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Characterization of nitrifying microbial community in a submerged membrane bioreactor at short solids retention times

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HIGHLIGHTS

- Solids retention time led to selection of different ammonia-oxidizing bacteria (AOB) in the MBR.
- All groups of AOB and nitrite-oxidizing bacteria (NOB) except *Nitrosolobus* and *Nitrococcus* were found in MBR.
- *Nitrosomonas* and *Nitrospira* were the dominant group of AOB and NOB in three reactors.
- The SRT had the strongest effect on the nitrifying bacterial community structure.

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ABSTRACT

This study investigated the nitrifying bacterial community in membrane bioreactor (MBR) at short solids retention times (SRTs) of 3, 5 and 10 days. The denaturing gradient gel electrophoresis results showed that different types of ammonia-oxidizing bacteria (AOB) can survive at different operating conditions. The diversity of AOB increased as the SRT increased. The real-time PCR results showed that the *amoA* gene concentrations were similar when MBRs were stabilized, and it can be a good indicator of stabilized nitrification. The results of clone library indicated that *Nitrosomonas* was the dominant group of AOB in three reactors. The microarray results showed that *Nitrospira* was the dominant group of nitrite-oxidizing bacteria (NOB) in the system. All groups of AOB and NOB except *Nitrosolobus* and *Nitrococcus* were found in MBR, indicated that the nitrifying bacterial community structure was more complicated. The combination of some molecular tools can provide more information of microbial communities.

1. Introduction

In recent years, membrane bioreactors (MBRs) have begun to operate successfully in municipal or industrial wastewater treatment plants worldwide. The MBR process has advantages over the conventional activated sludge (AS) in terms of effluent quality, reliability, footprint size, and possibly lower sludge production. In the MBR process, the membrane replaces the sedimentation tank for solids-liquid separation. Since the membrane retains virtually all solids in the reactor, including bacteria and viruses, the solids retention time (SRT) can be increased to very high values to maintain high biomass concentrations, reduce solids production and minimize reactor volume. As a result, biomass (mixed liquor) concentrations in MBRs increase at higher SRT and the increase frequently creates serious problems. High aeration rates are required to provide adequate oxygen supply due to increased biomass concentration and decreased mass transfer efficiency. Effective membrane scouring is difficult to achieve because of increased mixed liquor viscosity. MBR operation at shorter SRT may be a prudent option for facilities that wish to avoid these problems (Dizge et al., 2013; Han et al., 2005).

When an MBR is operated at a short SRT, nitrification becomes a challenge, because autotrophic nitrifiers are very slow growing microorganisms. Nitrification is performed by two different bacterial groups including ammonia-oxidizing bacteria (AOB) that are responsible for oxidization of ammonium to nitrite, and nitrite-oxidizing bacteria (NOB) that convert nitrite to nitrate. The functional *amoA* gene is considered to be a suitable marker to identify AOB due to their primer specificity (McTavish et al., 1993). Only limited information is available about the effect of SRT on the nitrifying microbial community in MBR, especially for short SRT (Duan et al., 2009; Whang et al., 2012). Previous studies of bacterial communities in MBR used mostly a single method such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment

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length polymorphism (T-RFLP), 16S rDNA clone library, high density microarray or respiratory quinones profile (Ahmed et al., 2007; Chang et al., 2011; Cicek et al., 2001; Tan et al., 2008). However, the results obtained with one molecular method can be incomplete as the whole complexity of microbial communities cannot be captured. Only dominant species in a community are displayed with the DGGE gels. Limited DNA sequence information obtained from these relatively short bands can underestimate the specificity of the phylogenetic identification. (Gilbride et al., 2006). Cloning is time and labor intensive and less appropriate for analyzing larger sets of samples and complex bacterial community (Wojnowska-Baryla et al., 2010). Microarrays cannot identify new microorganisms since the probes must be designed using identified bacterial genomic sequence (Xia et al., 2010). To capture the whole complexity of microbial communities, multiple molecular methods should be used at the same time to complement each other.

Thus, the objective of this study is to clarify the structure and composition of nitrifying bacteria communities in a submerged membrane bioreactor at short solids retention time, using several combined molecular techniques, and to compare the results with other common techniques. PCR-DGGE was used to investigate the AOB community changes in three MBRs during their operation. Cloning and sequencing of the *amoA* gene was applied to analyze the difference of AOB microbial populations. The concentration of *amoA* gene was quantified by real-time PCR. The domain AOB and NOB species were monitored with a high density microarray.

2. Methods

2.1. Bench-scale submerged membrane bioreactors (MBR)

Three lab-scale aerobic submerged membrane bioreactors were operated in parallel at SRT 3 days (reactor S3), 5 days (reactor S5) and 10 days (reactor S10) to treat synthetic municipal wastewater. The synthetic wastewater was composed mainly of acetate and corn starch as carbon sources. The influent contained 505.4 mg/L Sodium Acetate, 30 mg/L Corn Starch, 50 mg/L Yeast Extract, 133.75 mg/L NH_4Cl, 30.8 mg/L KH_2PO_4, 71 mg/L $MgSO_4{\cdot}7H_2O$, 19.3 mg/L $CaCl_2{\cdot}2H_2O$, 17.4 mg/L $FeSO_4{\cdot}7H_2O$, 0.07 mg/L $CuCl_2{\cdot}2H_2O$, 0.126 mg/L $MnCl_2{\cdot}4H_2O$, 0.132 mg/L $ZnSO_4{\cdot}7H_2O$, 0.03 mg/L$Na_2MoO_4{\cdot}2H_2O$, 0.0248 mg/L H_3BO_3 and 0.0332 mg/L KI. A hollow-fiber membranes (Sterapore LHEM03334, Mitsubishi Rayon, Japan), with a total area of 300 cm^2, were installed in a 4 L aerobic aeration tank. The membranes were made of polyethylene with the nominal cutoff size of 0.4 μm. Continuous aeration was provided underneath the membranes to supply dissolved oxygen maintaining concentration of about 9 mg/L and to prevent membrane fouling. The pH in the three reactors was maintained between 6.8 and 7.4 with NaOH and HCl addition. The temperature was kept at approximately 20 °C (room temperature). All MBRs were operated in a constant flux mode at the same hydraulic retention time (HRT) of 6 h. The target SRTs were maintained through direct removal of sludge from the bioreactor on a daily basis.

2.2. Analytical methods for water quality parameters

Influent and permeate quality as well as transmembrane pressure were monitored routinely. Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were measured by Standard Methods (APHA et al., 1998). Total chemical oxygen demand (COD), total nitrogen and ammonia–nitrogen were measured according to manufacturer's instructions using Hach Method 8000, 8039 and 8008, respectively.

2.3. Denaturing gradient gel electrophoresis (DGGE) analysis

Genomic DNA was extracted in triplicate from a 2 mL sample of suspended solids using a QIAamp DNA Stool Mini Kit (Qiagen, CA, USA) as described in the manufacturer's instructions. A two-step nested PCR procedure described in a previous paper was adopted to run PCR-DGGE (Muyzer et al., 1993). The primer sets *amoA*1f-*amoA*2r and *amoA*1fGC-*amoA*2r were used for the *amoA* gene amplification. The primer sets CTO189f-CTO654r and 357fGC-518r were used for the *Beta-proteobacteria* of AOB amplification.

The PCR reaction was carried out in 50 μL using a DNA thermocycler (Eppendorf, Germany). The PCR mixture contained 1.25 U of Taq polymerase (Promega, WI, USA), 1×PCR buffer, 2 mM $MgCl_2$, 0.5 μmol of each primer, each deoxynucleoside triphosphate at a concentration of 200 μM, and 40 ng of template DNA. The thermal profile used for the *amoA*1f-*amoA*2r, *amoA*fGC-*amoA*2r and CTO189f-CTO654r was: 2 min at 94 °C; 35 cycles of 45 s at 94 °C, 60 s at 48 °C, and 45 s at 72 °C; and a final extension for 10 min at 72 °C. The thermal profile used for the 357fGC-518r was: 2 min at 94 °C; 35 cycles of 60 s at 94 °C, 60 s at 55 °C, and 60 s at 72 °C; and a final extension for 10 min at 72 °C. The sizes of PCR products were assessed by Agilent 2100 Bioanalyzer (Agilent, CA, USA).

DGGE was performed with the D-code System (Bio-Rad, USA) according to manufacturer's instructions. The PCR products were loaded onto 8% (for *Beta-proteobacteria*) and 6% (for *amoA* gene) polyacrylamide (37.5:1, acrylamide/bisacrylamide) with 1xTAE buffer. The polyacrylamide gels were made with a linear denaturing gradient ranging from 35–60% (100% denaturing gradient contains 7 M urea and 40% formamide) for *Beta-proteobacteria* and 30–55% for *amoA* gene. Electrophoresis was run at a constant voltage of 75 V for 15 h. Subsequently, the gels were silver stained according to the procedure as description and pictures were taken immediately. The obtained DGGE patterns were subsequently analyzed with the NTSYS software version 2.10e. Shannon–Weaver diversity index (H') was used to evaluate the structural diversity between microbial communities of different SRT (Shannon, 1963) as:

$$H' = -\sum_{i=1}^{s}(p_i)(\ln p_i)$$

where H' is the Shannon biodiversity index, P_i is the ratio of one the abundance of a specific group of bacteria to total microorganisms, and s is the total number of microbial species in the samples.

2.4. AmoA gene libraries and phylogenetic analysis

Biomass samples taken after 40 days operation from each reactor were used to build cloning libraries. Total extracted DNA was used as a DNA template in the PCR reaction with primers *amoA*1f and *amoA*2r. The PCR products were ligated into a PCR 2.1-TOPO vector and transformed into TOP 10 *Escherichia coli* competent cells following the manufacturer's instructions (Invitrogen, CA, USA). Ampicillin and x-gal were used to screen for colonies with plasmids. For each sample, approximately 100 colonies with the correct inserts were screened and amplified with specific primers M13f and M13r (94 °C for 2 min, followed by 25 cycles of denaturation at 94 °C for 60 s, annealing at 55 °C for 60 s and extension at 72 °C for 2 min, and final extension for 5 min at 72 °C). The positive *amoA* PCR products were then used for screening the genetic diversity by using RFLP with HaeIII (GG/CC) (NEB, MA, USA) restriction enzyme digestion reactions. Colonies demonstrating identical restriction patterns were considered to be the same operational taxonomic units (OTUs). Two representative clones from each