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Nitrogen removal and microbial community structure in membrane bioreactors with addition of alkali-rice straw

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ABSTRACT

Effective carbon source plays a key role in denitrification process in membrane bioreactors (MBRs). In this study, alkali-rice straw was applied to a submerged hybrid MBR to achieve the nitrogen removal enhancement. It was found that total nitrogen (TN) removal of MBR with 1.4 g/d alkali-rice straw addition was 60.8% ± 1.5%, much higher than 44.5% ± 2.3%, 28.9% ± 5.4% from the MBR with 1.4 g/d normal rice straw addition and with 3.0 g/L PVC carrier addition, respectively, but lower than 77.5% ± 3.8% from the MBR with 4.0 g/d ethanol addition. However, chemical oxygen demand (COD) removal of MBR with 1.4 g/d alkali-rice straw addition was 94.6% ± 0.2% higher than 93.2% ± 0.3%, 91.8% ± 0.2% and 90.0% ± 0.4% from the MBR with 1.4 g/d rice straw addition, with 3.0 g/L PVC carrier addition, and with 4.0 g/d ethanol addition, respectively. Though the effect of alkali-rice straw on carbon release was not comparable with ethanol, it could have longer carbon releasing period to improve the carbon utilization rate, provide space for bacterial attachment, and promote the granular biofilm formation to reduce biofouling and increase COD removal efficiency. With alkali-rice straw addition, the dominant microbial community (*Curvibacter, Nitrosomonas, Pseudomonas, Magnetospirillum, Paracoccus*) in membrane bioreactor (MBR) was different from that with addition of other carbon source. Among these bacteria, the denitrifiers were dominantly expressed by nirS gene, and quantitative polymerase chain reaction of nirS gene in MBR with alkali-rice straw addition showed that its abundance was greatly higher than that with common rice straw addition. In summary, alkali-rice straw could serve as effective carbon source to enhance TN removals in MBRs.

Keywords: Alkali-rice straw; Carbon source; Denitrification; Membrane bioreactors; Microbial community structure

1. Introduction

Nitrogen is one of the major nutrients present in urban wastewater, which can cause serious problems in ecosystems [1]. Biological denitrogenation is a reliable method for removing nitrogen from wastewater performed by two major stages, where ammonia is first converted to nitrate/nitrite (nitrification), and further transformed to nitrogen gas (denitrification) needing organic carbon source to process. However, the lower C/N ratio of real sewage (<5) restricts the effect of denitrification by heterotrophic bacteria [2]. Several external carbon sources have been applied to enhance denitrification rate and ethanol, e.g., it is used as commercial carbon source to drive the high-rate denitrification process [3]. Moreover, the supply of liquid carbon source can contribute to the formation of soluble microbial products, which is a significant component of effluent organic matter for membrane fouling and flux decline in wastewater reclamation/reuse applications [4]. To reduce the expense of external carbon supply, agricultural wastes, such as retinervus, corncob, and rice straw, can be used as alternative carbon sources for the denitrification and effective membrane fouling control [5].

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A previous study of submerged hybrid membrane bioreactors (SBMBRs) focused on the effect of various addition of agricultural wastes on improving denitrification, and it was found that these agricultural wastes, especially rice straw, could improve denitrification efficiency up to 140.1 mg NO_3^-–N/g [6]. Although rice straw predominantly contains multiple carbon source, e.g., cellulose (32%–47%), hemicellulose (19%–27%), and lignin (5%–24%), the first-stage hydrolysis hardly achieved high yield of corresponding sugars. These sugars actually need to be fermented to simple carbohydrates, such as ethanol or xylitol for the facilitation of carbon utilization [7]. The method to facilitate the conversion of rice straw to products with simple structure is needed to improve carbon utilization efficiency. Some research showed that physical pretreatment of rice straw could intensively convert recalcitrant lignocellulose structure into reactive cellulosic intermediates before enzymatic hydrolysis [8], and bioethanol produced from various lignocellulosic rice straw is now regarded as the most potential carbon resource to help denitrification [9].

Alkali-rice straw can enhance effective carbon source release for denitrification in SBMBRs compared with rice straw [10]. However, there is less information about the effect of addition of alkali-rice straw on the performance of SBMBRs. The application of molecular biology methods is needed to determine the influence of this novelty carbon source on denitrogenation bacteria and study the effect of alkali-rice straw on SBMBRs. In the previous study, the nitrification performance and microbial community structure of SBMBRs with straw rice addition were compared with supply of other agricultural wastes [6]. It was found that the SBMBR with rice straw addition had most effective total nitrogen (TN) removal rate. Fluorescence in situ hybridization indicated that ammonia oxidizing bacteria, *Nitrosomonas* and denitrifying bacteria were the dominating bacteria in the bioreactors.

In this study, alkali-rice straw was evaluated in an SBMBR by studying its effects on nitrogen removal and analyzing characteristics of predominant bacteria populations/functional enzymes in the system. This study will compare with traditional SBMBRs under a series of operation (i.e., addition of ethanol, rice straw, and PVC carrier). The correlation between the nitrogen removal rate and functional enzymes was constructed, and microbiological mechanism of nitrogen removal with addition of alkali-rice straw was explored.

2. Materials and methods

2.1. MBRs setup and operation

Four 10 L tubular MBRs were operated with continuous mode (Fig. 1), and the carbon source additions were different for each MBR: alkali-rice straw (MBR_1), rice straw (MBR_2), ethanol (MBR_3), and PVC carriers (MBR_4). The MBR_1 and MBR_2 were to identify the different ways of carbon release between alkali-rice straw and rice straw; MBR_3 was to use ethanol to compare with MBR_1 and MBR_2 to test impact of different carbon source on bioethanol release; the PVC without carbon release ability was applied in MBR_4, which served as blank experiment to compare with MBR_1 and MBR_2 to test carbon releasing effect of (alkali) rice straw. The membrane component material was filamentous hollow fiber membrane (inner and outer diameter: 0.65 and 1 mm, Tianjin Motimo Membrane Technology Ltd., Tianjin, China). The membrane area was 1–1.5 m^2/piece; the water yield was 15–22 L/(m^2·h); the hydraulic retention time was 12 h. The four MBRs were inoculated with 80 mL sludge from the anaerobic digester in a local wastewater treatment plant (South City Municipal Wastewater Treatment Plant, Nanjing, China), and the solid retention time was 30 d. All reactors used blast aeration with the aeration rate of 700 m^3/d, and rotary flow meter was used to maintain dissolved oxygen concentration (at 3.0 mg/L). These four MBRs were operated at pH of 6.8 ± 0.2. The water temperature was maintained at 25°C ± 1°C. The same synthetic wastewater was adopted for all four reactors, with chemical oxygen demand (COD), NH_4^+–N, and total phosphorus controlled at 200.0 ± 20.0 mg/L, 40.0 ± 4.0 mg/L, and 6.4 ± 0.5 mg/L, respectively. An ethanol dose of 2.0 and 4.0 g/d was added. The (alkali) rice straw adding in MBRs was from 0.7 to 1.4 g/d. These carbon source dosages were determined by our previous studies on carbon release from agricultural wastes [6]. The pretreatment method for rice straw was as followed: the rice straw of 20 g was dried in air, and soaked with 2% sodium hydroxide of 400 mL for 24 h in beaker of 500 mL at room temperature. After finishing soaking, the rice straw will be repeatedly washed with distilled water to neutralize the pH. Subsequently, the rice straw will be dried under the temperature of 35°C in the oven, and stored in room temperature before being placed into MBRs. After about 2 months of start-up period, all four MBRs were then operated for 80 d through two stages: in the stage I (0–33 d), the dosage of (alkali) rice straw and ethanol was 0.7 and 2.0 g/d for MBR_1/MBR_2 and MBR_3, respectively. In the stage II (33–80 d), the dosage of (alkali) rice straw and ethanol was 1.4 g/d and 4.0 g/d for MBR_1/MBR_2 and MBR_3, respectively.

2.2. Measurement and analysis

NH_4^+–N, NO_3^-–N, TN, and COD were measured according to Chinese State Environmental Protection Agency (SEPA) Standard Methods (Chinese SEPA, 2002). The surface morphology of (alkali) rice straw was analyzed by scanning electron microscope (SEM; Quanta 2000 Scanning Electron Microscope, USA). The slurry mixture samples (5 mL/time) were taken every 2 d, and the COD concentration was analyzed to investigate the carbon release behavior before and after alkali treatment. Denitrification potential (DP) represented the amount of oxidized nitrogen reduced by the consumption of a given quantity of carbon by activated sludge [6], which was the sum of nitrogen removed by all the time intervals (one time interval defined as the time needed for NO_3^-–N concentration decreasing from 100.0 mg/L to lower than 20.0 mg/L by using 200 mL denitrifying sludge). For each interval, the removed nitrogen could be calculated as follows:

$$DP = \left[NO_3^- - N\right]_{inf} - \left[NO_3^- - N\right]_{eff} + 0.6\left\{\left[NO_2^- - N\right]_{inf} - \left[NO_2^- - N\right]_{eff}\right\} \quad (1)$$

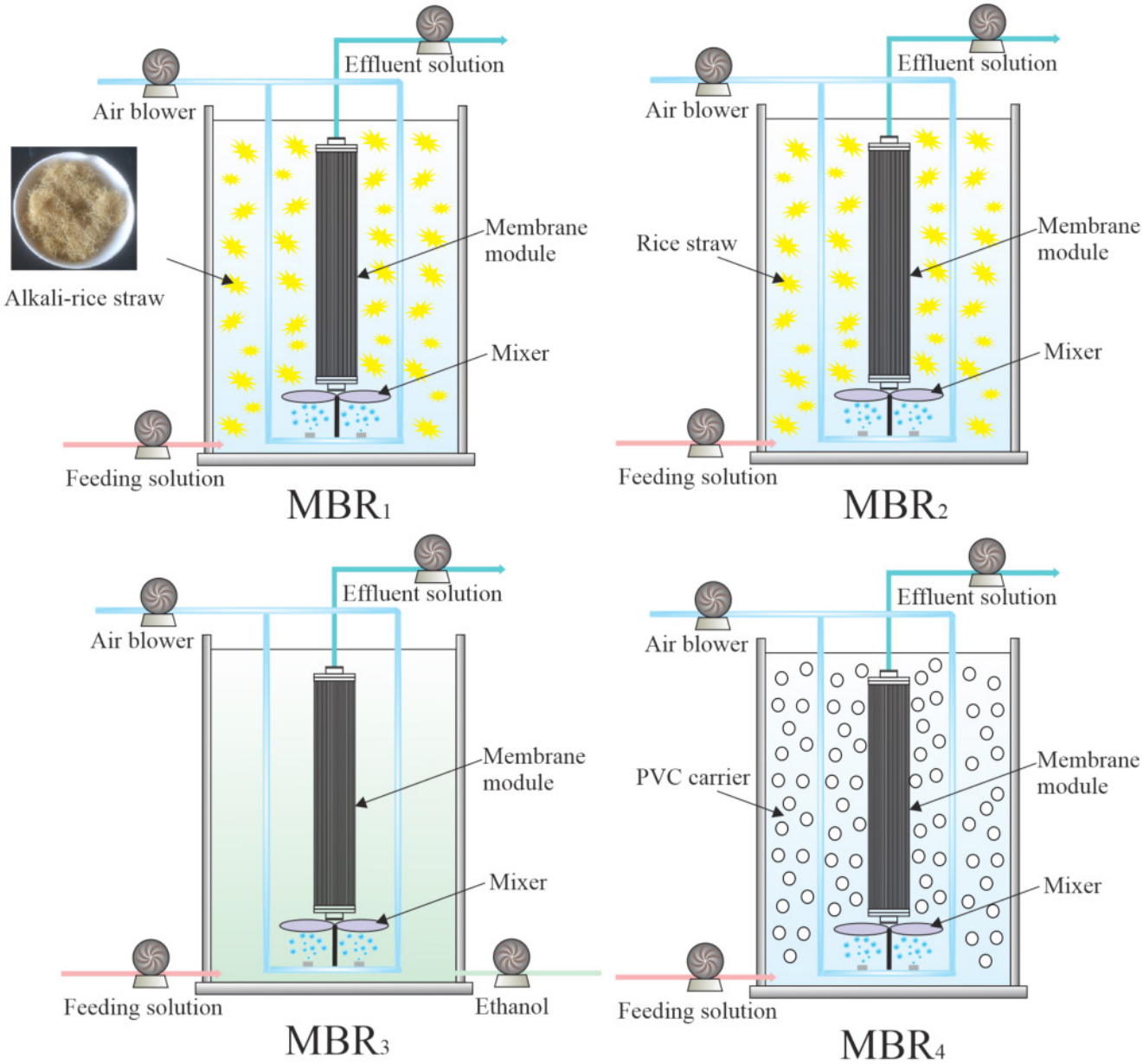

Fig. 1. The schematic graph of four MBRs, which were addition of alkali-rice straw (MBR_1), rice straw (MBR_2), ethanol (MBR_3), and PVC carriers (MBR_4). Notes: Rice straw and ethanol serve as carbon source; PVC serves as carrier; MBR_1 and MBR_2 have both carbon source and carrier, while MBR_3 only has carbon source and MBR_4 only has carrier to compare with MBR_1 and MBR_2 on carbon releasing effect of (alkali) rice straw.

2.3. DNA extraction, PCR-DGGE, cloning and sequencing

The way generally adopted by molecular biology is to use the functional polymerase chain reaction (PCR) primer based on the functional gene and 16S rDNA PCR primer to detect denitrogenation bacteria. Sludge samples were collected from each reactor, immediately concentrated by centrifugation at 12,000 rpm (Bio-Rad, Shanghai, China) for 5 min at 4°C. DNA isolation from the mixed sludge in MBRs was performed by using Ezup® column Soil Genomic DNA exaction kit (Sangon Biotech, Shanghai, China). For PCR purposes, the DNA concentration was measured by UV-9100 spectrophotometer (Lab Tech Ltd., Beijing, China), and adjusted to become a concentration of 100 ng/μL. PCR amplification of 16S rDNA gene, amoA gene, and nirS gene were performed in an Applied Biosystem 2720 Thermocycler. For identification of unique bacterial species, primers (PRBA338F, PRUN518R) were designed from the species-specific region on the 16S rDNA [11]. For the quantification of amoA nitrifiers and nirS denitrifiers, the primers amoA-1F (GGGGTTTCTACTGGTGGT)/amoA-2R (CCCCTCKGSAAAGCCTTCTTC) and nirS Cd3AF (5′-AACGYSAAGGARACSGG-3′)/nirS R3cd (5′-GASTTCGGRTGSGTCTTSAYGAA-3′) were used, respectively [12,13]. All primers were synthesized by Sangon (Sangon Biotech, China). The PCR solution preparation and program can be seen in supporting information.

The fragments amplified by PCR were analyzed by denaturing gradient gel electrophoresis (DGGE) for qualitative analysis. The amplified PCR products were pooled and resolved on DGGE gels using a BioRad DCode™ Universal Mutation Detection System (Bio-Rad, USA). The DGGE result can be applied to determine the dominant species, with the detailed procedures described in supporting information. The brightest DNA in DGGE indicated the dominant species, which was amplified again for sequence analysis, using BLASTN facility available in NCBI GenBank database (http://www.ncbi.nlm.nih.gov/nuccore), to find out the closest relative strains to represent the dominant species.

2.4. Quantification of functional gene

Quantitative PCR was used to quantitatively investigate the changes of these functional enzymes in long-term cultivations of MBRs. Based on 16S rDNA analysis, amoA/nirS gene served as a representative functional gene of nitrifiers/denitrifiers to make quantitative analysis. PCR products for the preparation of quantitative PCR standards were cloned into the PCR®2.1-TOPO® plasmid vector and *Escherichia coli* TOP10 cells using the TOPO-TA cloning vector kit according to manufacturer's instructions (Invitrogen , Shanghai, China). Clones containing recombinant plasmids were examined for the presence of the appropriate insert by PCR using the corresponding primers and subsequently sequenced (IIT GmbH, Geschäftsbereich Biotech, Germany). Amplification of qPCR products was carried out with an ABI Step One Plus by using SYBR green as the detection system. Thermal cycling conditions for the amplifying reactions were same as previous PCR condition. Melting curves were routinely checked to confirm purity of the amplified products. An external standard curve that showed the relationship between copy numbers and C_T values was generated with serial dilutions of a known copy number of nirS genes. The increment in fluorescence vs. reaction cycle was plotted and the threshold cycle (C_T) which could be related to the log of the target gene copy number. All measurements were done in triplicate.

3. Results and discussion

3.1. Characterization of alkali-rice straw

The carbon source for rice straw was cellulose/hemicellulose coated by lignose, which could not be easily biodegraded by microorganism. The dissolvable cellulose was slowly released to the solution, and the biodegradable hemicellulose was slowly biodegraded by attached microorganism. According to Fig. 2, alkali treatment strongly affected the type and constituent of carbon source released from rice straw, and its surface properties. The content of cellulose was significantly increased after soaking ($p < 0.05$, one-tailed two-sample *t*-test with unequal variance at $\alpha = 0.05$ for all the following statistical tests; Fig. 2(A)) due to the fact that sodium hydroxide pretreatment could reduce the extensive interactions among cellulose, hemicellulose, and lignose, which finally decreased the barrier nature of lignin to help enzyme to get access into carbohydrates and facilitated cellulosic ethanol production [14]. Thus, the smooth surface became rough and multiporous after alkali treatment, the content of cellulose was significantly increased and there was more space for bacteria to attach and grow (Fig. 2(B)).

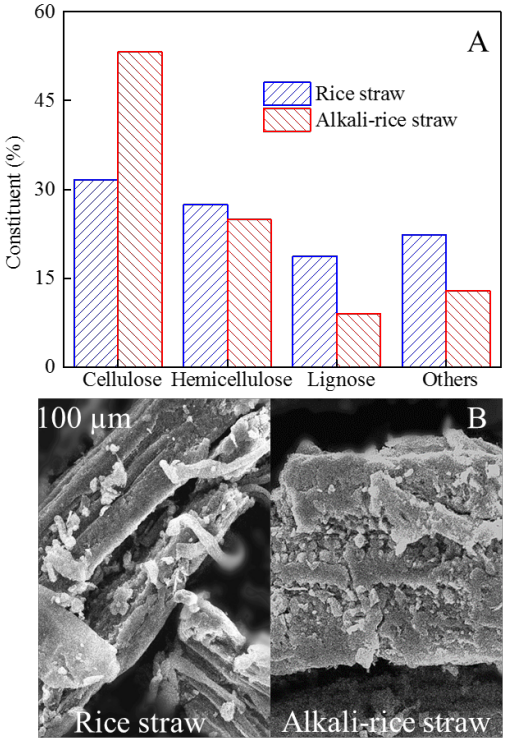

Fig. 2. Characterization of constituent and surface before and after alkali treatment: (A) constituent analysis and (B) SEM images.

There were two stages during the carbon release process: in the first stage (0–10 d), (alkali) rice straw showed a quick release of organic matter mainly coming from soluble organic matter; following that (10–30 d), COD release rate experienced a slow rate and stabilized on day 24, while alkali-rice straw had a higher COD release rate during stage II ($p < 0.05$; Fig. 3(A)). The difference implicated that alkali treatment could enhance insoluble carbon release process. Because of heterotrophic process of denitrification, it was expected that the ethanol addition could help achieve the highest DP, followed by alkali-rice straw and rice straw ($p < 0.05$), while for PVC carriers, no DP was detected (Fig. 3(B)). On one hand for ethanol addition, the DP dramatically increased from 0.0 to 395.3 mg/g within 15 d, but this maximum DP became flatten out after 15 d. On the other hand, the DP of (alkali) rice straw could still raise after 15 d, indicating its strong potential to become better everlasting carbon source to promote the denitrification process. Interestingly, although the COD release rate with alkali-rice straw addition was slightly higher than that with rice straw, the denitrification rate was significantly improved ($p < 0.05$). The reason was possible that after alkali treatment, the electron donor releasing from rice straw could be directly used by denitrifiers and this will be explained and proved in the following sections.

3.2. Performance of membrane bioreactors

The COD removals were affected by the addition of carbon source, and the MBR_1 could achieve a higher removal

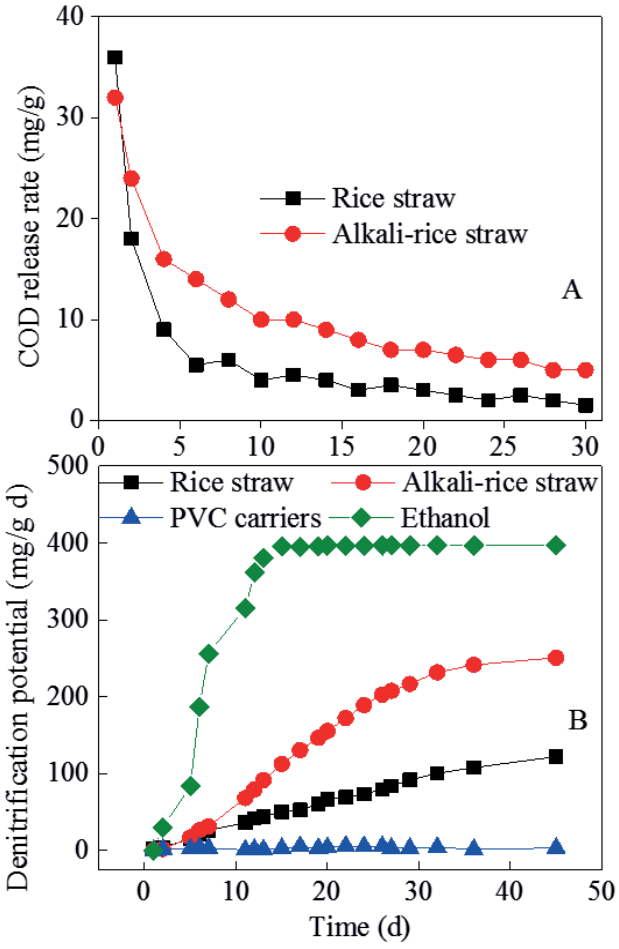

Fig. 3. Variation of COD release rate before and after alkali treatment (A) and denitrification potential under four operations (B).

efficiency, followed by MBR_2, MBR_4, and MBR_3 ($p < 0.05$), indicating that alkaline treatment for rice straw could enhance COD removal performance. The removal of NH_4^+–N in four MBRs were irrespective of carbon source ($p > 0.05$), but all of the reactors achieved high removal efficiency. For MBR_4, TN removal and nitrate generation became stable after 40 d, and they were affected by the type and dosage of carbon source (Fig. 4). For example, with dosage of alkali-rice straw increased from 0.7 to 1.4 g/d, the concentration of nitrate and TN in effluent decreased from 21.5 to 13.7 mg/L and 25.5 to 16.0 mg/L, respectively. During the stabilization stage II, clearly the MBR_3 could achieve a higher TN removal, followed by MBR_1, MBR_2, and MBR_4 ($p < 0.05$). Meanwhile, with the improvement of TN removal efficiency, the nitrate generation in effluent was reduced. The result difference has four implications: first, the removal of generated nitrate nitrogen was the key point for TN removal in MBRs; second, heterotrophic denitrifying bacteria existed to help reduce TN; third, rice straw carriers could release effective carbon to enhance TN removal compared with PVC carriers; fourth, suitable pretreatment could significantly improve TN removal in MBRs.

3.3. Microbial community structure

The above results have demonstrated that the alkali-rice straw (of the MBR_1) could improve TN removal efficiency in MBRs. Next, we attempted to understand why alkali-rice straw could result in such improvement via microbial community analysis. Fig. 5 shows microbial community structure analyzed by DGGE profiles of total 16S rDNA from four reactors during the stabilization stage II. Totally, 11 clones from DGGE gel which matched known diversity of total bacterium were amplified with the specific primers (Table 1). Taxonomic analyses using the NCBI classifier grouped these sequences into three phyla (alphaproteobacteria, betaproteobacteria, and gammaproteobacteria) and nine classes (*Burkholderiales, Nitrosomonadales, Pseudomonadales, Rhodospirillales, Rhodobacterales, Rhizobiales, Rhodocyclales, Hydrogenophilales, and Sphingomonadales*). On the other hand, the microbial distribution in the sludge from four experimental groups changed corresponding to carbon source. The *Aquaspirillum delicatum* group was only present in MBR_1 and capable of utilizing carboxylic and oxalic acids. All the reactors contained the class of *Nitrosomonadales* and *Pseudomonadales*, whereas the *Nitrosomonas* group was more abundant in MBR_3 due to the fact that the group was not only responsible for the oxidation of ammonia to nitrite, but also capable of oxidizing various hydrocarbon compounds such as methane, methanol, and benzene [15]. The *Magnetospirillum* and *Paracoccus* group which could accelerate the denitrification were abundant in MBR_1 and MBR_3 [16,17]. With the alkali treatment applied (MBR_1), the amount of cellulose used by *Aquaspirillum delicatum* group were distinctly increased, resulting in abundant low-molecular-weight compound production, which could be effectively used by special denitrifiers, e.g., *Magnetospirillum* and *Paracoccus*. The *Nitrobacter* group depending on the presence of oxygen for the oxidation of nitrite to nitrate was especially abundant in MBR_3, reasonably explaining that the nitrate removal rate was significantly improved with the increased ethanol dosage. The *Dechloromonas* group only existing in MBR_4 was not growth-supportive ethanol and glucose [18]. The *Thiobacillus and Sphingomonas* were only abundant in MBR_2. *Sphingomonas* was well adapted for the degradation of high-molecular-weight polycyclic aromatic hydrocarbons and other aromatic contaminants, contributing to the degradation of lignose to glucose [19], and the production of simple hydrocarbons is favorable for the growth of *Thiobacillus* capable of denitrification [20].

3.4. Correlation between nitrogen removal and bacterial functional enzyme structure

On the basis of DGGE profiles of total 16S rDNA, the strains were classified into three categories: (1) the bacteria able to degrade hydrocarbons into low-molecular-weight or carbon dioxide; (2) the bacteria responsible for the oxidation of ammonia to nitrite/nitrate and (3) the bacteria for denitrification. Autotrophic ammonia monooxygenase (amoA) was a key enzyme in the nitrification pathway, catalyzing the oxidation of ammonia to nitrite [21]. The dissimilatory denitrifiers in our reactors were mainly encoded by cytochrome cd1 nitrite reductase (nirS) catalyzing the one-electron reduction of nitrite to nitric oxide [22]. Thus, an SYBR Green I real-time

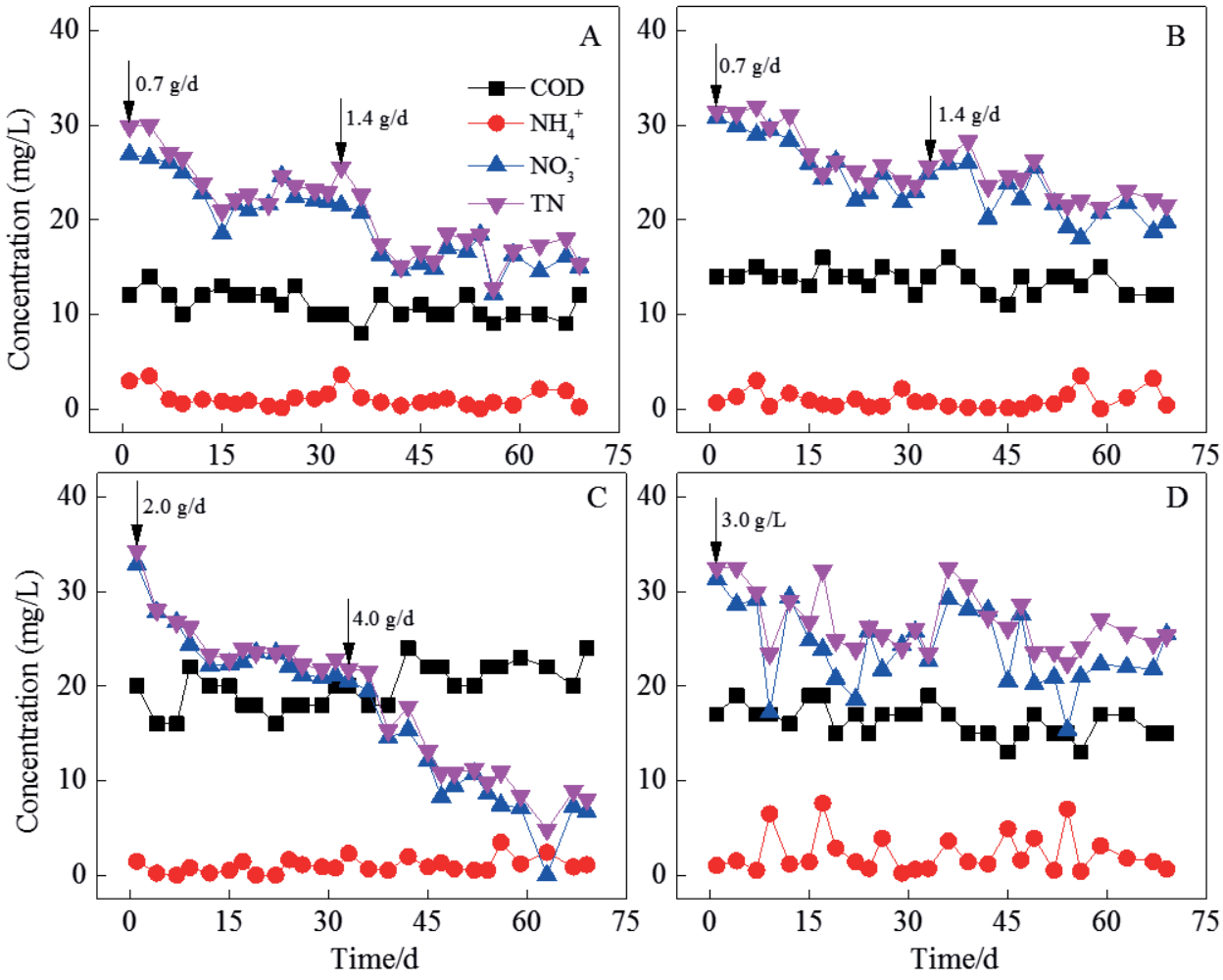

Fig. 4. The concentration of COD, NH_4^+, NO_3^-, and TN in the effluent with four reactors: (A) MBR_1, (B) MBR_2, (C) MBR_3, and (D) MBR_4. The dosage of alkali-rice straw, rice straw, ethanol, and PVC carriers are indicated by the arrows.

PCR protocol that combined several previously described primer sets targeting amoA/nirS genes of nitrifiers/denitrifiers was developed to explore the correlation between nitrogen removals and nitrifying/denitrifying community structure. Plasmids containing cloned amoA/nirS genes were used to generate a standard curve relating C_T to the number of gene copies. The same linear response ($R^2 > 0.99$) was observed for all plasmids between C_T and the $\log_{10}$ number of amoA/nirS copies over six orders of magnitude, ranging from 10^2 to 10^7 gene copies per microliters DNA (Fig. 6). A single melting peak of real-time PCR data corresponding to the standard DNA was observed for all sludge samples.

The amoA gene was less affected by the type of the carbon source, and the amount of amoA gene in the MBR_3 was slightly higher than that of the other three MBRs (Fig. 6(A)). However, based on the high removal rate of ammonia nitrogen in all MBRs, ammonia oxidation seemed not to be the limiting factor for total nitrogen removal, and only using amoA gene could not reflect the impact of microbial community difference on the nitrogen removal difference. nirS gene may become alternative indicator for nitrogen removal efficiency, because nirS gene was strongly related to carbon source utilization rate. The density of nirS followed the trend of nitrate concentration comparison among all MBRs. Thus, the MBR_3 had the highest nirS density, followed by MBR_1, MBR_2, and MBR_4 ($p < 0.05$), but the MBR_3 had poor COD removal efficiency (Fig. 4). The reason was that though ethanol addition could enhance the growth of denitrifiers (the density of nirS: "MBR_3" > "MBR_1" > "MBR_2" > "MBR_4"), the excess carbon source released from ethanol could not be completely utilized by denitrifiers or other heterotrophic bacteria, leading to a waste of carbon source (COD removals: "MBR_1" > "MBR_2" > "MBR_4" > "MBR_3"). Alkali treatment could effectively improve carbon release from rice straw to enhance the growth of denitrifiers, inherently resulting in the significant improvement of nitrate removal efficiency (MBR_1" > "MBR_2"), and this effective carbon source could also promote the growth of other heterotrophic bacteria to efficiently achieve higher COD removal efficiency (MBR_1" > "MBR_2"). The nirS density increased from 1.2×10^{19} to 3.0×10^{19} during initial 12 d with 2.0 g/d ethanol addition, but this increase became much slower from 13 to 33 d. When the dosage increased to 4.0 g/d, the similar trend was detected, but the increased rate during initial 12 d was unexpectedly lower than that with ethanol addition at 2.0 g/d.

Interestingly, this "increasing and flattened" trend of nirS density was not applied for MBRs with (alkali) rice straw, indicating that (alkali) rice straw could slowly release carbon source for denitrification. The slow release rate could result in less requirement of carbon source addition and lower cost. Compared with ethanol, the pore structures of (alkali) rice straw provided space for bacterial attachment and growth, resulting in the formation of granular biofilm with high potential to effectively inhibit membrane fouling.

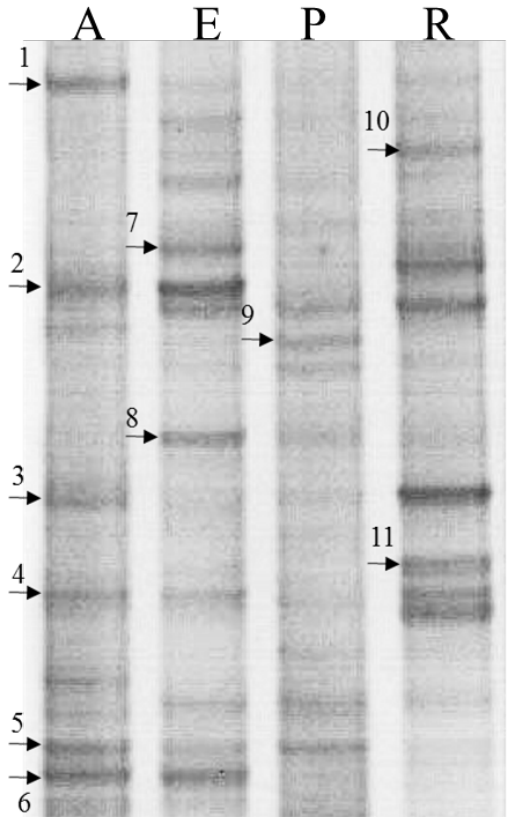

Fig. 5. DGGE profiles of total 16S rDNA from four reactors: A (MBR_1), E (MBR_3), P (MBR_4), and R (MBR_2). Band position was highlighted with a numbered arrow.

Therefore, this finding has three important implications to MBR development and operation. First, the alkali-rice straw addition can provide effective carbon source to enhance the denitrification process. Although its effect does not reach the amount of carbon releasing from ethanol, the alkali-rice straw can consistently release carbon source, which greatly improves the utilization rate of carbon source. Second, alkali

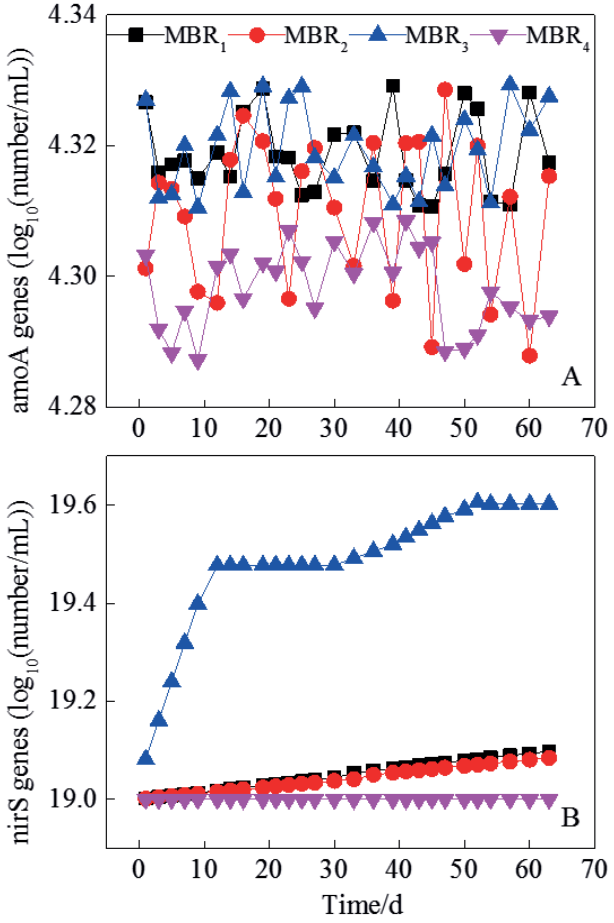

Fig. 6. Variation in amoA gene (A) and nirS gene (B) copy number (log_{10}) per milliliter of mixed liquid with four reactors.

Table 1
Sequence analysis of major 16S rDNA DGGE bands and their closest phylogenetic match found in the GenBank database

Band	Accession number	Closet match	Phylum	Match (%)
1	AF078756	*Aquaspirillum delicatum*	Betaproteobacteria	100
2	JX545090	*Nitrosomonas eutropha*	Betaproteobacteria	98
3	GU563981	*Pseudomonas aeruginosa*	Gammaproteobacteria	100
4	D17515	*Magnetospirillum magneticum*	Alphaproteobacteria	100
5	HE814023	*Pseudomonas stutzeri*	Gammaproteobacteria	100
6	AM230889	*Paracoccus denitrificans*	Alphaproteobacteria	100
7	FM161767	*Pseudomonas* sp.	Gammaproteobacteria	99
8	AF363288	*Nitrobacter winogradskyi*	Alphaproteobacteria	100
9	AM230915	*Dechloromonas* sp.	Betaproteobacteria	100
10	NR076380	*Thiobacillus denitrificans*	Betaproteobacteria	100
11	DQ076162	*Sphingomonas* sp.	Alphaproteobacteria	100

treatment is a simple but very effective pretreatment method to increase the amount of carbon source for the growth of denitrifiers. Last but not the least, the increased pore structures provide more space for bacterial attachment and growth after alkali treatment, improving TN removal efficiency improved and reducing membrane fouling.

4. Conclusions

This study has demonstrated the advantages of MBRs with addition of alkali-rice straw in terms of nitrogen removal and microbial community structure, compared with addition of rice straw, ethanol, and PVC carriers for MBR applications.

- With alkali treatment, the barrier nature of lignin minimize enzyme was inhibited, and cellulosic ethanol production was facilitated. Thus, the denitrification rate was significantly improved.
- (Alkali) rice straw could serve as a better everlasting carbon source to promote the process of denitrification compared with addition of ethanol.
- The removal of COD and NH_4^+–N in four MBRs were irrespective of carbon source, and both achieved high removal efficiency. However, TN removal and nitrate generation were directly affected by the type and dosage of carbon source. For example, with dosage of alkali-rice straw increasing from 0.7 to 1.4 g/d, the concentration of nitrate and TN in effluent decreased from 21.5 to 13.7 mg/L and 25.5 to 16.0 mg/L, respectively.
- The DGGE profiles of total 16S rDNA showed that microbial community structure in four MBRs was affected by the type of carbon source resulting in different TN removals.
- The amoA gene was less affected by the type of the carbon source, but the type and dosage of carbon source utilization had great effect on the amount of nirS gene, and thus the density of nirS could indicate the nitrogen removal efficiency.

Acknowledgments

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Supporting information

Description for PCR and real-time PCR

Plasmid DNA was extracted with a Plasmid Mini Kit (Invitrogen). The purified recombinant plasmid DNA serially diluted in double-distilled water to a final concentration ranging from 104 to 109 copies of genome equivalent/mL. 4 μL aliquots of each dilution were used for real-time PCR in triplicates to create standard curve and used as quantification standards for adenovirus in experimental samples.

A PCR mix of 25 μL containing 1 × PCR buffer (Fermentas, Vilnius, Lithuania), 2.7 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 2.5 g bovine serum albumin, 1% (vol/vol) formamide, 2.5 U Dream Taq polymerase (Fermentas), 0.2 M primers, and template DNA was prepared. Thermal cycling was carried out by using an initial denaturation step of 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30s, annealing at 60°C for 30s, and elongation at 72°C for 30s. Cycling was completed by a final elongation step of 72°C for 15 min. The PCR products were examined on 1.5% (w/v) ethidium bromide-stained agarose gels. To melt profile and avoid complete denaturing of the amplified fragments, a 33-bp GC-clamp was attached to the end of the primer.

PCR products of 16S rDNA were loaded onto polyacrylamide gradient gels with a denaturing gradient from 45% to 65% (100% denaturant contains 7 M urea and 40% formamide). Electrophoreses were run at 125 V for 8 h at a constant temperature of 60°C. The Quantity One software package (Bio-Rad, Hercules, CA, USA) was used to analyze DGGE profiles. The gel was stained in 1 × TAE buffer containing SYBR green before visualizing on a UV transilluminator and photographed. The brightest DGGE bands were cut out to determine which species were dominant in each reactor. The bands were then eluted in DNA-free water overnight at 4°C and reamplified using the same set of primers as for the first PCR.