WATER RESEARCH 67 (2014) 345–354

Available online at www.sciencedirect.com

ScienceDirect

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

GeoChip-based analysis of microbial community of a combined nitritation-anammox reactor treating anaerobic digestion supernatant

Jian Zhao [a], *Jiane Zuo* [a,*], *Xiaolu Wang* [a], *Jia Lin* [a], *Yunfeng Yang* [a], *Jizhong Zhou* [a,b], *Houjuan Chu* [a], *Peng Li* [a]

[a] *State Key Joint Laboratory of Environment Simulation and Pollution Control, School of Environment, Tsinghua University, Beijing 100084, China*

[b] *Institute for Environmental Genomics and Department of Botany and Microbiology, University of Oklahoma, Norman, OK 73019, USA*

ARTICLE INFO

Article history:
Received 27 May 2014
Received in revised form
2 September 2014
Accepted 23 September 2014
Available online 2 October 2014

Keywords:
GeoChip 4.0
Microbial community
Combined nitritation-anammox
Anaerobic digestion supernatant

ABSTRACT

A combined nitritation-anammox reactor was established to treat anaerobic digestion supernatant. The reactor achieved a nitrogen loading rate of 0.5 kg N/(m^3·d) and total nitrogen removal efficiency of 85% after 140 days' operation. To examine the microbial community responsible for the process, GeoChip 4.0, a high-throughput, microarray-based metagenomic tool, was adopted to measure microbial functional potential under different percentages of digestion supernatant. Intriguingly, our results showed that microbial community composition in a stably functioning bioreactor were significantly different under varying environmental conditions. Functional gene diversities decreased with increasing percentages of digestion supernatant. Genes involved in organic remediation and metal resistance were highly abundant, revealing new metabolic potentials in addition to nitrogen and carbon removal. Compared to the significant decrease of genes involved in denitrification and nitrification caused by inhibition of the digestion supernatant, relative abundances of genes for anammox remained relatively stable. This could be partially attributed to the protection of biofilm, which was vital for the stable performance of nitrogen removal. In addition, nitrogen compounds, C/N ratio and the operation parameters (pH and temperature) were the key variables shaping the microbial community, contributing to a total of 76.64% of the variance of the reactor.

1. Introduction

In recent years, the combined nitritation-anammox process (Sliekers et al., 2002; Joss et al., 2011) has been considered a promising alternative to conventional nitrogen removal because it is cost-saving and energy-efficient. Nitritation-anammox has been applied to treat wastewater with high concentration of ammonia and relatively low COD, mainly included anaerobic digestion supernatant (Zhang et al., 2012a; Daverey et al., 2013a), landfill leachate (Wang et al., 2010) and optoelectronic industrial wastewater (Daverey et al., 2013b).

* *Corresponding author*. Tel.: +86 10 62788505; fax: +86 10 62772455.
E-mail addresses: jiane.zuo@tsinghua.edu.cn, zhao-j07@mails.tsinghua.edu.cn (J. Zuo).
http://dx.doi.org/10.1016/j.watres.2014.09.029
0043-1354/

The mechanisms underlying the stable, efficient performance of the combined nitritation-anammox reactor treating anaerobic digestion supernatant might be manifold. This process is primarily related to nitrogen cycling such as nitrification, denitrification, and anammox. Furthermore, microbial genes related to carbon cycling, metal resistance and organic remediation among others should also be examined because of the relatively high concentration of total organic carbon (TOC), heavy metals (Demirel et al., 2013) and organic pollutants like pesticide (Chowdhury et al., 2013; Kupper et al., 2008) in the digestion supernatant. In previous studies, molecular technologies such as denaturing gradient gel electrophoresis (DGGE) (Zhang et al., 2012a), fluorescent in situ hybridization (FISH) (Vazquez-Padin et al., 2010) and q-PCR (Keluskar et al., 2013) were applied to study microbial communities associated with the combined nitritation-anammox reactor. These approaches have provided important insights into the key species and their niches, however, comprehensive microbial community analyses and the functional potential related to the reactor performance besides nitritation and anammox remained little understood.

In recent years, a high-throughput metagenomic technology named GeoChip has been proven to be a powerful tool for functional profiling of microbial communities (Lu et al., 2012a; Yang et al., 2013). GeoChip 4.0 contains a variety of probes for major microbial functions, such as nitrogen cycling, carbon cycling, metal resistance, organic remediation and stress response (He et al., 2012a).

This study aimed to reveal how the microbial functional communities in a combined nitritation-anammox system respond to the increasing percentage of complex raw digestion supernatant, which contained complicated contaminants, while maintaining stable nitrogen removal functioning. Therefore, a combined nitritation-anammox reactor was set up and fed by gradually increasing percentage of anaerobic digestion supernatant. GeoChip 4.0 was adopted to examine the microbial community, aimed to address the following questions: How were the overall functional diversity, composition and structure of the microbial community affected during reactor operation as the percentage of digestion supernatant increased? What were the key functional genes underlying process performance? What were the key parameters shaping the microbial communities? The results would be beneficial to determine the status of reactor and figure out the main factors to regulate the reactor to achieve stable and efficient operation.

2. Materials and methods

2.1. Reactor setup and operational conditions

An SBR (sequencing batch reactor) with a working volume of 5 L was used for the combined nitritation-anammox process. The reactor was made by polymethyl methacrylate with an inner diameter of 10 cm, as depicted in Fig. S1. Sponges were used as the biomass carrier and the packing rate was 40% (v/v). A thermostat water jacket kept the temperature around 33 °C during operation. The exchange volume ratio of the SBR was maintained at 40%. Compressed air was supplied via a diffuser at the bottom of the reactor to keep the dissolved oxygen around 2 mg/l. The SBR was operated in cycles consisted of 4 phases: feeding, reaction, settling and decanting phase with durations of 10, 420, 20 and 10 min, respectively (Fig. S2). The pH values ranged from 7.3 to 7.9 and were maintained by adding $NaHCO_3$ and HCl.

The reactor was seeded with 2 L anammox sludge from an anammox reactor in the same laboratory and 2 L nitrification seed sludge which was taken from the aeration tank of a municipal wastewater treatment plant in Beijing. The volatile suspended solids (VSS) of anammox sludge and nitrification sludge were 2500 and 3000 mg/l, respectively. During the start-up period, the SBR was fed with synthetic wastewater: NH_4HCO_3 200–400 mg N/l, KH_2PO_4 15 mg P/l, $NaHCO_3$ 200–350 mg/l, Trace element solutions I and II 1.25 ml/l (Molinuevo et al., 2009). The reactor achieved the NLR (nitrogen load rate) of 0.49 kg N/(m3•d) and the corresponding NRR (nitrogen removal rate) of 0.39 kg N/(m3•d) after 37-day operation and operated for another 10 days (30 cycles), maintaining stable and efficient performance.

After the start-up of reactor, the digestion supernatant from an anaerobic digester treating food and fruit/vegetable waste was added into the influent within day 48–140. Along the addition of digestion supernatant, the operation was divided into 5 periods in which the ammonia from the addition of digestion supernatant supplied 20%, 40%, 60%, 80% and 100% of the ammonia in the influent, respectively. Meanwhile, the total ammonia concentration in the influent was kept around 400 mg/l throughout the operation for the comparative study between different periods (to achieve this, the digestion supernatant was diluted and added into the influent to contribute specific percentage of ammonia and the shortage in ammonia was supplemented by chemicals addition to keep the ammonia in the influent around 400 mg/l). For each period, the reactor could accommodate the varied influent conditions gradually and on the achievement of steady performance, the reactor would operate for another 30 cycles before the sampling for microbial community analysis.

Samples of influent and effluent were collected on a daily basis and were analyzed immediately to determine ammonia, nitrite, nitrate and TOC concentrations using standard methods (APHA, 2005). The heavy metals, volatile organic compounds, volatile fatty acids (VFAs) and soluble chemical oxygen demand (sCOD) of the digestion supernatant was measured using APHA Standard Methods (2005). The dissolved oxygen (DO), temperature and pH of reactor were measured using a Hach HQ30d (Hach Inc., USA). The digestion supernatant was derived from an anaerobic digester in the same laboratory periodically and then stored at 4 °C for the addition to the influent. The stored digestion supernatant was replaced each period to avoid changes in composition due to biological activity or other factors. For this reason, the various compounds companied with digestion supernatant would vary between different periods to some certain extent. The average concentration of ammonia, TOC, soluble COD and suspended solids (SS) in the digestion supernatant were determined as 813 ± 59 mg/l, 341 ± 134 mg/l, 985 ± 217 mg/l, 535 ± 148 mg/l, respectively.

Operation of the reactor was divided into 6 periods based on the different percentages of digestion supernatant addition: the start-up period (P0), the periods in which 20% of the total ammonia was contributed by digestion supernatant (P20), 40% (P40), 60% (P60), 80% (P80) and 100% (P100). During the last 3 days of periods P40, P60, P80 and P100, the biofilm samples were collected each day for GeoChip analysis. The last 7–10 days of each period were considered as the steady operation status, thus the average values of the reactor performance in previous 7 days prior to the sampling were determined as the environmental variables and were summarized in Table S1.

2.2. DNA extraction, purification, labeling and hybridization

DNA extraction and hybridization were performed essentially as described previously (Yang et al., 2013; Lu et al., 2012b). DNA was extracted using a freeze-grinding method (Zhou et al., 1996) and then quantified by a NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). DNA was labeled with the fluorescent dye Cy-5 using a random priming method and then purified with QIA quick purification kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and dried at 45 °C for 45 min (ThermoSavant, Milford, MA, USA). Subsequently, labeled DNA was resuspended in 120 μl hybridization solution and hybridizations were performed with a MAUI hybridization station (BioMicro, Salt Lake City, UT, USA) according to the manufacturer's recommended method. Microarray was scanned by a NimbleGen MS200 scanner (Roche, Madison, WI, USA) and signal intensities were subsequently quantified.

2.3. Data analysis

The data processing was conducted as previously described (Yang et al., 2013). Spots with signal to noise ratio (SNR) < 2, signal intensity < 1000 were removed. Data normalization was based on logarithm transform, calculating relative abundance in each sample, then scaled up by average (Mean Ratio). Genes were considered positive if its corresponding probes were positive in at least 2/3 of replicate sets.

Shannon and Simpson Indices were calculated using the GeoChip data analysis pipeline (http://ieg.ou.edu/) to indicate the functional gene diversity. Selection for canonical correspondence analysis (CCA) modeling was conducted by an iterative procedure of eliminating redundant environmental variables based on variance inflation factors (VIF). Mantel test, CCA and variance partition analysis (VPA) were used to link the microbial community and the environmental variables. For the dissimilarity test of adonis, Bray–Curtis distance was used to calculate the dissimilarity distance matrices from GeoChip data. The similarity test between GeoChip and environmental variables was conducted by calculating Euclid distances among samples and plotted to calculate Pearson correlation. Detrended correspondence analysis (DCA), Mantel test, CCA and VPA were performed by the vegan package in R 2.15.0.

3. Results

3.1. Reactor performance

The reactor showed the capacity of nitrogen removal immediately after inoculation since total nitrogen decreased in the effluent compared to in the influent (Fig. S3). During the first 3 days of operation, NH_4^+ removal was observed and a small quantity of NO_3^- accumulated. After 37 days' operation, the ammonia in the influent increase to 389.5 mg/l, and the average concentrations of ammonia, nitrite and nitrate in the effluent were 20.5 mg/l, 0.3 mg/l and 56.0 mg/l, respectively. The reactor maintained the stable and efficient performance for another 10 days to day 47, and the nitrogen loading rate (NLR) and corresponding nitrogen removal rate (NRR) were kept around 0.50 kg N/(m^3·d) and 0.41 kg N/(m^3·d) (Fig. S3). The reactor showed stable ammonia removal efficiency of 95% and total nitrogen (TN) removal efficiency around 80%, indicated that a quick start-up of combined nitritation-anammox process was achieved.

From day 48–140, ammonia in the influent was maintained around 400 mg/l for the comparative study between different periods. The addition of the digestion supernatant was gradually increased so that the ammonia from the digestion supernatant contributed 20% (day 48–65), 40% (day 66–92), 60% (day 93–110), 80% (day 111–124) to 100% (day 125–140) of the total influent ammonia. As a result, the TOC in the influent ranged from 53 mg/l to 150 mg/l over the same period of time (the C/N ratio varied from 0.13 to 0.35 accordingly). The gradual increase of TOC resulted from both the increase in digestion supernatant added and the performance of the anaerobic digester the supernatant was collected from. From P0 to P80, the digester revealed stable and efficient performance. The effluent organics mainly consisted of refractory organics (32% of COD was determined as humid acids), and volatile fatty acids (the intermediates of anaerobic digestion) were undetected. While in P100, 150 mg/l VFAs residuals resulted from the fluctuant performance of previous anaerobic digester were introduced into the influent with addition of digestion supernatant. Nevertheless, the combined nitritation-anammox reactor still showed relatively stable and efficient nitrogen removal performance during the experiment, obtaining TN removal efficiencies at roughly 80% (Fig. S3). There was no obvious removal of organic carbon from period P20 to P80, while occasionally mild TOC increase in the effluent could be detected (Fig. S3). By contrast, due to the introduction of 150 mg/l VFAs in P100, about 33% of the TOC removal was obtained.

At the end of experiment, the average concentrations of NH_4^+, NO_2^- and NO_3^- in the effluent were 1.2 mg/l, 0.7 mg/l and 42.5 mg/l, and the TN removal efficiency went up to 89%, the highest throughout the operation. Both denitrification and anammox process occurred in the final period, while anammox process was the dominant pathway for nitrogen removal in the reactor according to the model calculation (Fig. S4). The procedures for model calculation were detailed described by previous research (Wang et al., 2010).

3.2. Overview of functional gene diversity and structure of microbial community

The combined nitritation-anammox reactor showed relatively stable and efficient performance throughout the operation, though the increased percentages of the digestion supernatant added into the influent caused significant differences between influent conditions at different periods (increased TOC, total suspended solid, heavy metals, pesticides and herbicides). To explore whether the microbial communities were significantly different during different periods in our reactor, and to elucidate the underlying mechanisms for stable nitrogen removal with increasing digestion supernatant, the biofilm communities were analyzed by GeoChip 4.0 at the end of period P40, P60, P80 and P100.

DCA was conducted to examine whether the structure of microbial community significantly changed at different periods. As shown in Fig. 1, samples were grouped by periods and samples from different periods were well separated from each other. Dissimilarity tests also demonstrated that microbial community structures were significantly different between periods (Table S2).

A total of 28575 genes were detected across all samples, ranging from 19910 to 23498 detected genes in each sample (Table 1). The number of genes detected in P100 were less than those in P40 ($P < 0.05$). In addition, Shannon and Simpson Indices were significantly lower in P100 compared to P40 ($P < 0.05$). The number of unique genes detected in each period increased from P40 to P60 and then decreased to their lowest level at P100. An average of 74.19% (70.08%–77.16%) of the genes was shared among the periods, and 16517 genes (57.80% of all detected genes) were present in all periods. Genes involved in organic remediation (3916 genes), stress (3308 genes), metal resistance (1894 genes), carbon cycling (1863 genes), and nitrogen cycling (1322 genes) were the majority of genes detected in all periods.

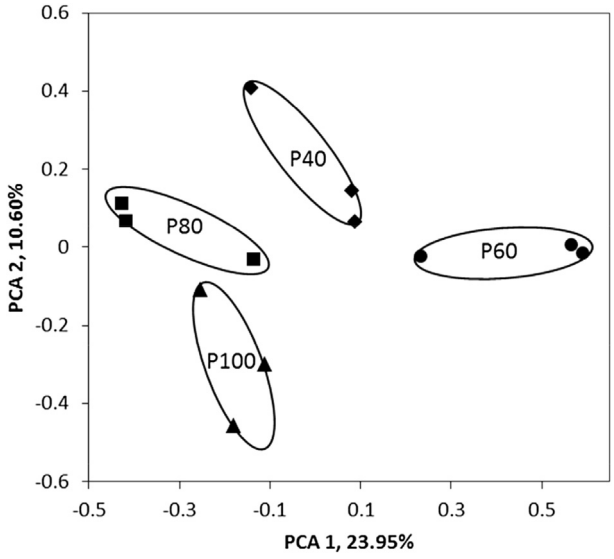

Fig. 1 – Detrended correspondence analysis (DCA) of GeoChip data. The values of Axis 1 and 2 represent the percentage of total variations that can be attributed to the corresponding axis.

3.3. Functional genes involved in carbon cycling

Because of the increasing percentage of digestion supernatant added to the influent, increasing concentrations of TOC, in which the majority were refractory organics, were introduced into the reactor. A large number of carbon cycling genes (3252) were detected across the samples (2468, 2656, 2653 to 2270 detected genes in P40, P60, P80 and P100, respectively) including genes for acetogenesis, carbon degradation, carbon fixation, methane oxidation and methane production. As shown in Fig. 2, the genes for carbon degradation decreased with the increasing percentage of the digestion supernatant in the influent. The majority of these genes showed a significant decrease in P100 compared to P40 ($P < 0.05$).

3.4. Functional genes involved in nitrogen cycling

With the addition of digestion supernatant, influent with high concentrations of nitrogen compounds (mainly ammonia) were introduced into the reactor. Relatively stable and efficient nitrogen removal performance was obtained throughout the operation. A total of 17 key gene families involved in nitrogen cycling processes including nitrogen fixation, ammonification, nitrification, denitrification, anammox, assimilatory N reduction and dissimilatory N reduction were detected by GeoChip 4.0.

The relative abundance of the genes involved in nitrogen cycling of initial period (P40) was compared to that of the final period (P100) (Fig. 3). The genes detected in nitrogen cycling all showed relative decrease in P100 compared to in P40. Among them, gene abundance for denitrification (*narG, nirK, norB and nosZ*) and nitrification (*amoA*) were significantly decreased with the increasing percentages of digestion supernatant added to the influent, ranging from 0.28% to 2.85%. The genes for assimilatory N reduction (*nasA*), dissimilatory N reduction (*nrfA*) and ammonification (*gdh*) also showed significant decrease. By contrast, the abundance of anammox (*hzo*) remained relatively stable throughout the operation.

3.5. Functional genes involved in metal resistance

Heavy metal pollution in the environments has become widespread in China with rapid industrialization, causing metal accumulation in crops and vegetables (Hao et al., 2011; Zhou et al., 2005). The relatively high concentration of various heavy metals can not be eliminated by anaerobic digestion, resulting in accumulation in the digestion supernatant (copper was detected with the highest level in this digestion supernatant, with concentration of 2.58 ± 0.05 mg/l). A total of 3217 genes involved in metal resistance were detected across the samples by GeoChip 4.0. The genes of metal resistance for aluminum, chromium, copper, mercury and tellurium were detected in all samples and genes of metal resistance for copper and mercury were in high abundance.

3.6. Functional genes involved in organic remediation

Organic contaminants are frequently detected in vegetables (Chowdhury et al., 2013) and might persist after anaerobic digestion and consequently be present in the digestion

Table 1 – Overlapped genes, unique genes (number and percentages in bold) and diversity indices for each sample.

Unique and overlap genes	P40	P60	P80	P100
P40	**887(3.87%)**	19950(75.37%)	20165(77.16%)	18367(75.08%)
P60		**1862(7.92%)**	19729(72.17%)	17895(70.08%)
P80			**1424(6.09%)**	18599(75.27%)
P100				**336(1.69%)**
Total no. of genes detected	22911	23498	23383	19910
Shannon Index	9.94	9.93	9.95	9.78
Simpson Index	20638	20637	20978	17588

supernatant (CCl_4 and p-Isopropyltoluene were detected with concentration of 40.68 ± 0.01, 124.19 ± 0.01 μg/l, might mainly resulted from the residuals of pesticides and herbicides). A total of 6637 genes involved in organic remediation were detected across all samples by GeoChip 4.0. Genes for degradation of aromatics, chlorinated solvents, herbicide related compounds and pesticides related compounds were detected in all samples and genes for degradation of aromatics and herbicides related compounds were abundant. The gene *bclA* (benzoate coenzyme A ligase) responsible for degradation of aromatic carboxylic acids, the atrazine catabolic genes (*trzN, trzA and trzE*) and genes *phn* for degradation of organophosphonates were also found to be abundant.

3.7. *Relationship between community functional structure and reactor performance*

The GeoChip results indicated significant changes in functional gene diversity and abundance. To examine whether the differences in the microbial communities were mediated by environmental variables, statistical analysis was performed to explore possible relationships between the microbial community structure and composition with environmental variables. As shown in Fig. 4, there was a strong relationship between microbial community structure and environmental variables, which was further verified by Mantel tests showing significant correlations between microbial community structure with substrates in the influent, C/N ratio and ammonia removal efficiency, total nitrogen removal efficiency (Table 2). No significant correlation was detected between the microbial community structure and pH, DO, temperature or nitrate.

Canonical correspondence analysis (CCA) was performed to reveal the most significant environmental factors shaping the microbial community structure. Seven variables were selected out based on variance inflation factors: ammonia, nitrite, nitrate and C/N ratio in the influent, TOC in the effluent, pH and temperature. The CCA model was significant ($P = 0.01$) and explained 56.52% of the total variance (Fig. 5). Furthermore, C/N ratio in the influent was the major factor linking to the microbial community of P100 samples (the final period of the whole experiment), verifying the important impact of both carbon organics and nitrogen compounds on the microbial community.

Variance partition analysis (VPA) based on CCA was subsequently performed to dissect the contribution of nitrogen compounds (ammonia, nitrite and nitrate in the influent), operation parameters (pH and temperature) and C/N ratio (TOC in the effluent and C/N ratio in the influent) to the

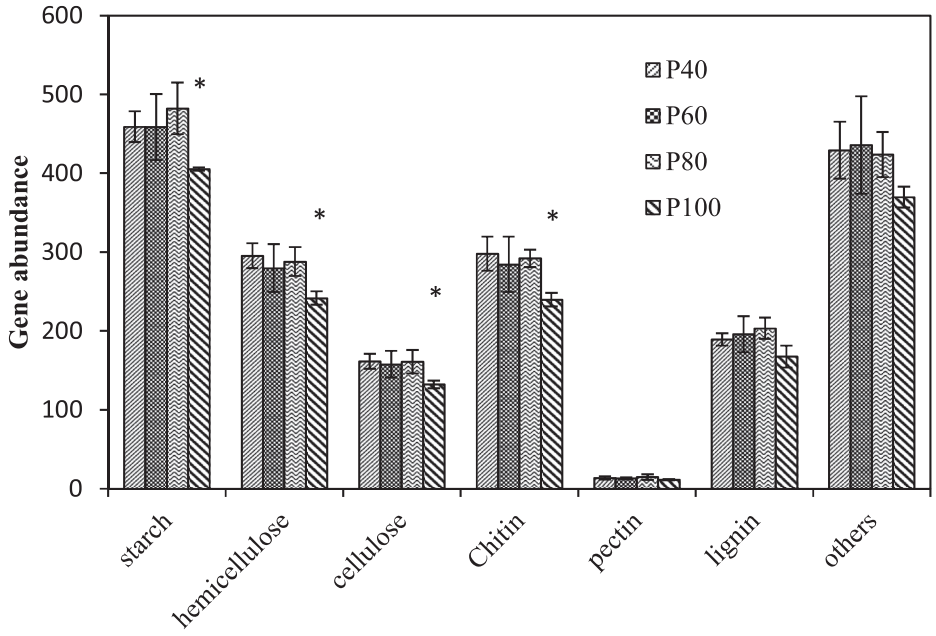

Fig. 2 – Relative abundance of the genes involved in complex carbon degradation among different periods of the bioreactor. The '*' indicates that this gene decreased significantly at P100 compared to the P40 ($P < 0.05$).

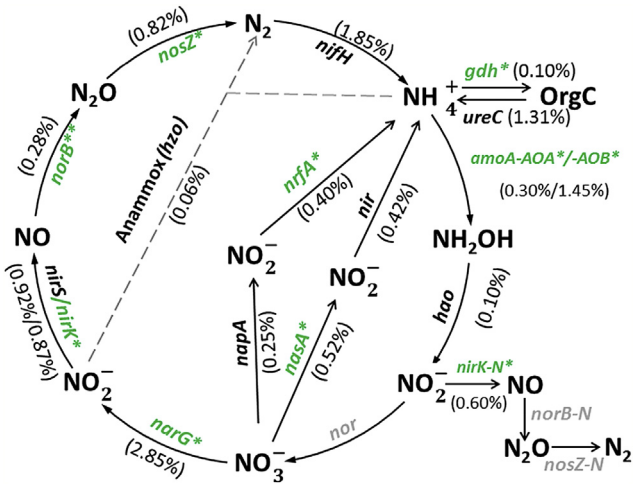

Fig. 3 – The relative change of the detected genes involved in nitrogen cycling throughout the operation of the bioreactor. The genes detected all presented relative decrease in P100 compared to in P40. The percentages in the bracket indicate fold changes in the normalized total intensity of the functional gene family between P40 and P100. Green color represents the decrease in gene abundance was significant. The grey-colored genes are not targeted by GeoChip 4.0. ***P < 0.01, **P < 0.05, *P < 0.1.

microbial community structure (Fig. 6). A total of 76.64% of community variations could be explained by these selected variables. Nitrogen compounds, C/N ratio and operation parameters contributed to 23.05%, 19.59% and 12.08%, respectively. Furthermore, C/N ratio and nitrogen compounds together, C/N ratio and operation parameters together contributed an additional 12.22% and 10.30% of the total variance, respectively, indicating that there were considerable interactions within these parameters.

4. Discussion

GeoChip is a powerful, high-throughput technology to target microbial functional genes, which represent metabolic potentials of microbial communities. Several recent studies have demonstrated that microbial functional genes can be used to indicate rates of environmental processes such as greenhouse gas emission or nitrification potentials (Liu et al., 2014; Zhao et al., 2014). Although abundance of messenger RNA (mRNA) might be a better indicator of functional activity, the profiling and quantification of mRNA is often difficult due to technical issues of interference from ribosomal RNA (rRNA) and transfer RNA (tRNA), strict transport and storage requirements, rapid turnover and severe instability rendering DNA profiling a more convenient and reliable alternative. However, there have been few GeoChip studies to examine the linkage between microbial community and bioreactor functioning, it is thus unclear whether GeoChip can be used to explain the performance of the combined nitritation-anammox reactor.

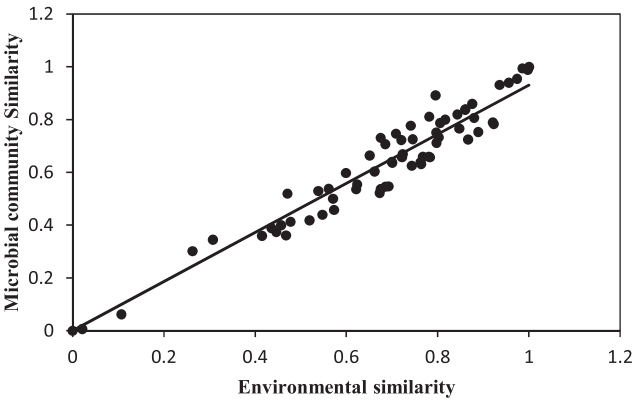

Fig. 4 – The correlation between GeoChip and environmental variables. Euclid distances among samples were calculated and plotted.

This reactor was established by introducing synthetic wastewater and then operated with increasing percentages of digestion supernatant for about 100 days. While a complex mixture of contaminants, including nitrogen compounds, organic carbon, heavy metals, suspended solid and anthropogenic contaminants, were introduced into the reactor, the relatively stable nitrogen removal capacity of 0.5 kg N/(m^3·d) and total nitrogen removal efficiency around 80% were maintained. Interestingly, the microbial community displayed significant differences with increasing percentages of digestion supernatant added to the influent, including a decrease in microbial functional abundance and diversity at the highest percentage of supernatant added. This decrease in diversity is likely caused by the loss of microorganisms unable to adapt to the changing conditions with the addition of digestion supernatant, which introduced high levels of ammonia, TOC, heavy metals, and organic pollutants in the influent that negatively affected the microorganisms (Ren-Cun et al., 2012). The reduction of diversity with the addition of digestion supernatant was also reported by previous research (Zhang et al., 2012b). The detection of high abundance of genes involved in organic remediation and metal resistance indicated the significant impacts of toxic compounds in trace amount (e.g. heavy metals and toxic organics), which should be also taken into consideration treating the digestion supernatant.

Surprisingly, there was no obvious removal of organic carbons except in P100, during which 150 mg/l VFAs residuals in the digestion supernatant were detected. This is most likely because most of the easily degradable organics were removed in the previous anaerobic digestion reactor and the TOC in the digestion supernatant that was added into the influent consisted mainly of refractory organics which was difficult to utilize by heterotrophic bacteria (Graja and Wilderer, 2001). In addition, bacteria release metabolic products (Xie et al., 2013; Ni et al., 2012a), which may increase TOC, compensating for TOC that was consumed. In the last period, P100, when TOC peaked and VFAs were detected in the influent, 33% TOC removal was detected. Nevertheless, the gene abundance for carbon degradation still presented significant decrease compared to the initial period. Therefore, despite the partial utilization of TOC in the last period, the adverse effects of digestion supernatant accumulated during the long-term operation and suppressed the microorganisms significantly,

Table 2 – Correlation between functional genes of microbial community and environmental variables as shown by Mantel tests.

Environmental variables	Whole genes		Genes involved in carbon cycling		Genes involved in nitrogen cycling	
	r	*p*	*r*	*p*	*r*	*p*
Inf. NH_4^+	0.8107	**0.003**	0.8019	**0.001**	0.7687	**0.002**
Eff. NH_4^+	0.4606	**0.034**	0.4476	0.057	0.4103	0.052
NH_4^+ removal efficiency	0.8036	**0.002**	0.7963	**0.002**	0.7573	**0.003**
Inf. NO_2^-	0.8853	**0.003**	0.8856	**0.002**	0.8934	**0.001**
Inf. NO_3^-	−0.4166	0.976	−0.4029	0.969	−0.3576	0.956
Eff. NO_3^-	0.0046	0.465	−0.0150	0.498	0.0452	0.379
TN removal efficiency	0.7646	**0.002**	0.7427	**0.004**	0.7469	**0.001**
pH	−0.0722	0.632	−0.0608	0.562	−0.0298	0.554
DO	−0.3569	0.918	−0.3736	0.930	−0.3111	0.868
T	−0.1462	0.664	−0.1569	0.682	−0.1636	0.663
Inf. TOC	0.6263	**0.026**	0.6187	**0.020**	0.5757	**0.025**
Eff. TOC	0.3023	0.129	0.3274	0.089	0.2429	0.140
Inf. C/N	0.5829	**0.029**	0.5771	**0.028**	0.5324	**0.038**
Eff. C/N	0.7513	**0.012**	0.7516	**0.007**	0.6841	**0.014**

Abbreviations: total nitrogen (TN); Dissolved oxygen (DO); Temperature (T); Total organic carbon (TOC). 'Inf.' and 'Eff.' refers to the substrate concentration in the influent and effluent, respectively. Values of significance at $P < 0.05$ are shown in bold.

determined as the critical factor shaping the microbial community.

There was evidence of a complete N cycle occurring in the reactors. Besides the nitritation and anammox processes, denitrifiers reduced the nitrate to nitrite and then to nitrogen gas with organic carbon. It was previously reported that anammox bacteria could use volatile fatty acids for dissimilatory N reduction (Kartal et al., 2007; Winkler et al., 2012). Other microorganisms could also reduce nitrate to form organic nitrogen through assimilatory N reduction. The

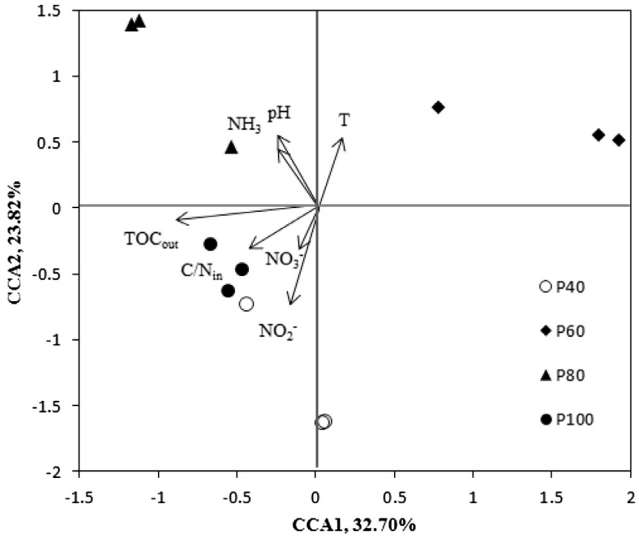

Fig. 5 – Canonical correspondence analysis (CCA) using all detected genes and seven selected variables. The percentage of variance explained by each axis is shown, and the relationship is significant ($P = 0.01$). The 'T', 'TOC_{out}', 'C/N_{in}', 'NH_3', 'NO_2^-', 'NO_3^-' indicated the temperature, TOC in the effluent, C/N ratio in the influent, the ammonia, nitrite and nitrate in the influent, respectively.

extracellular polymeric substances (EPS) and soluble microbial products (SMP) either produced by microorganisms or from hydrolysis of bacteria could be aminated to ammonia through ammonification (Ni et al., 2012a). Functional genes corresponding to each of these N cycle steps were detected by GeoChip 4.0. According to the model calculation (Fig. S4), stable nitrogen removal was achieved primarily by nitritation (*amoA*), anammox (*hzo*) and denitrification (*narG, nirK, norB and nosZ*), with anammox being the predominant approach. Due to the insufficiency in biodegradable organics and suppression of digestion supernatant added, the abundance of heterotrophic denitrifiers was significantly decreased. For the nitritation and anammox processes, sponge carriers were adopted in this research for ammonia oxidation (AOB) and anammox bacteria to form biofilms on. Previous studies have shown that AOB cells will form a layer around the anammox bacteria (Joss et al., 2011; Qiao et al., 2013), potentially protecting the anammox bacteria from adverse conditions such as the presence of toxic compounds or oxygen (Vazquez-Padin et al., 2010). This relationship explains the decrease in abundance of *amoA* genes from AOB while the abundance of *hzo* genes from anammox bacteria remained stable.

Since the anammox process was determined as the dominant approach for nitrogen removal (revealed by the model calculation), the stability of the anammox population in turn allowed for stable, efficient reactor performance throughout all the periods, despite significant succession of microbial community composition at different periods. This result demonstrates that the composition of most microbial groups is sensitive to digestion supernatant in the bioreactor, regardless of taxonomic breadth of the group. However, the stable performance raises the possibility that high functional redundancy exists in the bioreactor, that is, changes in microbial composition might not affect process rates. It is possible that the community at later periods contain taxa groups that are functionally redundant with those in the previous periods. Alternatively, taxa in different communities

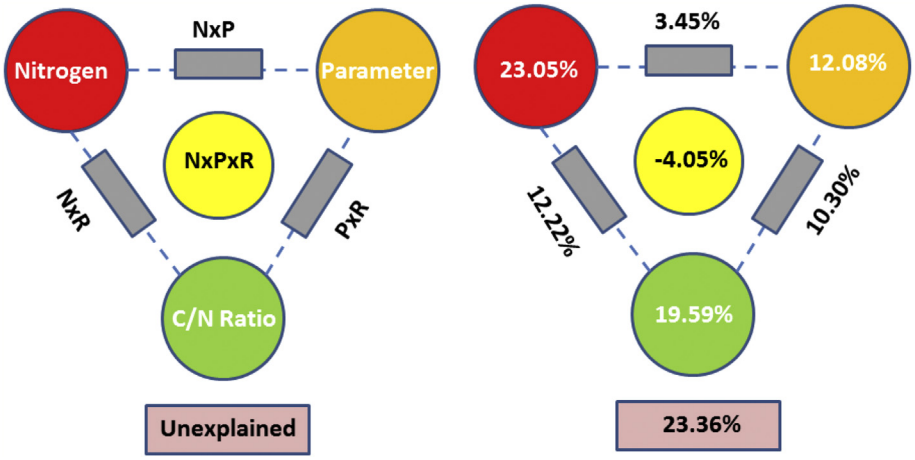

Fig. 6 – Variation partitioning analysis based on CCA (canonical correspondence analysis) that partitions relative influence of environmental variables on microbial community structure. Environmental variables are divided into groups of nitrogen compounds (ammonia, nitrite and nitrate in the influent), operation parameters (pH and temperature) and C/N ratio (TOC in the effluent and C/N ratio in the influent). The circles represent the specific variable groups by partitioning out the effects of the other groups. The squares represent the joint effect of the circles on either side of the square. The portion unexplained by any of the tested variables is represented by the rectangle at the bottom of the figure.

may function differently but have similar process rate when summed at the community level (Allison and Martiny, 2008). Additionally, considering the significant suppression of the overall microbial community diversity by increasing digestion supernatant, it may be inferred that there would be a threshold that the combined nitritation-anammox reactor could endure if the C/N ratio keep increasing and further researches would be recommended. Nevertheless, in this study, the C/N ratio of the influent increased to 0.35 at the end of experiment, equivalent to that of raw supernatant, and the combined nitritation-anammox could still present efficient and stable performance under this circumstance, indicating that this reactor was suitable to treat the digestion supernatant.

Our research revealed major environmental variables shaping the microbial community in a stably functioning bioreactor. Nitrogen compounds, C/N ratio and operation parameters showed significant correlation with the microbial community structure. Among them, C/N ratio additionally revealed considerable interactions with other two variable groups and was further determined as the major factor linking to the microbial community of P100 samples (the final period of operation) through CCA analysis, indicating that C/N ratio was a key environmental factor. The significant importance of C/N ratio to anammox-based process was also confirmed by previous study (Ni et al., 2012b). Notably, Mantel test revealed that the operation parameters could not significantly alter the microbial communities individually while the VPA analysis showed that there were significant interactions between operation parameters and C/N ratio, and the combination of these two groups further presented significant correlation with the microbial communities. Therefore, the operation parameters (pH and temperature) might shape the microbial community not directly but mainly through their influences on substrates availability in the influent, including adjusting ammonia-free ammonia (He et al., 2012b), nitrite-nitrous acid (Zhou et al., 2011) or the CO_2-bicarbonate-carbonate equilibrium (Ganigue et al., 2012). This was in accordance with previous studies suggesting that free ammonia and nitrous acid, which were determined by both the operation parameters (pH and temperature) and substrate concentration, influenced the nitritation and anammox processes directly (He et al., 2012b; Tao et al., 2012; Carvajal-Arroyo et al., 2013; Jaroszynski et al., 2012).

5. Conclusion

Here we used GeoChip 4.0 to analyze the microbial community of a combined nitritation-anammox reactor treating increasing percentages of digestion supernatant. The reactor with stable performance still showed significant change in the microbial community. The gene diversities and abundances decreased as the percentage of supernatant added to the influent increased. Nitrogen compounds, C/N ratio and operation parameters were determined to be key variables shaping the microbial community structure. Together, the results demonstrated the utilization of GeoChip-based technologies in examining microbial communities of a combined nitritation-anammox reactor treating anaerobic digestion supernatant and in-depth exploring into the mechanism and metabolic potential of the complicated bioreactor. The results of this study will provide important information for the establishment of a robust process and operational strategy for successful digestion supernatant treatment by the combined nitritation-anammox reactor.

Acknowledgement

This research was financially supported by the Mega-Projects of Science Research for Water Environment Improvement (No.

2012ZX07205-001). Thanks are due to Joy D. Van Nostrand of University of Oklahoma for valuable discussion and useful suggestions.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2014.09.029.

REFERENCES

APHA, 2005. Standard Methods for the Examination of Water and Wastewater, twenty-first ed. APHA, Washington, DC.

Allison, S.D., Martiny, J.B.H., 2008. Resistance, resilience, and redundancy in microbial communities. Proc. Natl. Acad. Sci. U. S. A. 105, 11512–11519.

Carvajal-Arroyo, J.M., Sun, W.J., Sierra-Alvarez, R., Field, J.A., 2013. Inhibition of anaerobic ammonium oxidizing (anammox) enrichment cultures by substrates, metabolites and common wastewater constituents. Chemosphere 91 (1), 22–27.

Chowdhury, M.A.Z., Fakhruddin, A.N.M., Islam, M.N., Moniruzzaman, M., Gan, S.H., Alam, M.K., 2013. Detection of the residues of nineteen pesticides in fresh vegetable samples using gas chromatography-mass spectrometry. Food Control 34 (2), 457–465.

Daverey, A., Hung, N.T., Dutta, K., Lin, J.G., 2013a. Ambient temperature SNAD process treating anaerobic digester liquor of swine wastewater. Bioresour. Technol. 141, 191–198.

Daverey, A., Su, S.H., Huang, Y.T., Chen, S.S., Sung, S.W., Lin, J.G., 2013b. Partial nitrification and anammox process: a method for high strength optoelectronic industrial wastewater treatment. Water Res. 47 (9), 2929–2937.

Demirel, B., Gol, N.P., Onay, T.T., 2013. Evaluation of heavy metal content in digestate from batch anaerobic co-digestion of sunflower hulls and poultry manure. J. Mater. Cycles Waste 15 (2), 242–246.

Graja, S., Wilderer, P.A., 2001. Characterization and treatment of the liquid effluents from the anaerobic digestion of biogenic solid waste. Water Sci. Technol. 43 (3), 265–274.

Ganigue, R., Volcke, E.I.P., Puig, S., Balaguer, M.D., Colprim, J., 2012. Impact of influent characteristics on a partial nitritation SBR treating high nitrogen loaded wastewater. Bioresour. Technol. 111, 62–69.

Hao, X.Z., Zhou, D.M., Wang, Y.K., Shi, F.G., Jiang, P., 2011. Accumulation of Cu, Zn, Pb, and Cd in edible parts of four commonly grown crops in two contaminated soils. Int. J. Phytoremediat 13 (3), 289–301.

He, Z.L., Van Nostrand, J.D., Zhou, J.Z., 2012a. Applications of functional gene microarrays for profiling microbial communities. Curr. Opin. Biotech. 23 (3), 460–466.

He, Y.L., Tao, W.D., Wang, Z.Y., Shayya, W., 2012b. Effects of pH and seasonal temperature variation on simultaneous partial nitrification and anammox in free-water surface wetlands. J. Environ. Manage. 110, 103–109.

Joss, A., Derlon, N., Cyprien, C., Burger, S., Szivak, I., Traber, J., Siegrist, H., Morgenroth, E., 2011. Combined nitritation-anammox: advances in understanding process stability. Environ. Sci. Technol. 45 (22), 9735–9742.

Jaroszynski, L.W., Cicek, N., Sparling, R., Oleszkiewicz, J.A., 2012. Impact of free ammonia on anammox rates (anoxic ammonium oxidation) in a moving bed biofilm reactor. Chemosphere 88 (2), 188–195.

Kartal, B., Kuypers, M.M.M., Lavik, G., Schalk, J., den Camp, H., Jetten, M.S.M., Strous, M., 2007. Anammox bacteria disguised as denitrifiers: nitrate reduction to dinitrogen gas via nitrite and ammonium. Environ. Microbiol. 9 (3), 635–642.

Kupper, T., Bucheli, T.D., Brandli, R.C., Ortelli, D., Edder, P., 2008. Dissipation of pesticides during composting and anaerobic digestion of source-separated organic waste at full-scale plants. Bioresour. Technol. 99 (17), 7988–7994.

Keluskar, R., Nerurkar, A., Desai, A., 2013. Development of a simultaneous partial nitrification, anaerobic ammonia oxidation and denitrification (SNAD) bench scale process for removal of ammonia from effluent of a fertilizer industry. Bioresour. Technol. 130, 390–397.

Lu, Z.M., He, Z.L., Parisi, V.A., Kang, S., Deng, Y., Van Nostrand, J.D., Masoner, J.R., Cozzarelli, I.M., Suflita, J.M., Zhou, J.Z., 2012a. GeoChip-based analysis of microbial functional gene diversity in a landfill leachate-contaminated aquifer. Environ. Sci. Technol. 46 (11), 5824–5833.

Lu, Z.M., Deng, Y., Van Nostrand, J.D., He, Z.L., Voordeckers, J., Zhou, A.F., Lee, Y.J., Mason, O.U., Dubinsky, E.A., Chavarria, K.L., Tom, L.M., Fortney, J.L., Lamendella, R., Jansson, J.K., D'Haeseleer, P., Hazen, T.C., Zhou, J.Z., 2012b. Microbial gene functions enriched in the Deepwater Horizon deep-sea oil plume. Isme J. 6 (2), 451–460.

Liu, S., Wang, F., Xue, K., Sun, B., Zhang, Y., He, Z., Van Nostrand, J.D., Zhou, J., Yang, Y., 2014. The interactive effects of soil transplant into colder regions and cropping on soil microbiology and biogeochemistry. Environ. Microbiol. (n/a-n/a).

Molinuevo, B., Garcia, M.C., Karakashev, D., Angelidaki, I., 2009. Anammox for ammonia removal from pig manure effluents: effect of organic matter content on process performance. Bioresour. Technol. 100 (7), 2171–2175.

Ni, B.J., Ruscalleda, M., Smets, B.F., 2012a. Evaluation on the microbial interactions of anaerobic ammonium oxidizers and heterotrophs in anammox biofilm. Water Res. 46 (15), 4645–4652.

Ni, S.Q., Ni, J.Y., Hu, D.L., Sung, S.W., 2012b. Effect of organic matter on the performance of granular anammox process. Bioresour. Technol. 110, 701–705.

Qiao, S., Tian, T., Duan, X.M., Zhou, J.T., Cheng, Y.J., 2013. Novel single-stage autotrophic nitrogen removal via co-immobilizing partial nitrifying and anammox biomass. Chem. Eng. J. 230, 19–26.

Ren-Cun, J., Guang-Feng, Y., Jin-Jin, Y., Ping, Z., 2012. The inhibition of the anammox process: a review. Chem. Eng. J. 197, 67–79.

Sliekers, A.O., Derwort, N., Campos-Gomez, J.L., Strous, M., Kuenen, J.G., Jetten, M.S.M., 2002. Completely autotrophic nitrogen removal over nitrite in one single reactor. Water Res. 36 (10), 2475–2482.

Tao, W.D., He, Y.L., Wang, Z.Y., Smith, R., Shayya, W., Pei, Y.S., 2012. Effects of pH and temperature on coupling nitritation and anammox in biofilters treating dairy wastewater. Ecol. Eng. 47, 76–82.

Vazquez-Padin, J., Mosquera-Corral, A., Campos, J.L., Mendez, R., Revsbech, N.P., 2010. Microbial community distribution and activity dynamics of granular biomass in a CANON reactor. Water Res. 44 (15), 4359–4370.

Wang, C.C., Lee, P.H., Kumar, M., Huang, Y.T., Sung, S.W., 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 (1–3), 622–628.

Winkler, M.K.H., Kleerebezem, R., van Loosdrecht, M.C.M., 2012. Integration of anammox into the aerobic granular sludge process for main stream wastewater treatment at ambient temperatures. Water Res. 46 (1), 136–144.

Xie, W.M., Ni, B.J., Seviour, T., Yu, H.Q., 2013. Evaluating the impact of operational parameters on the formation of soluble microbial products (SMP) by activated sludge. Water Res. 47 (3), 1073–1079.

Yang, Y.F., Wu, L.W., Lin, Q.Y., Yuan, M.T., Xu, D.P., Yu, H., Hu, Y.G., Duan, J.C., Li, X.Z., He, Z.L., Xue, K., van Nostrand, J., Wang, S.P., Zhou, J.Z., 2013. Responses of the functional structure of soil microbial community to livestock grazing in the Tibetan alpine grassland. Glob. Change Biol. 19 (2), 637–648.

Zhou, J.Z., Bruns, M.A., Tiedje, J.M., 1996. DNA recovery from soils of diverse composition. Appl. Environ. Microb. 62 (2), 316–322.

Zhou, D.M., Hao, X.Z., Wang, Y.J., Dong, Y.H., Cang, L., 2005. Copper and Zn uptake by radish and pakchoi as affected by application of livestock and poultry manures. Chemosphere 59 (2), 167–175.

Zhou, Y., Oehmen, A., Lim, M., Vadivelu, V., Ng, W.J., 2011. The role of nitrite and free nitrous acid (FNA) in wastewater treatment plants. Water Res. 45 (15), 4672–4682.

Zhang, Z.J., Li, Y.Y., Chen, S.H., Wang, S.M., Bao, X.D., 2012a. Simultaneous nitrogen and carbon removal from swine digester liquor by the Canon process and denitrification. Bioresour. Technol. 114, 84–89.

Zhang, Z., Li, Y., Chen, S., Wang, S., Bao, X., 2012b. Simultaneous nitrogen and carbon removal from swine digester liquor by the Canon process and denitrification. Bioresour. Technol. 114 (0), 84–89.

Zhao, M., Xue, K., Wang, F., Liu, S., Bai, S., Sun, B., Zhou, J., Yang, Y., 2014. Microbial mediation of biogeochemical cycles revealed by simulation of global changes with soil transplant and cropping. ISME J..