}d^{-1}$)	0.5	0.5	0.5	0.5
TN loading rate (kg N m^{-3} d^{-1})	0.27	0.11	0.06	0.06
COD (mg/L)	3839 ± 194	3456 ± 90	2831 ± 98	1572 ± 50
BOD_5 (mg/L)	657 ± 180	696 ± 101	561 ± 79	537 ± 9
TN (mg/L)	2154 ± 152	909 ± 104	327 ± 26	180 ± 9
NH_4^+-N (mg/L)	2039 ± 152	770 ± 94	265 ± 21	90 ± 6
pH	8.08 ± 0.13	7.41 ± 0.19	7.26 ± 0.15	7.24 ± 0.21
COD/TN	1.78 ± 0.06	3.85 ± 0.41	8.68 ± 0.51	8.76 ± 0.30
BOD_5/TN	0.30 ± 0.07	0.77 ± 0.06	1.71 ± 0.18	3.02 ± 0.09
Aeration rate in M zone (L h^{-1})	2500	2000	1500	800

Analytical methods The pH and DO were measured on site using a handheld analyser (HACH Sension378, HACH Company, USA). COD and TN were measured according to standard methods (17). BOD_5 was measured using a BOD_5 analyser (OxiTop, WTW, Germany). The ammonium nitrogen was measured using the distillation method (B-324, BüCHI, Switz); NO_2^--N and NO_3^--N were measured using a flow injection analyser (FIAstar 5000, FOSS, Denmark). Soluble total organic carbon (TOCs) was measured using an Apollo 9000 combustion TOC analyser (TeKmar, USA).

The calculated influent nitrogen concentration in B zone was related to the influent nitrogen concentration, recycle ratio (R) and recycle nitrogen concentration (18). The calculated influent N of B zone is given by Eq. 1.

$$\text{Calculated influent N of B zone} = (\text{influent N} + \text{recycle N} \times R)/(1 + R) \quad (1)$$

Increasing the pH value could convert the ammonium nitrogen to free ammonia. The free ammonia (FA) concentration is calculated by Eq. 2 (19).

$$FA(mg/L) = \frac{[NH_4^+ - N] \times 10^{pH}}{e^{\{\frac{6344}{273+t}\}} + 10^{pH}} \quad (2)$$

The biodiversity of the samples was calculated with Shannon–Weiner biodiversity index formula by Eq. 3 (20).

$$H = -\sum_{i=1}^{s} P_i \times \log P_i \quad (3)$$

DNA extraction and PCR-DGGE analysis Total DNA from the MLSS samples was extracted using a commercially available kit according to the manufacturer's instructions (Qiagen, USA). The concentration and quality of the DNA was determined at A260 nm and A280 nm using a spectrophotometer (Bio-Rad, USA). The PCR amplification was performed using a C1000 thermal cycler (Bio-Rad). The AOB gene was amplified using primer pairs of amoA-1F (with a GC-clamp) and amoA-2R as described by Rotthauwe et al. (21). Denaturing gradient gel electrophoresis (DGGE) was performed using the Dcode system (Bio-Rad). The PCR amplicons were separated using a 6% polyacrylamide gel in 0.5× TAE buffer with a linear denaturing gradient from 30% to 60%. The gel was run at 60°C and 85 V for 16 h and stained with ethidium bromide (0.5 mg/L) for 20 min. Then the gel was washed with 400 mL distilled water for 10 min and photographed using a MiniLumi gel documentation system (DNR, Israel).

Sequencing and phylogenetic analysis The bands on the gel were excised and transferred to sterile 2-mL tubes. After the addition of 40 μL of distilled water, the samples were incubated at 37°C overnight. The supernatants containing the DNA recovered from bands were re-amplified using the amoA-1F/amoA-2R primer pair without a GC-clamp, as described above. The fresh PCR products were then cloned into a pGEM-T vector (Tiangen, China) and transformed into *Escherichia coli* DH5α cells (Tiangen, China). Colonies with inserts were randomly selected and used to extract plasmid DNA. The inserts were sequenced using the universal primer pair T7/SP6 on an ABI 377 DNA sequencer (Applied Biosystems, USA).

The obtained sequences were checked for chimeras using Mothur 1.13.0 (http://www.mothur.org/). The sequences used for phylogenetic affiliation were identified using NCBI BLAST. ClustalX was used to align the amoA gene sequences, and the software MEGA 4 was used to construct the phylogenetic tree with a neighbour-joining bootstrap analysis with 1000 replicates.

Quantitative real-time PCR analysis Real-time PCR was performed using an IQ5 system (Bio-Rad, USA). The amplification was conducted in a 25 μL volume containing the following reagents: 12.5 μL of SYBR green mixture (Qiagen, Germany), 100 nM of each primer and 1.0 μL of template DNA. The population size of the AOB was determined by calculating the copy numbers of the amoA gene with the primer pairs of amoA-1F (without a GC-clamp) and amoA-2R. Furthermore, the 16S rRNA of total bacteria copy number was also detected for normalization with primer pairs of Bac1 and Bac2 (22). The PCR efficiency (E), calculated using the equation $E = (10^{-1/slope} - 1) \times 100$, was close to 100%.

Statistical analyses DGGE profiles were converted into matrix data based on the number of bands and their relative intensities among the individual samples using the software Quality One v4.5 (Bio-Rad, USA). The correlation between environmental factors and the AOB community was evaluated using canonical correspondence analysis (CCA) multivariate statistics with Canoco 4.5 software.

Nucleotide sequence accession numbers The partial amoA gene sequences generated in this study were deposited in GenBank under accession numbers KF828886–KF828900.

RESULTS

Nitrogen removal in the MBR The effluent nitrogen concentrations of MBR, including the NH_4^+-N, NO_2^--N and NO_3^--N concentrations, are shown in Table 2. The influent and effluent nitrogen

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