Bioresource Technology 159 (2014) 266–271

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Nitrogen removal pathway of anaerobic ammonium oxidation in on-site aged refuse bioreactor

Chao Wang [a], Youcai Zhao [b], Bing Xie [a,*], Qing Peng [a], Muhammad Hassan [a], Xiaoyuan Wang [a]

[a] Shanghai Key Lab for Urban Ecological Process and Eco-Restoration, Department of Environmental Science & Technology, East China Normal University, Shanghai 200062, China
[b] State Key Laboratory of Pollution Control and Resource Reuse, School of Environmental Engineering and Science, Tongji University, Shanghai 200092, China

HIGHLIGHTS

- Pollutants were effectively removed by on-site aged refuse bioreactor in landfill.
- *amoA*, *nirS* and anammox 16S rRNA gene were found to coexist in every bioreactor.
- Ratios of functional genes and pollutants removal performance were closely related.
- Anammox provides an alternative pathway for nitrogen management in landfill.

ARTICLE INFO

Article history:
Received 19 December 2013
Received in revised form 19 February 2014
Accepted 21 February 2014
Available online 1 March 2014

Keywords:
Landfill leachate
Aged refuse bioreactor
Nitrogen removal
Anammox

ABSTRACT

The nitrogen removal pathways and nitrogen-related functional genes in on-site three-stage aged refuse bioreactor (ARB) treating landfill leachate were investigated. It was found that on average 90.0% of COD_{Cr}, 97.6% of BOD_5, 99.3% of NH_4^+–N, and 81.0% of TN were removed with initial COD_{Cr}, BOD_5, NH_4^+–N, and TN concentrations ranging from 2323 to 2754, 277 to 362, 1237 to 1506, and 1251 to 1580 mg/L, respectively. Meanwhile, the functional genes *amoA*, *nirS* and anammox 16S rRNA gene were found to coexist in every bioreactor, and their relative proportions in each bioreactor were closely related to the pollutant removal performance of the corresponding bioreactor, which indicated the coexistence of multiple nitrogen removal pathways in the ARB. Detection of anammox expression proved the presence of the anammox nitrogen removal pathway during the process of recirculating mature leachate to the on-site ARB, which provides important information for nitrogen management in landfills.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Sanitary landfilling is currently recognized as the most common ultimate disposal method for municipal solid waste (MSW) worldwide. The sanitary landfill method not only presents economical advantages but also permits the decomposition of waste under controlled conditions until the MSW is eventually transformed into relatively inert, stabilized material (Renou et al., 2008). However, landfill leachate, which is generated during the biodegradation of MSW and the percolation of rainwater during landfilling, is a heavily polluted wastewater. It is generally enriched in numerous organic, inorganic, ammonium, and toxic constituents, and its characteristics vary significantly with increasing disposal time (Foo and Hameed, 2009). Mature leachate, which is achieved in landfills after operation for more than 5 years, is characterized by high ammonium (NH_4^+) content and low biodegradability of organics (low ratio of biochemical oxygen demand (BOD_5)/chemical oxygen demand (COD)), making its treatment challenging, especially *in situ* treatment (Cortez et al., 2010; Kurniawan et al., 2006).

An aged refuse bioreactor (ARB), filled with 8- to 10-year-old refuse excavated from a landfill as a filter medium, has been shown to be a highly efficient method for treating landfill leachate, both at the lab-scale and the field-scale (Zhao et al., 2002; Xie et al., 2010). The primary pollutant removal pathways in ARB treatment of landfill leachate involve degradation by aerobic microorganisms in the surface layer and anaerobic microbes beneath the surface, adsorption of refuse particles, ion exchange, and chelation (Long et al., 2008). Previous studies have also verified that the high level of nitrogen pollutant removal in ARB treatment is achieved through a wide range of numerous microorganisms present in the aged refuse (Li et al., 2010; Song et al., 2011; Xie et al., 2012). With regard to nitrogen removal, it is a common notion that nitrogen-removing functional microbes such as ammonia-oxidizing bacteria (AOB) and denitrifying bacteria play a vital role in both nature and

* Corresponding author. Tel./fax: +86 21 54341276.
E-mail address: bxie@des.ecnu.edu.cn (B. Xie).

http://dx.doi.org/10.1016/j.biortech.2014.02.093
0960-8524/© 2014 Elsevier Ltd. All rights reserved.

engineered systems. However, considering the characteristics of mature leachate, the removal rate of total nitrogen (TN) in ARB treatment of mature leachate will be restricted by the low biodegradable organic matter content of the leachate according to the traditional nitrification/denitrifaction pathway. Thus, multiple nitrogen removal pathways may exist in ARBs.

Recently, different nitrogen removal mechanisms such as autotrophic denitrification and anaerobic ammonium-oxidation (anammox) have been demonstrated in the nitrogen cycle and have received widespread attention because they provide an alternative nitrogen removal pathway in environments with a low carbon to nitrogen ratio (Daverey et al., 2013; Sliekers et al., 2002). The anammox process, which combines ammonium as an electron donor and nitrite as an electron acceptor to form nitrogen, is universally believed to require approximately equimolar concentrations of ammonium and nitrite (Jetten et al., 2001). Previous studies have reported contradictory results on the presence of the anammox process in landfills that conduct leachate recirculation. Price et al. (2003) suggested that the existence of the anammox process remains uncertain in the conditions of leachate recirculation to the bioreactor after the conversion of ammonium to nitrate in an ex situ aerobic reactor. However, Valencia et al. (2011) confirmed that the introduction of small quantities of oxygen promotes the growth of anammox bacteria, and the presence of the anammox process contributes to nitrogen removal in landfill bioreactors. In addition, our previous work also verified the presence of anammox bacteria in a lab-scale ARB and revealed that multiple nitrogen removal pathways coexist when leachate was pretreated using shortcut nitrification and the influent ratio of ammonium and nitrite was approximately 1 (Xie et al., 2013). Although simultaneous partial nitrification, anammox, and denitrification (SNAD) were found in a single liquor bioreactor for nitrogen removal (Chen et al., 2009; Kumar and Lin, 2010), for an on-site ARB in which the environment is aerobic at the surface and gradually becomes anaerobic deeper within the reactor (Han et al., 2013; Wang et al., 2013), the existence of anammox bacteria and the anammox nitrogen removal pathway remain uncertain when nitrogen compounds exist primarily in the form of ammonium in influent leachate. Nonetheless, it will be helpful to understand the different nitrogen removal pathways that occur in landfill processing if anammox bacteria are present in on-site ARBs.

The purpose of this study was to investigate the nitrogen removal efficiency of on-site ARBs at the Shanghai Laogang landfill and the corresponding expression patterns of nitrogen-removing functional genes (*amoA*, *nirS*, and anammox 16S rRNA) using quantitative polymerase chain reaction (qPCR). It is expected that the results will reveal a potential alternative nitrogen removal pathway in *in situ* landfill bioreactors.

2. Methods

2.1. On-site ARB

The Shanghai Laogang MSW plant (31°00′N, 120°52′E), located north of Hangzhou Gulf and south of the mouth of the Yangtze River in China, is the largest landfill in Asia. It receives 10,000 tons of MSW and produces nearly 3000 m^3 leachate per day (Ding et al., 2007; Li et al., 2009). Aged refuse was excavated from a single landfill compartment that has been closed for nearly 10 years and sieved to less than 40 mm before use as the packing material (Zhao et al., 2002). Some basic characteristics of the aged refuse were: volatile suspended solids (VSS) of 10–12%, moisture content of 12–15%, and density of 1.60–1.75 g/cm^3. Three-stage horizontal and tower ARBs were constructed on-site to treat the landfill leachate.

In the three-stage horizontal ARB, the surface area of the first aged refuse bed (bioreactor) was 2300 m^2, and that of the additional two beds was 2000 m^2. The vertical height of each bed was about 3 m. The leachate was sprayed over the bed through holes in the influent tube, and the effluent was collected and pumped to the second and third beds. Effluent from each bed was collected in corresponding collecting basins approximately 30 m^3 in volume. In the three-stage tower ARB, which was constructed above ground, the first bed was on the top and the third one was on the bottom. The vertical height of each tower ARB was approximately 1 m, which was less than that of the horizontal ARB. The leachate was pumped and sprayed over the first tower bed and then infiltrated into the sequenced beds by gravity. (Images of the horizontal and tower ARBs are shown at Fig. S1(a) and (b), respectively, in the Supplementary Material). The characteristics of the leachate from Shanghai Laogang MSW were as follows: COD_{Cr} of 2322.9–2754.4 mg/L, BOD_5 of 276.5–361.9 mg/L, NH_4^+–N of 1237.2–1506.2 mg/L, NO_2^-–N of 0.5–4.7 mg/L, NO_3^--N of 13.7–46.1 mg/L, TN of 1251.3–1580.5 mg/L, pH between 7.9 and 8.2, and oxidation/reduction potential (ORP) of −300 to −340 mV.

The ARBs were operated under a hydraulic loading rate (HLR) between 10 and 20 L/m^3 d, and the field temperature varied between 20 °C and 30 °C during the study period. The wastewater samples were collected over 10 weeks for water quality analysis, and at the end of the experiment, aged refuse was sampled for microbiological analysis. For the horizontal ARB, the influent, first effluent, second effluent, final effluent, and triple aged refuse samples of each bioreactor (1 m below the surface using screw drilling, Fig. S2 of Supplementary Material) were sampled. For the tower ARB, because the space between the first and second stage was very small, and it is not always possible to get samples from the second bed, only the influent, third effluent, and duplicate aged refuse samples of first and third beds were collected (0.20 m below the surface).

2.2. Analytical methods

2.2.1. Wastewater quality

The influent and effluent parameters such as COD_{Cr}, BOD_5, NH_4^+–N, NO_2^-–N, NO_3^-–N, and TN were measured according to the standard methods (APHA, 1998).

2.2.2. Microbiological analysis

2.2.2.1. DNA extraction and plasmid standard preparation. Samples of 0.6 g aged refuse were used for total DNA extraction. Genomic DNA was extracted using MoBio Ultra-Clean™ soil DNA isolation kits (MoBio Laboratories Inc., CA, USA) according to the manufacturer's protocol. Total 16S rRNA gene, *amoA*, *nirS*, and anammox 16S rRNA gene were amplified using the following PCR procedure. Each 50-μL PCR reaction mixture was composed of 5 μL 10 × Buffer, 4 μL (25 mmol/L) Mg^{2+}, 1 μL (5 mmol/L) dNTP, 0.5 μL (5 U) Taq polymerase, 1 μL of each primer, 1 μL DNA template, and 36.5 μL ddH_2O. The PCR products were analyzed using 1% agarose gel electrophoresis.

The V3 region of 16S rRNA of bacteria was amplified with the outer 27F (AGAGTTTGATCMTGGCTCAG)/1492R (TACGGYTACCTT GTTACGACTT) (Xu et al., 2008) and inner F357 (CCTACGGGAGGC AGCAG)/R518 (ATTACCGCGGCTGCTGG) (Muyzer et al., 1993) primers. The PCR program for each 27F/1492R primer pair was: initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 s, and annealing for 30 s. The first cycle was at 65 °C, with the temperature in each of the next 11 cycles decreasing by 0.5 °C. The next 23 cycles were carried out at 59 °C, followed by extension at 72 °C for 1 min, and final extension at 72 °C for 7 min. The PCR

program for primers F357-GC/R518 was as follows: initial denaturation for 3 min at 96 °C, 35 PCR cycles (94 °C for 30 s, 58 °C for 45 s, and 72 °C for 40 s), and then a final extension step at 72 °C for 7 min.

Gene expression in AOB was studied by amplifying *amoA* using the primers amoA-1F (GGGGTTTCTACTGGTGGT) and amoA-2R (CCCCTCKGSAAAGCCTTCTTC) (Nicolaisen and Ramsing, 2002). The PCR procedure was 15 min at 95 °C, 35 cycles at 94 °C for 60 s, 57 °C for 45 s, 72 °C for 45 s, and then 72 °C for 7 min.

Expression of denitrifying bacteria was studied by amplifying *nirS* using the primer pair of *nirS* 4F (TTC(A/G)TCAAGAC(C/G)CA(C/T)CCGAA)/*nirS* 6R (CGTTGAACTT(A/G)CCGGT) (Tamegai et al., 2007). The PCR procedure was as follows: initial denaturation at 95 °C for 15 min, denaturation at 95 °C for 30 s, and annealing for 40 s. The first cycle was at 60 °C, with the temperature in each of the next 9 cycles decreasing by 0.5 °C. The next 25 cycles were carried out at 55 °C, followed by extension at 72 °C for 1 min, and final extension at 72 °C for 7 min.

Anammox expression was studied by amplifying the 16S rRNA gene using the primer set of Anammox 1F (GGATTAGGCATGCAAGTC)/Anammox 2R (TCTGTATTACCGCGGCT) (Xie et al., 2013). The PCR procedure was 95 °C for 5 min, 94 °C for 30 s, 50 °C for 60 s, 72 °C for 60 s, and final extension at 72 °C for 60 s.

The purified PCR products were ligated into the pGEM-T Easy plasmid vector (Promega, USA) and transformed into DH-5α cells. Following overnight incubation, positive recombinants were recognized and screened for the presence of an insert by PCR using the primers and procedures described above. Plasmid DNA was isolated using the Plasmid Kit (Shanjing Molecular Biological Technology Co., Shanghai, China) according to the manufacture's instruction. The plasmid DNA from the clone was used as a standard for the qPCR assays. Plasmid DNA concentration was determined on a biophotometer.

2.2.2.2. qPCR. qPCR assays were performed using SYBR Green I and conducted using an FTC2000 fluorescence real-time PCR system (Funglyn Biotech Inc., Canada). Each 50-μL reaction mixture contained 25 μL 2 × PCR buffer, 1 μL of each primer (25 μM), 0.5 μL SYBR Green I fluorescent dye, 2 μL DNA template, and diethylpyrocarbonate (DEPC) water. The copy number of the target gene was calculated based on the concentration of plasmid DNA and its molecular weight. Ten-fold serial dilutions of a known copy number of the plasmid DNA were prepared as an external standard. The melting curve correlation coefficients of the total 16S rRNA gene and functional genes were all above 0.999. The results are expressed as gene copies per gram of dried aged refuse.

2.2.3. Statistical correlation analysis

The relationships between the performance of the ARBs and the functional gene expression levels were studied. All the statistical analyses were performed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA), and $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Pollutant treatment performance of on-site bioreactor

The pollutant removal performance of the three-stage horizontal ARB at the Shanghai Laogang MSW plant was investigated for 10 weeks. As shown in Fig. 1, during 10 weeks of operation, the influent COD_{Cr} varied from 2323 to 2754 mg/L, and the effluent COD_{Cr} of the first, second, and third bioreactors were 372–990, 308–371, and 205–288 mg/L, respectively. The average removal rates of the respective bioreactors were 78.9%, 7.4%, and 3.7%, showing that most COD was removed in the first stage bed, whereas less removal was achieved in the second and third beds. The total COD removal rate reached, on average, 90.0%. The average effluent BOD_5 concentrations of the first, second, and third beds were 66.1, 22.4, and 7.6 mg/L, respectively. Greater than 97.6% of BOD_5 was removed when the initial concentration ranged from 277 to 362 mg/L, similar to the removal of COD_{Cr}, indicating that most of the biodegradable organic compounds also were removed by the first bed. These results imply that this on-site ARB demonstrated excellent organic compound removal ability, which agrees with the high efficiency in organic matter removal reported by other studies. Li et al. (2010) reported that COD removal by an ARB increased from 64% to 93% and BOD_5 removal increased from 95.8% to 99.8%. Notably, the average BOD_5/COD values of the first, second, and third effluents were 0.13, 0.07, and 0.03, respectively, which indicates the low biodegradability and relatively high refractory organic matter content of the effluent leachate (Erses et al., 2008). The less degradable organic substances in the leachate will make the carbon source scarce for denitrification.

The influent NH_4^+–N concentration ranged from 1237–1506 mg/L, and the average effluent NH_4^+–N concentration in the first, second, and third effluents was 133.1, 24.5, and 9.1 mg/L respectively. A total removal rate of 99.3% was achieved, and the NH_4^+–N removal was achieved primarily in the first-stage bed as well. During the entire period, the final effluent NH_4^+–N concentration could meet the 25 mg/L standard of the Chinese Emission Standards for pollution control on MSW landfill sites (GB16889-2008). These results demonstrated that the on-site ARB was extremely efficient in treating NH_4^+–N. A high NH_4^+–N removal rate also was obtained when treating leachate using an ARB in lab- and pilot-scale experiments when the bioreactor was operated under aerobic and semi-aerobic conditions (Xie et al., 2010; Sun et al., 2011), suggesting that ammonia could be easily transferred if operation conditions permit in ARBs.

The original NO_2^-–N concentration was maintained at a very low level, 2 mg/L, compared with NH_4^+–N, while in the first effluent, the NO_2^-–N concentration was increased to the peak value of 154–189 mg/L and then decreased in the second and third effluent. For NO_3^-–N, the effluent concentration showed an increasing trend. The original concentration was also low, i.e., 26.6 mg/L, and gradually increased to the final effluent concentration of 344 mg/L. The variation in NO_2^-–N and NO_3^-–N suggested that the on-site ARB offered strong nitrification, but denitrification was somehow restricted due to the less usable carbon source.

An average TN removal rate of 70.9% was achieved with an initial concentration between 1251 and 1580 mg/L. The TN concentration in the first, second, and third effluents were 757–826, 504–610, and 379–392 mg/L, respectively, and the average removal rates of each respective bioreactor were 39.1%, 19.6%, and 12.1%. Unlike COD, BOD_5, and NH_4^+–N; TN removal was not overwhelmingly achieved in the first bed, and every bed significantly contributed to TN removal. The nitrogen in the final effluent mainly existed in the form of NO_3^-–N.

Meanwhile, it can be seen from Table 1, the tower ARB performed with basically the same organic pollutant removal efficiency as that of the horizontal ARB ($P > 0.05$), and the COD removal rate was 80.2–90.8%. However, for TN removal, the average removal rate of the tower ARB was 81.0% (ranged from 77.0–85.1%), which was higher than that of the horizontal ARB ($P < 0.05$). The structure of the tower bioreactor may contribute to the relatively high TN removal performance, because leachate was exposed to better alternating aerobic and anaerobic environments due to the low vertical height of each tower ARB compared with horizontal dimension.

According to traditional nitrification/denitrification, the theoretical amount of carbon source required for complete denitrifica-

Table 1
Pollutant removal performance of the tower ARB.

Water parameters	COD_{Cr}	BOD_5	NH_4^+-N	TN
Influent concentration (mg/L)	2323–2754	277–362	1237–1506	1251–1580
Effluent concentration (mg/L)	226–325	7–12	5–21	284–315
Removal rate (%)	80.2–90.8	95.3–98.5	98.6–99.4	77.0–85.1

tion is 2.86 mg COD/mg NO_3^-–N (Wong and Lee, 2011). Supposing all COD in the influent is biodegradable, approximately 700 mg/L TN could be removed by the nitrification and denitrification pathway in this study, which accounts for nearly 50% of influent TN. However, not all COD_{Cr} are biodegradable, and the BOD to COD ratio is much lower in mature landfill leachate. Therefore, the TN removal efficiency of the Laogang on-site ARB cannot be achieved simply via the traditional nitrification/denitrifaction according to the removal amounts of COD_{Cr}, BOD_5 and TN described previously. Other nitrogen removal pathways also must exist in the ARB. To better understand the microbiological mechanism of the on-site ARB, aged refuse was sampled for functional gene analysis after 10 weeks of ARB operation.

3.2. Nitrogen-removing functional gene analysis

Table 2 shows the qPCR results for total 16S rRNA genes and nitrogen-removing functional genes, *amoA*, *nirS*, and anammox 16S rRNA, in the three-stage horizontal ARB and the first and third stages of the tower ARB. The number of total 16S rRNA genes in each bed was 10^9–10^{10} copies/g.

For the horizontal ARB, the first bed had the highest *amoA* expression at 3.46×10^8 copies/g, whereas at the second and third beds, *amoA* expression was decreased to the magnitude of 10^7 copies/g. Combined with the results showing that the first bed had the highest NH_4^+–N removal efficiency of 90.5% and followed by 7.7% for the second bed and 1.1% for the third bed, these results indicate that the first-stage bed contributed largely to the nitrification of ammonium nitrogen via a relatively greater quantity of AOB. Expression of *nirS* remained at a stable level of 10^7 copies/g at all beds. The amount of *nirS* expression is relatively less than that of *amoA*, and one reason may be that the number and activity of denitrifying bacteria are affected by the level of organic matter (Beauchamp et al., 1989). Because the low BOD_5/COD ratio (less than 0.13), the amount of biodegradable organic carbon is low in the leachate, and denitrification in the horizontal ARB was somehow inhibited. Another reason could be that *nirS* may not be the predominant denitrifying gene in ARBs (Braker et al., 1998). The anammox 16S rRNA gene was found in all ARBs, and its quantity maintained around 1 to 2×10^7 copies/g, showing the presence of anammox bacteria in the on-site ARB. Thus, these results may provide an alternative nitrogen removal pathway under the condition of a low C/N ratio even when the influent nitrogen is mostly ammonium. Therefore, it can be safely concluded that the anammox process did occur in the on-site ARB and contributed to nitrogen removal in addition to traditional nitrification/denitrification. For the tower ARB, the quantity of nitrogen-removing functional genes in the first stage bed was even higher than that in the horizontal ARB, especially the anammox 16S rRNA gene, coupled with its higher TN removal efficiency, suggesting the tower ARB shows better nitrogen removal ability.

Meanwhile, the percentage of each nitrogen-removing functional gene among all 16S rRNA genes was calculated. As shown in Table 3, the ratio of *amoA* expression in the first bed is much higher than that of others, and other functional genes account for less than 1%, except the anammox 16S rRNA gene in the third stage tower ARB. In comparing the horizontal and tower ARBs, the proportions of three functional genes in the first and third stage tower ARB are higher than those in the horizontal ARB ($P < 0.05$). At the first bed, the proportions of *nirS* and anammox 16S rRNA gene in the tower ARB are higher than those in a horizontal ARB, although the proportion of *amoA* is lower. At the third bed, although there were no significant differences in the anammox gene ratio, the other two ratios of functional genes in a tower ARB were significantly higher. These results suggest that the tower bioreactor is more suitable for the existence of different nitrogen-removing functional microbes, which may be the reason for its better nitrogen removal performance.

The process of leachate trickling down through the ARB by gravity supported the formation of an aerobic–anoxic–anaerobic environment, which provided a favorable condition for attached bacterial growth (Han et al., 2013). The qPCR results suggest that during the process of recirculating leachate to the biofilter, ammonium in the influent was converted to nitrite, and in some anaerobic areas, such as at the surface or within the aged refuse pellet, nitrite could be denitrified through anammox or short-denitrifica-

Table 2
qPCR results for nitrogen-removing functional genes in different ARBs.

Location	Total 16S rRNA gene (copies/g)	*amoA* (copies/g)	*nirS* (copies/g)	Anammox 16S rRNA gene (copies/g)
First-stage horizontal ARB	(3.92 ± 1.31)[a] $\times 10^9$	$(3.46 \pm 1.28) \times 10^8$	$(1.72 \pm 0.68) \times 10^7$	$(2.13 \pm 1.06) \times 10^7$
Second-stage horizontal ARB	$(1.60 \pm 0.72) \times 10^{10}$	$(9.00 \pm 0.58) \times 10^7$	$(2.86 \pm 1.68) \times 10^7$	$(2.30 \pm 0.27) \times 10^7$
Third-stage horizontal ARB	$(1.08 \pm 0.38) \times 10^{10}$	$(5.44 \pm 0.28) \times 10^7$	$(1.39 \pm 0.54) \times 10^7$	$(1.43 \pm 0.15) \times 10^7$
First-stage tower ARB	7.68[b] $\times 10^9$	5.09×10^8	6.06×10^7	1.77×10^8
Third-stage tower ARB	7.20×10^9	6.81×10^7	2.25×10^7	8.22×10^6

[a] Represents average ± standard deviation (S.D.).
[b] Average of two samples.

Table 3
Proportions of nitrogen-removing functional genes of horizontal ARB and tower ARB.

Proportion (%)	Horizontal ARB			Tower ARB	
	First stage	Second stage	Third stage	First stage	Third stage
amoA/total 16S rRNA genes	8.82	0.56	0.50	6.63	0.95
nirS/total 16S rRNA genes	0.44	0.18	0.13	0.79	0.31
Anammox 16S rRNA gene/total 16S rRNA genes	0.54	0.14	0.13	2.30	0.11

tion. The presence of an anammox nitrogen removal pathway during the process of recirculating mature leachate to an on-site ARB provided important information for nitrogen management in landfill processing (Price et al., 2003; Chen et al., 2009).

3.3. Comparison analysis of pollutant removal performance and nitrogen-removing functional genes

After the comprehensive analysis of influent parameters shown in Fig. 1 and the expression levels of nitrogen-removing functional genes at each horizontal ARB (Table 2), we found that the first bed had the highest ammonium removal efficiency and the greatest *amoA* gene proportion. However, the ammonium removal performance of the second and third beds was decreased significantly, and at the same time the *amoA* gene ratio also obviously decreased. The proportions of *nirS* and anammox 16S rRNA gene gradually decreased with each stage, and the same trend was observed for the TN removal rate variation between beds. The correlation coefficient for ammonium removal and *amoA* gene was 0.998 ($P < 0.05$), whereas the coefficient for the *nirS* and anammox proportions with TN removal was 0.981. These contrasting results suggest that the proportion of nitrogen-removing functional genes is closely related to the pollutant removal performance of each ARB.

Additionally, the influent of the second and third beds had approximately equal concentrations of ammonium and nitrite, implying that a high concentration of ammonium nitrogen in the original leachate can be transformed into equimolar concentrations of ammonium and nitrite during recirculation of leachate to the ARB. These findings provide the prerequisite of the anammox process (Jetten et al., 2001; Daverey et al., 2012). At the same time,

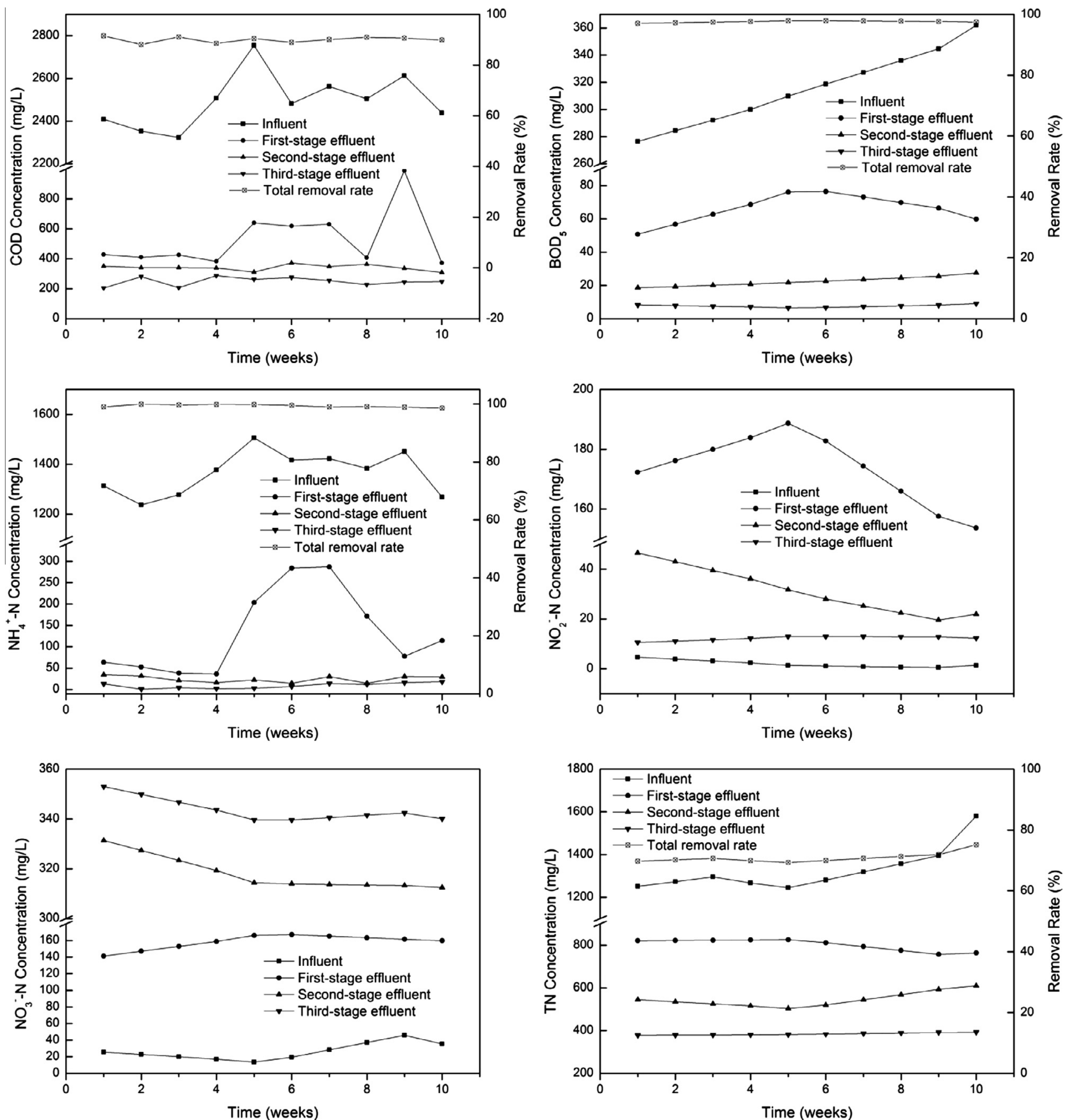

Fig. 1. Pollutant removal efficiency of on-site horizontal ARB.

aged refuse is characterized by a complex particle surface and diversified, attached microorganisms, which may provide a suitable micro-environment for anammox. Fig. 3S in Supplementary Material shows the significantly porous surface before running and the richness of the biofilm after leachate recirculation for several weeks. The results of the present study illustrate that anammox can happen at any suitable interface of the aged refuse wherever it is located in the ARB.

Previous studies confirmed that SNAD can happen in well-mixed sequential batch reactors (SBRs) treating industrial wastewater and landfill leachate (Wang et al., 2010; Lan et al., 2011). Furthermore, the stable detection of *amoA*, *nirS*, and anammox 16S rRNA gene within every bioreactor in this study also proved the existence of SNAD in on-site ARB, and these functional microorganisms accomplished nitrogen transfer and removal together. Nowadays, with more attention paid to nitrogen pollution control in environmental protection, as an essential component of nitrogen-removing functional microbes, anammox bacteria are expected to contribute greatly to nitrogen removal in *in situ* landfill management.

4. Conclusions

In this study, 90.0% of COD_{Cr}, 97.6% of BOD_5, 99.3% of NH_4^+–N, and 81.0% of TN were removed in on-site ARBs in a Laogang MSW plant treating mature landfill leachate, which showed excellent performance in pollutant removal. The proportions of nitrogen-removing functional genes *amoA*, *nirS*, and anammox 16S rRNA gene were found to be closely related to the pollutant removal performance of the corresponding bioreactor. The anammox 16S rRNA gene was detected in all bioreactors, indicating that SNAD can coexist in an ARB, and anammox may represent an alternative pathway for nitrogen removal in a landfill bioreactor.

Acknowledgements

This work was supported by Natural Science Foundation of China (31370510, 20977031), and Shanghai project of international cooperation of science and technology (13520720600).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2014.02.093.

References

APHA-AWWA-WEF, 1998. Standard Methods for the Examination of Water and Wastewater, 20th ed. United Book Press, USA.

Beauchamp, E.G., Trevors, J.T., Paul, J.W., 1989. Carbon sources for bacterial denitrification. Adv. Soil Sci. 10, 113–142.

Braker, G., Fesefeldt, A., Witzel, K., 1998. Development of PCR primer systems for amplification of nitrite reductase genes (nirK and nirS) to detect denitrifying bacteria in environmental samples. Appl. Environ. Microbiol. 64, 3769–3775.

Chen, H.H., Liu, S.T., Yang, F.L., Xue, Y., Wang, T., 2009. The development of simultaneous partial nitrification, ANAMMOX and denitrification (SNAD) process in a single reactor for nitrogen removal. Bioresour. Technol. 100, 1548–1554.

Cortez, S., Teixeira, P., Oliveira, R., Mota, M., 2010. Ozonation as polishing treatment of mature landfill leachate. J. Hazard Mater. 182, 730–734.

Daverey, A., Su, S.H., Huang, Y.T., Chen, S.S., Sung, S., Lin, J.G., 2013. Partial nitrification and anammox process: a method for high strength optoelectronic industrial wastewater treatment. Water Res. 47, 2929–2937.

Daverey, A., Su, S.H., Huang, Y.T., Lin, J.G., 2012. Nitrogen removal from opto-electronic wastewater using the simultaneous partial nitrification, anaerobic ammonium oxidation and denitrification (SNAD) process in sequencing batch reactor. Bioresour. Technol. 113, 225–231.

Ding, Z.H., Tang, Q.H., Liu, C.E., Wang, W.H., Zhuang, M., Lin, Y.M., 2007. Distribution and ecological effect of mercury in Laogang landfill, Shanghai, China. J. Environ. Sci. 19, 200–204.

Erses, A.S., Onay, T.T., Yenigun, O., 2008. Comparison of aerobic and anaerobic degradation of municipal solid waste in bioreactor landfills. Bioresour. Technol. 99, 5418–5426.

Foo, K.Y., Hameed, B.H., 2009. An overview of landfill leachate treatment via activated carbon adsorption process. J. Hazard Mater. 171, 54–60.

Han, Z.Y., Liu, D., Li, Q.B., 2013. A removal mechanism for organics and nitrogen in treating leachate using a semi-aerobic aged refuse biofilter. J. Environ. Manage. 114, 336–342.

Jetten, M.S.M., Wagner, M., Fuerst, J., van Loosdrecht, M., Kuenen, G., Strous, M., 2001. Microbiology and application of the anaerobic ammonium oxidation ('anammox') process. Curr. Opin. Biotechnol. 12, 283–288.

Kumar, M., Lin, J.G., 2010. Co-existence of anammox and denitrification for simultaneous nitrogen and carbon removal—strategies and issues. J. Hazard Mater. 178, 1–9.

Kurniawan, T.A., Lo, W., Chan, G., 2006. Physico-chemical treatments for removal of recalcitrant contaminants from landfill leachate. J. Hazard Mater. 129, 80–100.

Lan, C.J., Kumar, M., Wang, C.C., Lin, J.G., 2011. Development of simultaneous partial nitrification, anammox and denitrification (SNAD) process in a sequential batch reactor. Bioresour. Technol. 102, 5514–5519.

Li, H.J., Gu, Y.Y., Zhao, Y.C., Wen, Z.P., 2010. Leachate treatment using a demonstration aged refuse biofilter. J. Environ. Sci. 22, 1116–1122.

Li, H.J., Zhao, Y.C., Shi, L., Gu, Y.Y., 2009. Three-stage aged refuse biofilter for the treatment of landfill leachate. J. Environ. Sci. 21, 70–75.

Long, Y., Guo, Q.W., Fang, C.R., Zhu, Y.M., Shen, D.S., 2008. In situ nitrogen removal in phase-separate bioreactor landfill. Bioresour. Technol. 99, 5352–5361.

Muyzer, G., De Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695–700.

Nicolaisen, M.H., Ramsing, N.B., 2002. Denaturing gradient gel electrophoresis (DGGE) approaches to study the diversity of ammonia-oxidizing bacteria. J. Microbiol. Methods 50, 189–203.

Price, G.A., Barlaz, M.A., Hater, G.R., 2003. Nitrogen management in bioreactor landfills. Waste Manage. 23, 675–688.

Renou, S., Givaudan, J.G., Poulain, S., Dirassouyan, F., Moulin, P., 2008. Landfill leachate treatment: review and opportunity. J. Hazard Mater. 150, 468–493.

Sliekers, A.O., Derwort, N., Gomez, J.L., Strous, M., Kuenen, J.G., Jetten, M., 2002. Completely autotrophic nitrogen removal over nitrite in one single reactor. Water Res. 36, 2475–2482.

Song, L.Y., Shi, L., Zhao, Y.C., Li, H.J., 2011. Novel engineering controls to increase leachate contaminant degradation by refuse: From lab test to *i* in situ engineering application. Ecol. Eng. 37, 1914–1919.

Sun, Y., Sun, X., Zhao, Y., 2011. Comparison of semi-aerobic and anaerobic degradation of refuse with recirculation after leachate treatment by aged refuse bioreactor. Waste Manage. 31, 1202–1209.

Tamegai, H., Aoki, R., Arakawa, S., Kato, C., 2007. Molecular analysis of the nitrogen cycle in deep-sea microorganisms from the Nankai Trough: genes for nitrification and denitrification from deep-sea environmental DNA. Extremophiles 11, 269–275.

Valencia, R., van der Zon, W., Woelders, H., Lubberding, H.J., Gijzen, H.J., 2011. Anammox: an option for ammonium removal in bioreactor landfills. Waste Manage. 31, 2287–2293.

Wang, C.C., Lee, P.H., Kumar, M., Huang, Y.T., Sung, S., Lin, J.G., 2010. Simultaneous partial nitrification, anaerobic ammonium oxidation and denitrification (SNAD) in a full-scale landfill-leachate treatment plant. J. Hazard Mater. 175, 622–628.

Wang, C., Xie, B., Han, L., Xu, X.F., 2013. Study of anaerobic ammonium oxidation bacterial community in the aged refuse bioreactor with 16S rRNA gene library technique. Bioresour. Technol. V145, 65–70.

Wong, B.T., Lee, D.J., 2011. Denitrifying sulfide removal and carbon methanogenesis in a mesophilic, methanogenic culture. Bioresour. Technol. 102, 6673–6679.

Xie, B., Lv, B.Y., Hu, C., Liang, S.B., Tang, Y., Lu, J., 2010. Landfill leachate pollutant removal performance of a novel biofilter packed with mixture medium. Bioresour. Technol. 101, 7754–7760.

Xie, B., Lv, Z., Hu, C., Yang, X.Z., Li, X.Z., 2013. Nitrogen removal through different pathways in an aged refuse bioreactor treating mature landfill leachate. Appl. Microbiol. Biotechnol. 97, 9225–9234.

Xie, B., Xiong, S.Z., Liang, S.B., Hu, C., Zhang, X.J., Lu, J., 2012. Performance and bacterial compositions of aged refuse reactors treating mature landfill leachate. Bioresour. Technol. 103, 71–77.

Xu, H.X., Wu, M., Wang, X.G., Yang, J.Y., Wang, C.S., 2008. Bacterial diversity in deep-sea sediment from northeastern Pacific Ocean. Acta Ecol. Sin. 28, 479–485.

Zhao, Y.C., Li, H., Wu, J., Gu, G.W., 2002. Treatment of leachate by aged-refuse-based biofilter. J. Environ. Eng. 128, 662–668.