Denitrification and microbial community in MBBR using A. donax as carbon source and biofilm carriers for reverse osmosis concentrate treatment

Li Li1,2,3, Guokai Yan1,2,3, Haiyan Wang1,2,3,*, Zhaosheng Chu1,3,4, Zewen Li1,2,3, Yu Ling1,2,3, Tong Wu1,2,3

1. State Key Laboratory of Environmental Criteria And Risk Assessment, Chinese Research Academy of Environmental Sciences, Beijing 100012, China
2. Engineering Center for Environmental Pollution Control, Chinese Research Academy of Environmental Sciences, Beijing 100012, China
3. National Engineering Laboratory for Lake Pollution Control and Ecological Restoration, Chinese Research Academy of Environmental Sciences, Beijing 100012, China
4. State Environmental Protection Key Laboratory for Lake Pollution Control, Chinese Research Academy of Environmental Sciences, Beijing 100012, China

ABSTRACT

In this study, raw Arundo donax (A. donax) pieces were applied as carbon source and biofilm carriers for denitrification in a lab-scale moving bed biofilm reactor (MBBR) for the treatment of reverse osmosis concentrate gathered from local wastewater reuse plant. At stable phase (about 60 days), efficient denitrification performance was obtained with 73.2% ± 19.5% NO3−-N average removal and 8.10 ± 3.45 g N/(m3·day) NO3−-N average volumetric removal rate. Mass balance analysis showed that 4.84 g A. donax was required to remove 1 g TN. Quantitative real-time PCR analysis results showed that the copy numbers of 16S r-RNA, narG, nirS, nosZ and anammox gene of carrier biofilm and suspended activated sludge in the declination phase (BF2 and AS2) were lower than those of samples in the stable phase (BF1 and AS1), and relatively higher copy numbers of nirS and nirK genes with lower abundance of narG and nosZ genes were observed. High-throughput sequencing analysis was conducted for BF2 and AS2, and similar dominant phyla and classes with different abundance were obtained. The class Gammaproteobacteria affiliated with the phylum Proteobacteria was the most dominant microbial community in both BF2 (52.6%) and AS2 (41.7%). The PICRUSt prediction results indicated that 33 predictive specific genes were related to denitrification process, and the relative abundance of 18 predictive specific genes in BF2 were higher than those in AS2.

© 2019 The Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences. Published by Elsevier B.V.
Introduction

Moving bed biofilm reactor (MBBR) was established by AnoxKaldnes™ based on the combination of conventional activated sludge process and biofilm process in 1989 with the characteristics of bearing high treatment loading, small reactor space demand and high biomass (Odegaaard et al., 1994). It has been proven that MBBR is an effective biotechnology for the treatment of nitrogen contaminated water through heterotrophic denitrification process (Kopec et al., 2018). The carrier material, structure and pore space configuration as well as the biofilm surface morphology affect the substrate transportation to the biofilm microbial community by influencing the flow velocity, and subsequently affect the nitrogen removal efficiency (Young et al., 2016). Polyethylene, polypropylene, polyurethane foams and haydite were commonly used in treatment of different kinds of nitrogen contaminated wastewater (Yuan et al., 2015). Composite-refined diatomaceous earth, polymeric nano-fibrous material, bioplastic based product, granular activated carbon, sand and home-made suspended ceramic product were also applied as carriers for nitrogen removal in MBBR systems (Dong et al., 2011). The application of novel economic carriers has been becoming the research focus for MBBR development (Dong et al., 2011).

Reverse osmosis (RO) technology is in incremental applications to produce high quality water from full scale municipal wastewater treatment plant (WWTP) effluent (Shannon et al., 2008). However, the RO concentrate (ROC) contains most contaminants and nutrients filtered out from the secondary effluents (Pradhank et al., 2016), whose genotoxicity and potential environmental risks are greatly increased (Umar et al., 2015). Cost-effective treatment methods for nitrogen removal from ROC is urgently needed to meet the nitrogen discharge standards and then reduce the environmental risks. Various technologies are applied for nitrogen removal from ROC, such as coagulation, UV/H2O2, ozonation, Fenton oxidation and biological processes (Umar et al., 2015). Nevertheless, few studies are carried out for the MBBR treatment of ROC.

Heterotrophic denitrification is the dominant biological process for nitrogen removal from ROC with nitrate (NO3−) as the key nitrogen form (Umar et al., 2015), and its efficiency is affected by external carbon sources. Traditional liquid carbon sources such as ethanol, methanol, acetate and glucose are commonly used (Sun et al., 2017), and polyhydroxyalkanoates, polybutylene succinate and starch/polyvinyl alcohol polymer blends are reported both as solid carbon provider and biocarrier (Xu et al., 2018). Woodchip, corncob rice straw, retinervus luffae fructus and wheat straw (Yang et al., 2015) are also used as carbon sources. Bamboo charcoal and bamboo powder blended with other materials are applied as carbon source and biofilm carrier in denitrifying filters (Cao et al., 2016; Liu et al., 2018).

Arundo donax (A. donax) is widely distributed in fresh or moderately saline damp soils of mild temperate, subtropical and tropical regions, and the abundant cellulose and hemicellulose in its stalk could be easily degraded, thus makes it feasible to use raw A. donax pieces as carriers and carbon source for MBBR. Analysis of microbial community and its functions as well as gene copies are helpful to investigate the contaminant removal mechanism during biological processes. The microbial communities and gene copies are analyzed for various carriers and carbon sources in MBBR (Torresi et al., 2018). However, the differences of microbial communities and gene copies between the biofilm and the suspended sludge of MBBR are not studied, and the relationship of MBBR treatment efficiency and specific microorganism should be further illustrated.

In this study, raw A. donax pieces were used as carbon source and biofilm carriers in MBBR for the treatment of ROC gathered from the Dalton Filtration Reverse Osmosis (DFRO) unit of Beijing Cuifu Water Reuse Plant. DFRO is an advanced treatment unit for domestic wastewater reuse, which has lower operation pressure and cost compared with traditional reverse osmosis processes. The denitrification performance of A. donax-based MBBR was extensively explored, and mass balance was carried out. Furthermore, the denitrification gene copies and microbial communities in both biofilm and suspended activated sludge were analyzed and compared, and the correlations of specific bacteria functions and MBBR denitrification performance were also studied. Microbial community functions were predicted by PICRUSt based on high-throughput sequencing data.

1. Materials and methods

1.1. A. donax preparation and characterization

A. donax was harvested along Erhai littoral zone (25°36′–25°58′ N, 100°06′–100°18′ E) in July 2017. The aboveground parts of A. donax were cut into 2–3 cm pieces, then air-dried in the sun for 96 hr. When dried to relatively constant mass, the pieces were put into sealed bag and then reserved in dry and ventilated place.

Certain essential physical chemical parameters were measured to determine the compositions variation of A. donax and calculate the mass balance during the operation. A. donax pieces were cleaned and oven-dried at 40°C to constant mass, milled and screened through 60-mesh sieve, and then its cellulose, hemicellulose and lignin content were quantified using Van Soest’s method (Hang et al., 2017). Total carbon (TC) and total nitrogen (TN) were determined by elemental analyzer (Thermo Flash 2000 CHNS/O, Thermo Fisher Scientific, USA), and total phosphorus (TP) was analyzed by Inductively Coupled Plasma Optical Emission Spectrograph (ICP-OES optima 8000, Perkin Elmer, USA). A. donax carriers were sampled before operation and after 170 days operation for compositions analysis.

1.2. Experimental set-up and operation design

1.2.1. Experimental set-up

Lab-scale plexiglas column with 12 L effective volume (25 cm in height and 25 cm in inner diameter) was set up as anoxic MBBR (Fig. 1). ROC was continuously supplied from the bottom of anoxic MBBR by peristaltic pump (BT100-1L, Baoding Lange Constant Pump Company, China) and then overflowed from
the top with 12 hr hydraulic retention time (HRT). A. donax carriers were filled about 30% (V/V), and electric mechanical agitator was used to keep the suspension of carriers and sludge. Temperature in the bioreactor was controlled at 23–30°C by heating rods. The anoxic MBBR was covered by black shade to prevent photosynthesis disturbance. The experiments were carried out for 170 days, and the MBBR operation could be divided into three phases, i.e. inoculation phase (day 1–42), stable phase (day 43–98) and declination phase (day 99–170).

1.2.2. Sludge inoculation and operation design
The anoxic MBBR was inoculated by activated sludge taken from the anoxic tank of a municipal WWTP in Beijing, China. After inoculation, the mixed liquid suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS) and MLSS/MLVSS of the MBBR were 3544 mg/L, 1897 mg/L and 0.54, respectively.

The influent ROC of the anoxic MBBR was generated from the DFRO unit of a municipal WWTP in Beijing, China, which produces high quality reclaimed water. Certain amount of NaNO2 and KH2PO4 were added in the influent ROC to keep nitrite (NO2−-N) at about 10 mg/L (NO2−-N loading rate of 0.2 g N/(m2 carrier·day)) and the ratio of TN to TP was 20:1. The influent ROC ingredients for anoxic MBBR are shown in Table 1.

### Table 1 – Characteristics of influent ROC.

<table>
<thead>
<tr>
<th>Operation phases</th>
<th>Operation time (day)</th>
<th>pH</th>
<th>NO3− -N (mg/L)</th>
<th>NO2− -N (mg/L)</th>
<th>NH4+ -N (mg/L)</th>
<th>COD (mg/L)</th>
<th>C/N ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculation phase</td>
<td>1–42</td>
<td>6.8–7.7</td>
<td>3.7 ± 2.5</td>
<td>7.3 ± 4.5</td>
<td>1.1 ± 0.7</td>
<td>28 ± 13.8</td>
<td>1.9 ± 1.2</td>
</tr>
<tr>
<td>Stable phase</td>
<td>43–98</td>
<td>7.3–7.4</td>
<td>9.7 ± 2.7</td>
<td>6.5 ± 2.1</td>
<td>2.3 ± 0.8</td>
<td>26.7 ± 3.3</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>Declination phase</td>
<td>99–170</td>
<td>7.0–8.0</td>
<td>13.2 ± 7.6</td>
<td>8.7 ± 8.5</td>
<td>1.3 ± 1.3</td>
<td>32.8 ± 25.4</td>
<td>1.4 ± 0.9</td>
</tr>
</tbody>
</table>

### Table 2 – Primer sequences of target genes for QPCR.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primers</th>
<th>Reverse primers</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>16Sr-RNA gene</td>
<td>ACTCTTACGGGAGGCGACAG</td>
<td>ATTACGGGCGAGCTGCTG</td>
<td>Noah et al. (2005)</td>
</tr>
<tr>
<td>anamox (PLA46F/AMX667R)</td>
<td>GAATTAGGACTGAACTC</td>
<td>ACGAGGACTCCCTCT</td>
<td>Wouter et al., (2007)</td>
</tr>
<tr>
<td>nirK (875/1040)</td>
<td>ATYYGGCAGCGCAYGGCGCA</td>
<td>GCTCTGGATCGACTTRTTGTT</td>
<td>Kim et al. (2011)</td>
</tr>
<tr>
<td>nirS (cd3af/R3cd)</td>
<td>GTSAACGTSAAGGARACSGG</td>
<td>GASTTCGCGTGCTTCTGGA</td>
<td>Satoshi et al. (2011)</td>
</tr>
<tr>
<td>nosZ (1527F/1773R)</td>
<td>CGCCTGCTCTGCACAGYCA</td>
<td>ATRTCGATCGCTGCTGTT</td>
<td>Stres and Murovec (2009)</td>
</tr>
<tr>
<td>narG (1960m2f/2050m2r)</td>
<td>TA(C/T)GT(G/C)GCCAG(G/A)AAA</td>
<td>CGTAAGAAAGCAG(T/C)GTT</td>
<td>Hang et al. (2017)</td>
</tr>
</tbody>
</table>
for bacterial 16S rRNA gene, _anammox_ bacteria and target genes ranged from 0.9949 to 0.9998, and the amplification efficiencies ranged from 84.8% to 97.3%.

1.3.2. High-throughput sequencing assay

Total genomic DNA of BF2 and AS2 was extracted using CTAB/SDS method. DNA concentration and purity were monitored on 1% agarose gels. DNA was diluted to 1 ng/μL using sterile water. 16S rRNA genes of 16S V3-V4 were amplified with primers 515F (5′-GTGCTACGGGAGGCAGCAG-3′) and 806R (5′-GACTACHVGGGTWTCTAAT-3′) with barcode. All PCR reactions were carried out by Phusion® High-Fidelity PCR Master Mix (New England Biolabs, USA).

The same volume of 1× loading buffer (SYB green contained) was mixed with PCR products, and then operated on 2% agarose gel for electrophoresis detection. Samples with bright main strip between 400 and 450 bp were selected for further experiments. PCR products were sequenced in an Illumina platform, and 250 bp paired-end reads were generated. The raw reads obtained from Illumina Hiseq2500 platform in this study were deposited in the NCBI sequence read archive under accession numbers SRR8607415 (sludge sample) and SRR8607416 (biofilm sample).

Paired-end reads were assigned to samples, and effective tags were obtained after filtering and chimeras removal using the QIIME (V1.7.0) quality controlled process and UCHIME algorithm. Sequences were assigned to the same operational taxonomic units (OTUs) at 97% identity, and Green Gene Database was used to annotate taxonomic information. Alpha diversity indices calculated by QIIME (Version 1.7.0) and displayed by R software (Version 2.15.3) were applied in the complexity analysis of species diversity. Alpha diversity indices were calculated according to reported formulas (Bing et al., 2016) and detailed in Appendix A.

1.3.3. PICRUSt prediction

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used to predict the microbial community functions in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database based on 16S rDNA data (Langille et al., 2013). The closed reference OTU table based on the Green Gene Database was uploaded to the online PICRUSt prediction website (http://huttenhower.sph.harvard.edu/galaxy), and the K numbers corresponding to the molecular functions of individual genes and proteins, which were stored in KEGG Orthology (KO) database, were obtained and analyzed.

1.4. SEM and analytical method

In the stable period, the _A. donax_ carriers were taken from reactor and cut into 5 mm × 5 mm pieces for attached biofilm morphology examination. The attached biofilm was immobilized by 2.5% neutral glutaraldehyde, washed by phosphoric acid buffer solution, dehydrated in increasing grades of ethanol, dried at critical point and then sprayed by gold before scanning electron microscope (SEM) observation.

Influent and effluent samples were collected every 3-4 days. Chemical oxygen demand (COD), ammonia (NH4-N), TP, MLSS and MLVSS of sludge after inoculation were analyzed according to the standard methods (Sun et al., 2017). NO3-N and NO2-N were analyzed using ion chromatography (DIONEX ICS-1000, Dionex Inc., USA) after 0.45 μm syringe tip-filter filtration (SCAA-201). Dissolved oxygen (DO) and pH were measured by DO meter (LD010103, HACH, USA) and pH meter (PHC10103, HACH, USA) using multi-parameters water analysis instrument (HQ-30d, HACH, USA).

1.5. Data analysis

The NO3-N removal efficiency and volumetric removal rate were calculated using the following equations:

\[
\text{NO}_3^-\text{N removal efficiency} = \frac{C_{\text{NO}_3^-\text{N,inf}} - C_{\text{NO}_3^-\text{N,eff}}}{C_{\text{NO}_3^-\text{N,inf}}} \times 100\%
\]

\[
\text{NO}_3^-\text{N volumetric removal rate} = \frac{(C_{\text{NO}_3^-\text{N,inf}} - C_{\text{NO}_3^-\text{N,eff}})}{V \times \text{HRT}}
\]

where, \( C_{\text{NO}_3^-\text{N,inf}} \) and \( C_{\text{NO}_3^-\text{N,eff}} \) are total inorganic nitrogen (TIN) concentration, NH3-N concentration, influent and effluent NO3-N concentrations, respectively. \( V \) (L) is the effective volume of the anoxic MBBR.

The NO2-N and TIN removal efficiency and volumetric removal rate were calculated using similar equations.

2. Results and discussion

2.1. Characteristics of _A. donax_

2.1.1. Compositions change of _A. donax_

Two pieces of raw and used _A. donax_ with similar shape and size were selected to measure the compositions (Table 3). Cellulose and hemicellulose degradation was reported as the carbon source for denitrification in constructed wetlands (Chen et al., 2011), so the cellulose (30.2%) and hemicellulose (32.2%) in the raw _A. donax_ pieces could be hydrolyzed and degraded as the extra carbon source for denitrifying bacteria in denitrification MBBR. After 170 days operation, cellulose, hemicellulose, lignin, TN and TC contents per gram of _A. donax_ were all reduced. It was worth noting that great hemicellulose decayed more than cellulose, lignin, TN and TC contents.
cellulose, which was coherent with other report (Hang et al., 2017).

2.1.2. Surface properties
The surface properties as contact angle and surface structure were analyzed by pendent drop method and SEM (Fig. 2a, b). The outer surface of A. donax was hydrophobic with 117.5 ± 2.5° (n = 43) average contact angle, and it could be seen by SEM that the outer surface was smoother than the inner surface. The inner surface and sectional surface of A. donax pieces were rougher, thus more suitable for microbial attachment and biofilm formation than the outer surface. SEM pictures revealed that the relatively regular protrusion and void in the raw A. donax inner surface was conducive to the microbial attachment (Yang et al., 2015).

2.2. Denitrification performance

2.2.1. NO3-N removal
The A. donax MBBR reached stable phase (day 43–98) after 42 days inoculation, and NO3-N was removed effectively (Fig. 3a). Although the influent ROC quality fluctuated, the MBBR kept satisfactory denitrification performance with 73.2% ± 19.5% average NO3-N removal efficiency and 8.10 ± 3.45 g N/(m3·day) average volumetric NO3-N removal rate. The results demonstrated that MBBR using A. donax pieces as carriers and carbon source for ROC treatment had relatively strong adaptability to the unstable ROC load, and the average effluent NO3-N kept at 1.82 ± 1.73 mg/L while the influent NO3-N was 5.87 ± 2.32 mg/L. After the stable phase, the NO3-N removal efficiency began to decline on day 108 and rapidly became negative, which might be caused by NO2-N oxidation as the dissolved oxygen (DO) in the reactor was 0.2 mg/L. The average NO3-N removal efficiency and average volumetric NO3-N removal rate in this study was close to that of the similar systems. 18%–95% nitrogen removal efficiency was obtained for a bioreactor using woodchip as solid carbon source with 27.5 ± 4.5 mg influent NO3-N/L and 7.2–51 hr HRTs, and the removal rate ranged from 8.0 to 18.0 g N-removed/(m3·woodchips·day) (Christianson et al., 2017). Filamentous bamboo was also applied as supplemented carbon source for denitrification enhancement and good denitrification effect were obtained in these experiments (Chu and Wang, 2016).

2.2.2. NO2-N removal
Unlike the obvious variation of NO3-N removal efficiency in different phases, the A. donax MBBR maintained comparable high NO2-N removal efficiency during the whole operation (Fig. 3b). During the stable phase (day 43–98), the average NO2-N removal efficiency and volumetric removal rate were 84.1% ± 8.0% and 7.64 ± 4.03 g N/(m3·day) under the condition of 4.4 ± 2.09 mg NO2-N/L influent. 83.4% ± 14.6% average NO2-N removal efficiency and 8.98 ± 5.36 g N/(m3·day) average volumetric removal rate were obtained during the whole operation. The above-mentioned high NO2-N removal performance might be resulted from the anammox process, because DO in the reactor (0.2 mg/L) was suitable for the growth of anammox bacteria. Combined with 54.5% ± 30.7% average removal efficiency and 1.94 ± 2.3 g N/(m3·day) volumetric

Table 3 – Compositions variation of A. donax.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cellulose (g/g)</th>
<th>Hemicellulose (g/g)</th>
<th>Lignin (g/g)</th>
<th>TC (g/g)</th>
<th>TN (g/g)</th>
<th>TP (g/g)</th>
<th>Sample weight (g/a piece)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td>0.37</td>
<td>0.32</td>
<td>0.20</td>
<td>0.48</td>
<td>0.01</td>
<td>4.3 × 10^−4</td>
<td>0.99</td>
</tr>
<tr>
<td>Used (after 170 days’ operation)</td>
<td>0.34</td>
<td>0.22</td>
<td>0.18</td>
<td>0.47</td>
<td>7.1 × 10^−3</td>
<td>3.7 × 10^−4</td>
<td>0.08</td>
</tr>
</tbody>
</table>

* g/g means g/g A. donax.

Fig. 2 – SEM micrographs of A. donax surface (a 4000×, b 1300×) and photo of A. donax piece (upper right corner of b) before biofilm inoculation.
removal rate of NH$_3$-N throughout the whole operation, the proportional NO$_2$-N removal amount could be calculated according to the anammox reaction process (Eq. (1)).

\[
\text{NH}_3 + 1.32\text{NO}_2 + 0.066\text{HCO}_3 + 0.13\text{H}^+ \rightarrow 1.02\text{N}_2 + 0.26\text{NO}_3 + 0.066\text{CH}_2\text{O} + 3\text{N}_2 + 2.03\text{H}_2\text{O}
\]  

(1)

Though the NO$_2$-N removal efficiency kept high throughout the whole experiment, slight variation could be observed. NO$_2$-N removal efficiency increased to 94.8% on day 51, kept stable (91.7% ± 8.0%) for a long time (day 51–130), and then declined to 66.8% ± 14.2%. NO$_2$ accumulation was occurred after 130 days operation, which was probably resulted from the depletion of A. donax endogenous carbon source. When NO$_2$ removal was incomplete due to inadequate carbon source, NO$_3$ would accumulate because of the different reduction rates of NO$_3$ and NO$_2$ reductase caused by electron donors’ competition (Ge et al., 2012).

2.2.3. TIN removal
At stable phase, the removal efficiency and volumetric removal rate of TIN were 69.8% ± 17.0% and 15.5 ± 4.02 g N/ (m$^3$·day) when the influent and effluent TIN were 11.26 ± 1.75 mg/L and 3.51 ± 2.29 mg/L, respectively (Fig. 3c). TIN removal efficiency began to decline on day 99, and the average TIN removal efficiency kept 31.1% ± 19.2% during declination phase, which might be owing to the NO$_3$-N increase caused by influent fluctuation and A. donax carbon source depletion. However, the TIN volumetric removal rate, which was 14.54 ± 9.61 g N/(m$^3$·day) at declination phase, remained relatively steady until day 142. The anammox reaction between NO$_2$-N and NH$_4$-N was the potential key factor for the above-mentioned phenomenon, which needs further investigation. Furthermore, the nitrogen release from A. donax would influence the TIN removal efficiency, which was concealed by the influent fluctuation.

2.2.4. NH$_3$-N and COD variation
The influent NH$_3$-N was low but fluctuated with an average value of 1.40 ± 1.17 mg/L, which was caused by the instability of DFRO unit and decomposition of organic nitrogen in A. donax (Fig. 4a). In the first 26 days, the influent NH$_3$-N was around 1.0 mg/L, then kept relatively high from day 27 to day 105 with the maximum value of 5.42 mg/L. Afterwards, the influent NH$_3$-N decreased to 1.0 mg/L again. The average NH$_3$-N removal efficiency and volumetric removal rate during the whole operation were 54.5% ± 31.2% and 1.94 ± 2.34 N/(m$^3$·d), which verified the existence of anammox reaction in the anoxic MBBR.

The effluent COD variation could be divided into two stages (Fig. 4b). In the first 13 days, the effluent COD averaged at 1441.0 ± 300.2 mg/L, then decreased gradually and kept at relatively stable level (20.2 ± 12.9 mg/L) from day 27. The rapid COD increase in the first 13 days was probably caused by the soluble organic matter release from A. donax carriers and the low carbon usage by the biofilm and sludge. Then much released carbon was consumed continuously by the matured biofilm and sludge, thus kept the COD at stable concentration. The results also indicated that the organic matter release rate from A. donax was high during the first 2 weeks and then began to decrease after that period. Other plant carbon source exhibited similar release trend with slight differences of rapid release time (Yang et al., 2015). Effluent COD before day 27 was high enough to cause secondary environmental pollution, which should be collected and partly recycled to the influent as external carbon source. Certain measures should be taken for the effluent before day 27 to meet the COD discharge limitation. Appropriate pretreatment, modification or combination with other materials of the plant carbon source could stabilize its carbon release (Liu et al., 2018), and such measures could also be applied to A. donax pieces for steady carbon release.

Fig. 3 – NO$_2$-N (a), NO$_3$-N (b) and TIN (c) removal efficiency of the A. donax MBBR.
At stable phase, the biofilm bacterial morphology on *A. donax* surface was observed by SEM (Fig. 5a, b). The surface of *A. donax* was covered by dense biofilm mainly consisted of cocci (0.5–2.0 μm), bacillus (0.5–1.0–1.5 μm) and filamentous bacteria, which was consistent with the microbial morphology in a packed-bed denitrification bioreactor using biodegradable polymer corncob as carbon source (Shen et al., 2013). Chu and Wang (2016) also reported that the filamentous, cocci and short rod bacteria were the main bacteria in three denitrification packed bed reactors, which were filled with PHBV, PHBV/starch and PHBV/bamboo powder (BF) blends as carbon source and biofilm carriers.

2.4.1. SEM

At stable phase, the biofilm bacterial morphology on *A. donax* surface was observed by SEM (Fig. 5a, b). The surface of *A. donax* was covered by dense biofilm mainly consisted of cocci (0.5–2.0 μm), bacillus (0.5–1.0–1.5 μm) and filamentous bacteria, which was consistent with the microbial morphology in a packed-bed denitrification bioreactor using biodegradable polymer corncob as carbon source (Shen et al., 2013). Chu and Wang (2016) also reported that the filamentous, cocci and short rod bacteria were the main bacteria in three denitrification packed bed reactors, which were filled with PHBV, PHBV/starch and PHBV/bamboo powder (BF) blends as carbon source and biofilm carriers.

2.4.2. Gene abundance

The copy numbers of 16S r-RNA gene (BF1: 2.9 × 10^10/g·BF, AS1: 4.5 × 10^10/g·AS, BF2: 1.3 × 10^10/g·BF, AS2: 3.7 × 10^9/g·AS) and *nirG* (BF1: 1.7 × 10^9/g·BF, AS1: 7.0 × 10^8/g·AS, BF2: 8.9 × 10^9/g·BF, AS2: 1.6 × 10^9/g·AS) in BF and AS of *A. donax* denitrification MBBR (Fig. 6) were similar with those of denitrifying MBBR (Torresi et al., 2018). and other plant-biomass carbon source denitrification systems (Arantzazu et al., 2011; Ji, 2018; Torresi et al., 2018). The copy numbers of *nirS* (BF1: 1.5 × 10^10/g·BF, AS1: 6.4 × 10^9/g·AS, BF2: 3.2 × 10^10/g·BF, AS2: 8.1 × 10^9/g·AS) and *nirK* (BF1: 2.2 × 10^9/g·BF, AS1: 2.5 × 10^9/g·AS, BF2: 2.3 × 10^9/g·BF, AS2: 4.5 × 10^8/g·AS) were in the same order of magnitude with those in wetland nitrogen removal system (Arantzazu et al., 2011), but the numbers of *nosZ* were lower than those reported in similar woodchip denitrification bioreactors (Ji, 2018). In this study, the copy numbers of *nosZ* (BF1: 3.0 × 10^9/g·BF, AS1: 1.7 × 10^9/g·AS, BF2: 8.7 × 10^9/g·BF, AS2: 4.3 × 10^8/g·AS) genes were 1 to 6 orders of magnitude lower than those of *nirS* and *nirK*, which indicated that possible N₂O accumulation occurred because the majority of NO₂-N reduced to N₂O by *nirS* and *nirK* could not be reduced completely without enough *nosZ* genes.

2.3. Mass balance calculation

Mass balance among nitrogen, carbon and *A. donax* was calculated after 170 days operation (Table 4). Influent and effluent TIN mass were calculated by the influent and effluent average TIN concentration multiplied by the operation days and daily wastewater treatment amounts. Influent and effluent total organic carbon (TOC) mass were obtained by similar method. TN (TC) mass released from *A. donax* carriers was generated from the weight and TN (TC) content of the raw and used *A. donax* in the MBBR reactor. TC and *A. donax* needed for 1 g nitrogen removal were calculated by the total removed TN mass as well as the total consumed TC and *A. donax* mass.

The calculation results indicated that 4.84 g *A. donax* was required for 1 g TN removal, which was similar to other studies, e.g., 4.72 g starch/polyacaprolactone for a packed-bed denitrification bioreactor (Shen et al., 2013), 4.77 g corncob for a corncob carbon source and bamboo charcoal filter combined nitrogen removal system (Cao et al., 2016). In consideration of the carbon loss and carbon consumption for bacteria assimilative growth in practical applications, more *A. donax* should be dosed than the theoretical value.

### Table 4 – Mass balance of nitrogen and carbon for *A. donax* MBBR denitrification.

<table>
<thead>
<tr>
<th>Item</th>
<th>Indexes</th>
<th>Mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nitrogen</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In</td>
<td>Influent TIN</td>
<td>85.36</td>
</tr>
<tr>
<td>–</td>
<td>TN released from <em>A. donax</em></td>
<td>4.08</td>
</tr>
<tr>
<td>Out</td>
<td>Effluent TIN</td>
<td>48.00</td>
</tr>
<tr>
<td>Removed</td>
<td>Total removed TN</td>
<td>41.44</td>
</tr>
<tr>
<td><strong>Carbon</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In</td>
<td>Influent TOC</td>
<td>70.30</td>
</tr>
<tr>
<td>–</td>
<td>TC released from <em>A. donax</em></td>
<td>195.93</td>
</tr>
<tr>
<td>Out</td>
<td>Effluent TOC</td>
<td>170.13</td>
</tr>
<tr>
<td>Consumed</td>
<td>Total consumed TC</td>
<td>96.11</td>
</tr>
<tr>
<td>Required/g N</td>
<td>TC</td>
<td>2.32</td>
</tr>
<tr>
<td></td>
<td><em>A. donax</em></td>
<td>4.84</td>
</tr>
</tbody>
</table>

* : TOC was calculated from the liner relation with COD (R^2 = 0.83).
RNA gene, narG, nirS and nosZ in BF2 were 55.2%, 47.8%, 79.3%, 97.1% and 99.7% less than those in BF1, respectively. Compared with AS1, the copy numbers of 16S r-RNA gene, narG, nirK, nirS and nosZ in AS2 decreased 91.7%, 77.7%, 82.5%, 87.4% and 97.4%, respectively. The decrease of all the above-mentioned genes in BF samples was lower than those in AS samples, which suggested that the biofilm microorganisms had higher resistance to unfavorable conditions to some extent.

The total copy numbers of denitrification genes (narG, nirK, nirS and nosZ) in BF1, BF2, AS1 and AS2 were 1.6 × 10^{10} copies/g·BF, 3.4 × 10^9 copies/g·BF, 6.7 × 10^9 copies/g·AS and 8.5 × 10^8 copies/g·AS, respectively. Both the copy numbers of denitrification genes in BF were higher than those in AS at two phases, which meant higher denitrifying microbial richness in BF than AS. When the MBBR was approaching the declination phase, the copy numbers of denitrification genes were decreased because of the insufficient carbon source.

The copy numbers of Anammox gene in BF1, BF2, AS1 and AS2 were 1.0 × 10^7 copies/g·BF, 3.1 × 10^4 copies/g·BF, 9.5 × 10^6 copies/g·AS and 2.1 × 10^4 copies/g·AS, respectively, which suggested the existence of anammox reaction and thus explain the phenomenon of Sections 2.2.2–2.2.4. The copy numbers of nirS and nirK were 1 to 3 orders of magnitude higher than those of narG in both BF and AS, which was probably caused by the relatively high influent NO_2^−-N (7.3 ± 4.5 mg/L) during inoculation phase.

### 2.4.3. Microbial community diversity

High-throughput sequencing assay was used to study the microbial diversity of BF2 and AS2 (the NCBI accession numbers of SRR8607415 for AS and SRR8607416 for BF). After low quality sequences and chimeras removal, 93,434 and 90,079 effective sequences were selected for further analysis in BF2 and AS2. The effective tags of BF2 and AS2 samples were clustered (with ≥97% similarity) into 1816 and 2260 OTUs.

Alpha diversity indexes of the microbial community diversity of BF2 and AS2 were listed in Table 5. The coverage indexes of BF2 and AS2 were 0.995 and 0.994, which were high enough to ensure the remarkable representativeness of the identified sequences for the majority of microbial diversity (Matar et al., 2017). ACE, Chao 1 and Shannon values (2487.4, 2455.5 and 8.2) of AS2 were higher than those (2034.1, 1975.6 and 7.8) of BF2, which indicated the relatively higher microbial diversity in AS2 than that in BF2 as reported by Bing et al.

### Table 5 – Alpha diversity analysis for BF2 and AS2 samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>OTUs</th>
<th>Shannon</th>
<th>ACE</th>
<th>Chao 1</th>
<th>Coverage</th>
<th>Simpson</th>
</tr>
</thead>
<tbody>
<tr>
<td>BF2</td>
<td>1816</td>
<td>7.78</td>
<td>2034.13</td>
<td>1975.61</td>
<td>0.995</td>
<td>0.016</td>
</tr>
<tr>
<td>AS2</td>
<td>2260</td>
<td>8.24</td>
<td>2487.36</td>
<td>2455.48</td>
<td>0.994</td>
<td>0.016</td>
</tr>
</tbody>
</table>
AS2 and BF2 had the same Simpson values, which meant similar microbial evenness. It was reported that multi-stage MBBR had higher microbial richness than single-stage MBBR (Torresi et al., 2018), which was consistent with the lower alpha diversity indexes of single-stage A. donax MBBR in this study. The results of 16S rRNA high-throughput sequencing suggested that there was minor difference of dominant groups between BF2 and AS2 (Fig. 7a). There were the same dominant phyla of Proteobacteria (64.3% and 50.1%), Bacteroidetes (7.9% and 5.5%), Firmicutes (7.6% and 12.1%), Chloroflexi (5.6% and 3.5%), Acidobacteria (4.4% and 3.7%) and Gemmatimonadetes (1.4% and 10.4%) with different amount in BF2 and AS2, however, the Actinobacteria (4.4%) only existed in BF and Planctomycetes (4.9%) only in AS. Obviously, Proteobacteria were the main dominant community in both BF (64.31%) and AS (50.1%), which were in accordance with the previous reports, e.g., 37%–89% (Biswas et al., 2014) and 85.5% (Shen et al., 2013) for different scale denitrification systems. Proteobacteria community were Gram-negative bacteria and contained large number of denitrificans (Bing et al., 2016), whose outer surface lipopolysaccharides could assist their attachment to the carrier surface (Biswas et al., 2014). Bacteroidetes and Firmicutes were found as dominant phyla in two full-scale MBBRs with suspended polyethylene carriers and other lab-scale denitrification reactors, and many strains of Bacteroidetes and Firmicutes were been identified as denitrificans (Biswas et al., 2014; Shen et al., 2013; Xu et al., 2018). The same predominant bacterial classes in BF and AS were Gammaproteobacteria (52.6%, 41.7%), Clostridia (5.6%, 6.4%), Anaerolineae (5.3%, 3.0%), Bacteroidia (4.8%, 3.4%), Deltaproteobacteria (3.3%, 3.9%) and Bacilli (1.6%, 4.9%) (Fig. 7b). However, the other dominant classes of Alphaproteobacteria (8.3%), unidentified_Actinobacteria (3.8%) and Deltaproteobacteria (3.3%) were only found in BF, and unidentified_Gemmimonadetes (10.4%) and Planctomycetaceae (3.5%) were only observed in AS. Gammaproteobacteria, Alphaproteobacteria, Deltaproteobacteria and Clostridia were also found as the dominant denitrification groups in other denitrification systems (Biswas et al., 2014; Bing et al., 2016). Types of carbon source, the availability of organic substrate and the stages of MBBR could influence the microbial communities of biofilm and suspended sludge (Torresi et al., 2018).

At genus level, the bacterial sequences in BF mainly belonged to Acinetobacter (10.9%), Denitratisoma (2.9%) and Pseudomonas (2.8%). However, Lactobacillus (4.4%), Denitratisoma (3.1%) and Sulfitordana (2.2%) were the dominant genera in AS. There were the same genera of Denitratisoma and Acinetobacter with different abundance in BF and AS, and other genera were different (Fig. 7c). Acinetobacter, which belonged to Gammaproteobacteria class, were the predominant bacteria in both biofilm and suspended sludge from MBBR (Biswas et al., 2014). Pseudomonas contained typical denitrification bacteria which participated in denitrification, nitrogen fixation and carbon degradation process, and were dominated in anoxic denitrification biofilm reactor (Bing et al., 2016). Fig. 7c suggested that Pseudomonas in BF were more abundant than those in AS, which might be resulted from higher consumption of A. donax released carbon source by Pseudomonas in BF than those in AS for the shorter transportation distance. Moreover, the stronger anoxic environmental condition in the BF inner surface of A. donax was relatively favorable for Pseudomonas growth. Lactobacillus were related to NO synthesis (Yarullina and Ilinskaya, 2007). Denitratisoma were typical heterotrophic bacteria affiliated with Proteobacteria phylum, which were detected in wastewater denitrification system (Zhang et al., 2017).

2.4.4. Functional prediction of microbial community
PICRUSt results illustrated that the Nearest Sequenced Taxon Index of BF2 and AS2 were 0.11 and 0.16 respectively, which were similar with those reported by Li et al. (2017). The K
number in BF (5716) was lower than that in AS (6019), which was corresponded with the lower OTU numbers of BF2 than AS2 in Section 2.4.3. For both BF2 and AS2, the predicted functions can be classified into 6 categories at KO level 1, 41 categories at KO level 2, and 295 (BF2) and 297 (AS2) categories at KO level 3. The most abundant functions at KO level 1, level 2 and level 3 were metabolism, membrane transport and transporters, respectively.

There were 33 predictive specific genes related to denitrification process in the prediction results, which included 21 genes about NO$_3^-$-N/NO$_2^-$-N reduction, 11 genes about NO reduction and 1 gene about N$_2$O reduction (Appendix A Table S1). The predictive denitrification functional genes included NO$_3^-$-N/NO$_2^-$-N reductase, NO$_3^-$-N/NO$_2^-$-N transporter, NO$_3^-$-N/NO$_2^-$-N response regulator, NO$_3^-$-N/NO$_2^-$-N sensor histidine kinase, NO reductase, NO dioxygenase, NO sensitive transcriptional repressor, N$_2$O reductase, etc. The total abundance of predictive denitrification functional genes and the relative abundance of 18 predictive specific genes in BF2 were higher than those in AS2, which suggested the higher denitrification capacity of BF2 than AS2. Moreover, the relative lower abundance of N$_2$O reductase and higher abundance of NO reductase illustrated the accumulation of N$_2$O in the A. donax denitrification MBBR. However, the prediction of denitrification was limited as the annotated anammox process is included in AS, and the abundance of narG and nosZ genes were higher than that of AS2, which indicated that the microorganisms in BF played more important role during denitrification.

3. Conclusions

Raw A. donax pieces could be used as carbon source and biofilm support to remove NO$_3^-$-N from ROC in a denitrification MBBR, the feasibility and microbial community characteristics of which were extensively investigated and demonstrated in this study. High denitrification capability (73.2% ± 19.5% NO$_3^-$-N removal efficiency and 8.10 ± 3.45 g N/(m$^3$·day) NO$_3^-$-N volumetric removal rate) were obtained at stable phase (60 days). The NH$_4^+$-N removal efficiency of 54.5% ± 31.2% was achieved due to the anammox process, which had been verified by the QPCR analysis. COD release from A. donax pieces was excessive in the first 13 days, which kept at relatively stable level from day 27.

All of the copy numbers of 16S r-RNA, narG, nirS, nosZ, and anammox genes in declination phase (BF2, AS2) were lower than those in stable phase (BF1, AS1). The copy numbers of nirK in BF1 and BF2 were closed, and the copy number of nirK in AS1 was higher than that in AS2. The copy numbers of narG, nirS/nosZ and anammox genes in BF were higher than those in AS, and the abundance of nirS and nirK genes were higher than that of narG and nosZ genes for both BF and AS. However, the microbial diversity of BF2 was relatively lower than that of AS2. Similar dominant phyla and classes with different abundance were obtained for BF2 and AS2. There were the same dominant phyla of Proteobacteria (64.3% in BF2 and 50.1% in AS2), Bacteroidetes (7.9% in BF2 and 5.5% in AS2) and Firmicutes (7.6% in BF2 and 12.1% in AS2), etc. The classes of Gammaproteobacteria (52.6% in BF2 and 41.7% in AS2), Clostridia (5.6% in BF2 and 6.4% in AS2), Anaerolineae (5.3% in BF and 3.0% in AS), etc., were also predominant for both BF2 and AS2.

However, the genera difference was found large for BF2 and AS2, and the denitrifying bacteria Acinetobacter (10.9%) and Denitratisoma (2.9%) were the predominant genera in BF2, while Lactobacillus (4.4%) and Denitratisoma (3.1%) were abundant in AS2. The PICRUSt prediction results illustrated that 33 predictive specific genes were related to denitrification process including NO$_3^-$-N/NO$_2^-$-N/NO/N$_2$O reductase and other denitrification-related genes. The relative abundance of 18 predictive specific genes in BF2 were higher than that in AS2, which indicated that the microorganisms in BF played more important role during denitrification.

Ethical statement

No conflict of interest exits in this manuscript. The work has not been published previously, and not under consideration for publication elsewhere. This article does not contain any studies with human participants and animals performed by any of the authors.

Acknowledgments

This work was supported by the National Major Science and Technology Program for Water Pollution Control and Treatment (Nos. 2017ZX07401003-05-01; 2014ZX07216-001) and the China Scholarship Council Foundation (No. 2011911098).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jes.2019.04.030.

REFERENCES


