Science of the Total Environment 612 (2018) 788–798

Contents lists available at ScienceDirect

Science of the Total Environment

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

Rapid startup of thermophilic anaerobic digester to remove tetracycline and sulfonamides resistance genes from sewage sludge

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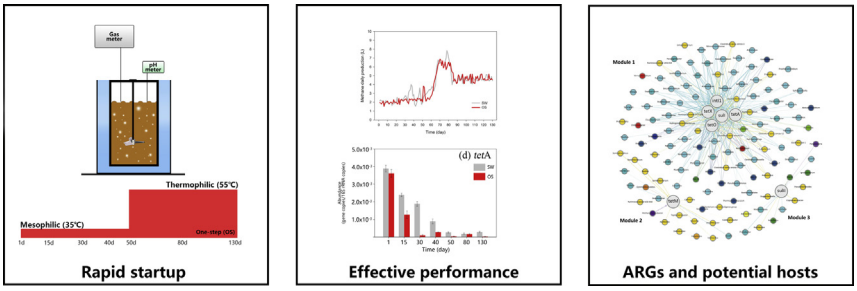
HIGHLIGHTS

- Rapid startup of thermophilic digester saved 20 days.
- Most antibiotic resistance genes were removed during thermophilic digestion.
- Network of antibiotic resistance genes and potential hosts were presented.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 20 June 2017
Received in revised form 27 August 2017
Accepted 29 August 2017
Available online xxxx

Editor: Simon Pollard

Keywords:
Anaerobic digestion
Sewage sludge
Antibiotic resistance gene
Thermophilic digester
Microbial community

ABSTRACT

Spread of antibiotic resistance genes (ARGs) originating from sewage sludge is highlighted as an eminent health threat. This study established a thermophilic anaerobic digester using one-step startup strategy to quickly remove tetracycline and sulfonamides resistance genes from sewage sludge. At least 20 days were saved in the startup period from mesophilic to thermophilic condition. Based on the results of 16S rDNA amplicons sequencing and predicted metagenomic method, the successful startup largely relied on the fast colonization of core thermophilic microbial population (e.g. *Firmicutes*, *Proteobacteria*, *Actinobacteria*). Microbial metabolic gene pathways for substrate degradation and methane production was also increased by one-step mode. In addition, real-time quantitative PCR approach revealed that most targeted tetracycline and sulfonamides resistance genes ARGs (*sul*I, *tet*A, *tet*O, *tet*X) were substantially removed during thermophilic digestion (removal efficiency > 80%). Network analysis showed that the elimination of ARGs was attributed to the decline of their horizontal (*int*I1 item) and vertical (potential hosts) transfer-related elements under high-temperature. This research demonstrated that rapid startup thermophilic anaerobic digestion of wastewater solids would be a suitable technology for reducing quantities of various ARGs.

1. Introduction

Bacteria becoming more resistant to various antibiotics has been recognized in recent years (Pruden et al., 2013). Although many actions have been taken to reduce the abuse of antibiotics, a continual increase in bacterial resistance to antibiotics was observed (Diehl and Lapara,

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http://dx.doi.org/10.1016/j.scitotenv.2017.08.295
0048-9697/

2010). One reason is that the resistance can be conferred among environmental bacteria through the transfer of antibiotic resistance genes (ARGs). Such bacteria can be considered as potential hosts to transfer resistance to pathogenic bacteria (Wright, 2007). Proliferation of ARGs among bacteria, such as *Enterobacteriaceae* (a broad-spectrum β-lactamase producing bacteria) and *Enterococcus* (a vancomycin-resistant bacteria), resulted in the increasing morbidity and mortality of infections from multiantibiotic-resistant pathogens (Wright, 2010).

Municipal sewage collected by wastewater treatment plants (WWTPs) is a pertinent reservoir for ARGs (Lapara et al., 2011; Yuan et al., 2016). Because sewage contained substantial quantities of antibiotics, antibiotic resistance bacteria (ARBs) and ARGs originating from human gastrointestinal tract, livestock and poultry farm or medical industry (Zhang et al., 2009). During wastewater treatment, some ARGs might proliferate in sewage sludge due to the preferential survival and selection pressure of ARBs (Munck et al., 2015). Several typical ARGs (e.g. tetracycline resistance genes and sulfonamides resistance genes) have been widely detected from worldwide WWTPs (Bing et al., 2015; Hou et al., 2016; Jiang et al., 2017; Zhang and Zhang, 2011). What's worse, ARGs originating from sewage sludge escaped from WWTPs facilities to soil system due to their land application of residual solids, such as a fertilizer or a soil conditioner (Ghosh et al., 2009a; Munir et al., 2011). Thus, WWTPs are important nodes to help slowing the spread of ARGs into downstream natural environment (Pruden et al., 2013; Zhang et al., 2015).

Anaerobic digestion (AD) has been widely used to dispose sewage sludge at WWTPs, due to the reduction of sludge volume, production of renewable energy (methane) and removal of pathogens (Gou et al., 2014; Xu et al., 2017). Nowadays, AD is also likely to reduce the persistent ARGs by some degree. However, conclusions are not universal in previous studies (Diehl and Lapara, 2010; Ghosh et al., 2009a; Ma et al., 2011a; Tian et al., 2015; Tian et al., 2016; Zhang et al., 2015). Some research reported ARGs were considerably removed during AD process. For example, Zhang et al. (2015) found that 8 out of 35 ARG types (including tetracycline resistance genes) could be removed >90%. Diehl et al. also suggested the ability of full-scale anaerobic digesters in removing tetracycline resistance genes (*tet*A, *tet*L, *tet*O, *tet*W, and *tet*X) (Diehl and Lapara, 2010). However, other studies indicated that some ARGs types were slightly reduced or even rebounded using anaerobic digesters (Ma et al., 2011b; Zhang et al., 2015). This may be attributed to the complex interactions among ARGs, microbes, digestion conditions, and related-metabolic pathways involved in AD process, which are still considered as a "black box". Horizontal gene transfer (by the transfer of mobile element carrying ARGs) and vertical gene transfer (by the proliferation of ARGs' hosts) between different bacterial cells were recognized as the main spread ways of ARGs (Sørensen et al., 2005). Therefore, studying the fate of ARGs and their horizontal/vertical transfer-related elements simultaneously can provide comprehensive insights into controlling mechanism of ARGs during AD process.

Temperature is one of critical parameters determining the stability and performance of AD. Studies suggested that high-temperature digestion is also able to inactivate ARB and ARGs in sludge (Diehl and Lapara, 2010; Ghosh et al., 2009a; Ma et al., 2011b). AD technology is usually conducted at mesophilic (ca. 35 °C) or thermophilic (ca. 55 °C) condition to supply an optimum parameter for the growth of different microbes (Diehl and Lapara, 2010). Prior studies indicated that thermophilic digestion generally outperforming mesophilic digestion in the reduction of ARGs (Diehl and Lapara, 2010; Tian et al., 2015; Tian et al., 2016). However, most of current full-scale digesters adopted mesophilic digestion. Expect for a higher operation cost, another main obstacle for the application of thermophilic digestion is the longer startup time (Tian et al., 2016). Since lacking of existing thermophilic seed sludge, it is usually required to domesticate from mesophilic to thermophilic condition in a full-scale digester, which was supposed to be time-consuming (Tian et al., 2015). A stable AD system highly depends on the complex interaction among different microorganisms participated in hydrolysis, acidogenesis, acetogenesis and methanogenesis processes (Rivière et al., 2009). Key to successful transformation depends on the formation of a mature thermophilic community for AD operation. But it is reported that abrupt temperature-raising will disturb original mesophilic population (e.g. methanogens) during domestication process, making the AD system unstable (Griffin et al., 1998).

To maintain the reactor's stability and save startup time, a rapid startup mode of thermophilic anaerobic digester is proposed in this study. Digestion performance is continuously monitored to compare the influence of different temperature-raising strategies. The fate of ARGs, potential hosts and function gene pathways involved in AD process are jointly investigated by real-time quantitative PCR (RT-qPCR), high-throughput sequencing (HTS) and predicted metagenomic method. This study will be helpful to quickly establish thermophilic AD reactors and effectively control proliferation of ARGs.

2. Material and methods

2.1. Preparation of digestion substrate

SW and OS were initially inoculated with 2 L anaerobic seed sludge and 1 L feed sludge. Seed sludge was collected from a mature mesophilic anaerobic digester (Yang et al., 2016). Daily feed sludge (a mixture of secondary sludge and dewatered sludge) was sampled from Yuelu WWTPs, Changsha. Main characteristics were summarized in Table 1.

2.2. Startup of thermophilic digesters

Experiments were conducted in two continuous stirred-tank reactors with 3 L working volume (mixing speed is 60 rpm with a cycle of 1 min on and 10 min off throughout the experiment). Two reactors were operated at a constant hydraulic retention time (HRT) of 15 days, 200 mL of digested/untreated sludge was removed/refilled every day to maintain a constant working volume (Jang et al., 2016). Reactors' temperature was controlled by circulating hot water using heating rod. Initially, both reactors were operated at 35 °C (mesophilic condition) for 30 days until biogas production stabilized. Then, reactor SW (step-wise mode) increased digestion temperature from 35 °C to 55 °C gradually during 20 days with the raising interval of 1 °C/d. On day 50, reactor OS (one-step mode) increased temperature from 35 °C to 55 °C directly. Lastly, reactors SW and OS were operated at thermophilic condition (55 °C) over 80 days to reach a steady state (Table 2).

Table 1
Main characteristics of seed sludge and feed sludge used in this study.

Item	Seed	Feed
Volume (mL d^{-1})	–	200
pH	7.21 ± 0.1	6.82 ± 0.1
SCOD (g L^{-1})	0.74 ± 0.3	14.7 ± 2.8
TS (g L^{-1} substrate)	37.9 ± 0.2	479.5 ± 8.3
VS (g L^{-1} substrate)	9.3 ± 1.1	142.4 ± 4.8
VS/TS (%)	24.6 ± 0.7	29.7 ± 0.5
VFAs (mg L^{-1})	878.5 ± 36	1219.4 ± 81
*sul*I[a]	ND	(1.42 ± 0.2) × 10^{-2}
*sul*II[a]	ND	(3.37 ± 0.6) × 10^{-3}
*tet*A[a]	ND	(4.13 ± 0.7) × 10^{-3}
*tet*L[a]	ND	(3.27 ± 0.6) × 10^{-3}
*tet*M[a]	ND	(4.39 ± 0.2) × 10^{-3}
*tet*O[a]	ND	(1.78 ± 0.4) × 10^{-2}
*tet*W[a]	ND	(5.98 ± 1.1) × 10^{-4}
*tet*X[a]	ND	(3.37 ± 0.9) × 10^{-4}

[a] The relative abundance of ARGs was normalized by total 16S rDNA gene numbers.

Table 2
General performance of two anaerobic digesters.

Item	Step-wise mode (SW)							One-step mode (OS)						
Time (day)	1	15	30	40	50	80	130	1	15	30	40	50	80	130
Temperature (°C)	35	35	35	35–55[a]	55	55	55	35	35	35	35	55	55	55
Accumulative methane (L)	2.1	30.2	62.8	93.3	115.8	264.5	496.3	2.0	28.9	60.2	82.5	105.3	245.5	483.0
VFAs (mg L^{-1})	951 (103)	1387 (82)	1251 (132)	2144 (69)	2391 (96)	737 (88)	886 (67)	1124 (42)	1187 (87)	1072 (72)	1258 (95)	1164 (83)	2714 (98)	995 (84)
pH	7.47 (0.13)	7.44 (0.08)	7.40 (0.11)	7.14 (0.02)	7.16 (0.12)	8.01 (0.09)	8.13 (0.07)	7.41 (0.02)	7.51 (0.08)	7.37 (0.02)	7.41 (0.06)	7.33 (0.05)	8.04 (0.11)	8.21 (0.04)
sCOD (mg L^{-1})	1191 (186)	1688 (188)	1527 (266)	3224 (185)	6034 (165)	4146 (263)	3782 (282)	1288 (106)	1228 (238)	1379 (336)	2659 (229)	2753 (300)	3125 (337)	3109 (352)
VS (g L^{-1})	6.8 (0.3)	6.6 (0.2)	16.7 (0.8)	16.8 (0.4)	24.6 (0.9)	27.0 (0.8)	25.9 (0.6)	6.3 (0.6)	6.1 (0.6)	15.0 (0.7)	24.1 (0.4)	20.2 (0.2)	25.9 (0.3)	25.4 (0.4)
TS (g L^{-1})	34.9 (1.0)	33.1 (0.7)	69.7 (1.4)	65.9 (1.4)	91.4 (0.9)	122.8 (2.1)	120.0 (1.7)	33.3 (1.8)	35.8 (1.9)	67.9 (2.2)	98.8 (2.4)	82.4 (1.9)	119.6 (2.2)	114.2 (2.1)
VS/TS[b] (%)	19.6 (1.2)	20.1 (1.1)	24.0 (1.4)	25.5 (1.4)	26.9 (1.3)	21.9 (1.2)	21.6 (1.4)	18.8 (1.4)	16.9 (1.6)	22.1 (1.5)	24.4 (1.4)	24.5 (1.3)	21.6 (1.2)	22.3 (1.3)

Shown data was mean value (standard deviation).
[a] Temperature increased from 35 °C to 55 °C during 31 d to 50 d, with an interval of 1 °C/d.
[b] TS = total solids; VS = volatile solids; sCOD = soluble COD.

2.3. Chemical analysis

Methane production, pH, soluble chemical oxygen demand (SCOD), total alkalinity (TA), volatile fatty acids (VFAs), total solids (TS), and volatile solids (VS) of the digested sample were chosen as the main digestion parameters to monitor reactors' performance. Digested samples were centrifuged at 8000 rpm for 10 min. Filtered (0.45 μm) supernatant was used for following chemical analysis. pH, sCOD, TA, TS and VS were measured as previously described (Gou et al., 2014). Analysis of VFAs components was carried out at a gas chromatograph (GC2010-plus, Shimazu) with a flame ionization detector (Yang et al., 2016). Organic matters of supernatant were measured by three-dimension excitation emission matrix fluorescence spectroscopy (3D-EEM) as reported by Huang et al. (2015). Biogas production was continuously collected in 20 L Haide gas-bags (Haide-tech, Dalian). Biogas components were determined by a gas chromatograph-mass spectrometer (GCMS-QP 2010, Shimadzu). All experiments were conducted in triplicates.

2.4. DNA extraction

Genomic DNA was extracted from 0.50 g of well-mixed sludge on 1 d, 15 d, 30 d, 40 d, 50 d, 80 d and 130 d from SW and OS reactors using the FastDNA® spin kit (MP bio, Santa Ana, USA). DNA extractions were performed in triplicate and composited to average out bias. Extracted DNA was diluted to 1 ng/μL using sterile water and qualified by 1% (w/v) agarose gels electrophoresis. All the DNA extracts were stored at −24 °C until further analysis.

2.5. Real-time quantitative PCR (RT-qPCR) analysis

RT-qPCR was used to quantify the most reported ARGs and related-mobile element in sewage sludge, including 6 genes encoding tetracycline resistance (*tet*A, *tet*L, *tet*M, *tet*O, *tet*W, and *tet*X), 2 genes encoding sulfonamides resistance (*sul*I and *sul*II), and 1 integrase gene of class 1 integrons (*intI*1), as well as microbial 16S rDNA genes for archaea/bacteria (as a measure of microbial biomass) (Diehl and Lapara, 2010; Ghosh et al., 2009a; Ju et al., 2016; Tian et al., 2016; Zhang et al., 2015). Each gene was amplified in triplicates from the extracted genomic DNA. A 20 μL reaction mixture contained 10 μL of 2× Power PreMix (Tiangen, China), 0.4 μL Rox solution, 1 μL template, 0.4 μL of forward/reverse primer, and 7.8 μL sterile water. Each RT-qPCR assay was performed using Bio-Rad thermocycler (iQ5, USA) by SYBR-Green method. A RT-qPCR run consisted of an initial denaturation at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 10 s and anneal/extension at a temperature specific for the target gene for 30 s. Standard curves (10-fold serial dilutions) and negative control were performed as Deng et al. (2015). To minimized the basis caused by different microbial abundance, gene quantity data was normalized by the 16S rDNA gene numbers (bacterial + archaeal). Additional information of primer pairs and annealing temperature for RT-qPCR is summarized in Table A.1.

2.6. High-throughput sequencing of V3–V4 region of 16S rDNA genes

16S rDNA amplicons were amplified by barcode-primer pair 515F (5′-GTGYCAGCMGCCGCGGTAA-3′)/907R (5′-CCYCAATTCMTTTR AGTTT-3′) targeting the V3–V4 hyper variable regions (Kuczynski et al., 2011). PCR program was described in our previous study (Yang et al., 2016). Sequencing library was constructed using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA). The high-throughput sequencing of qualified 16S rDNA amplicons library was performed on the Illumina HiSeq PE 250 platform. Each PCR reaction was performed in triplicates. Sequencing data was deposited to NCBI with accession No. SUB2653512. Barcoded tags were merged using FLASH (V1.2.7) and filtered using QIIME (V1.7.0) (Caporaso et al., 2010; Magoč and Salzberg, 2011). Finally, the chimera sequences were removed by comparing with the Gold database using UCHIME (Haas et al., 2011). Operational taxonomic units (OTUs) (97% identity) were clustered by UPARSE (v7.0.1001) using all effective tags. Phylogenetic taxonomy was assigned against the RDP classifier (Version 2.2,). Alpha diversity and richness indices were determined according to OTUs classification as described previously (Yang et al., 2016).

2.7. Metagenomic prediction

PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) was used to predict the potential metagenome of digestion microbes (Langille et al., 2013). PICRUSt was conducted on the Galaxy platform (Goecks et al., 2010) against the KEGG (Kyoto Encyclopedia of Genes and Genomes) database. Predicted functional gene (KEGG Ortholog, KO) abundances at L1-L3 levels were calculated. The difference of metagenome distribution in different samples were calculated by pair-sample *t*-test on SPSS (v.19.0).

2.8. Network analysis for ARGs and potential hosts

A co-occurrence network was generated to visualize the correlation between the targeted ARGs and potential hosts using the random matrix theory-based network inference method (Deng et al., 2012). All assigned OTUs at genus level were selected. Only the item with correlation index higher than 0.8 ($p < 0.05$) was considered as a potential host for a targeted gene. The co-occurrence networks were visualized by Gephi (version 0.9.1).

3. Results

3.1. General digestion performance

Operation temperature of two reactors was increased from 35 °C to 55 °C with step-wise (SW) and one-step (OS) strategies. General performance of AD is depicted in Fig. 1 and Table 2. Following the gradual increase of temperature from 35 °C to 55 °C during 31–50 d in SW, a significant fluctuation of methane production was observed. While both SW and OS showed similar trends of methane daily production during 1–30 d (stable mesophilic period), 65–85 d (thermophilic peak period), and 85–130 d (stable thermophilic period). Notably, OS achieved the thermophilic domestication during 51–65 d rapidly, and methane production reached its maximum value as well as SW (ca. 6860 vs. 6400 mL at 70 d). In SW and OS, the methane proportion on day 80 (62.3% vs. 64.7%) and accumulative methane yield (496 L vs. 483 L) reached similar condition at thermophilic digestion (Fig. 1-b and Table 2). Compared with gradual temperature-raising during day 31–80, a stable thermophilic digestion was rapidly established using one-step mode during 51–80 d. Both OS and SW reached similar methane production at effective thermophilic condition. Similar performance of SW and OS at thermophilic condition can be also supported by the results of 3D-EEM fluorescence spectroscopy (Fig. 2), which is used to characterize the capacity of substrates degradation (Huang et al., 2015). According to the region classification (I to V) by Chen et al. (2003), two peaks (A and B) were mainly identified from all samples in region I and IV, respectively. Peak A was mainly detected at excitation/emission (Ex/Em) wavelengths of 260–280/280–330 nm, which is in region IV (soluble microbial by-products, such as proteins and polysaccharides). While peak B was detected at Ex/Em of 230–240/290–330, which is stretched across region I (aromatic protein) and II (aromatic protein). The intensity values of peak A are always higher than peak B in two reactors. It is reported that the tightly bound extracellular polymerics (TB-EPS), rather than the loosely bound extracellular polymerics (LB-EPS), showed higher intensity values for peak A (Huang et al., 2015). Consequently, peak A and B were mainly associated with microbial metabolites products, and derived from TB-EPS of the digested sludge. On day 80, the fluorescence of peak A and B reached similar value and location in two reactors. With digestion-time prolonged to day 130, no significant change of fluorescence value was observed. Results suggested that reactor OS reached stable condition of substrates degradation on day 80 as well as SW, which means a thermophilic digestion reactor can be quickly launched by one-step manner in this study. From the results, at least 20 days can be saved in gradual temperature-raising period (31–50 d). Notably, temperature change had a more substantial effect on VFAs accumulation. Total VFAs was accumulated following the increasing of temperature immediately (30–50 d in SW and 50–80 d in OS) (Table 2). Maximum concentrations of VFAs reached approximately 2400 and 2700 mg L^{-1} in SW and OS, respectively. After that, the concentrations of VFAs began to decrease and stabilized on day 130. It is suggested that quick increasing of digestion temperature can lead to the accumulation of VFAs, finally might result in a failure of digestion process.

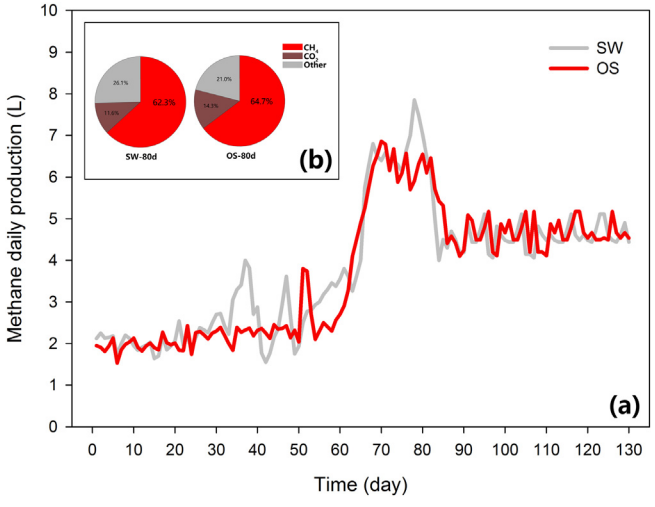

Fig. 1. Comparison of methane yield in two reactors, (a) methane daily production and (b) biogas composition on day 80.

3.2. Change of microbial community across temperature-raising process

High-throughput sequencing of 16S rDNA amplicons yielded 1,645,229 (combined percent: 75.05%) valid reads (average length 412 bp). OTUs were identified into 52 phyla, 92 classes, 162 orders, 316 families, and 571 genera. Relative abundance showed archaeal groups did not change greatly (Fig. 3). The phylum *Euryarchaeota* member was dominated in 5 identified archaeal phyla with a stable range of 80–90%. While members from bacteria are strongly sensitive to temperature-raising in OS and SW. Members of the phyla *Proteobacteria* (accounting for 44.09% of total reads, averaged), *Chloroflexi* (19.92), and *Firmicutes* (16.14%) were dominated in mesophilic condition from two reactors. After 31 d in SW, the relative abundance of *Proteobacteria* and *Chloroflexi* markedly decreased. *Firmicutes* formed a predominant composition on 80 d. With the additional operation time for 50 days (80 d–130 d) at thermophilic condition, no significant change of bacterial community was observed. In OS, the bacterial community remained a stable composition during 1 d–50 d. After that, the microbial community structure in OS rapidly formed similar microbial community structure with SW on 80 d. A core bacterial community consisted of 4 phyla were found in all thermophilic samples (80–130 d), including *Firmicutes* (75.20%), *Proteobacteria* (9.15%), *Actinobacteria* (4.67%), and *Chloroflexi* (3.03%), expect for the detection of *Synergistetes* in SW (Fig. 3-a). These phyla accounted for >90% of the total 16S rDNA sequences.

3.3. Predicted metabolic functions using PICRUSt

Metagenomic prediction using PICRUSt based on 16S rDNA gene sequences revealed the most detected function genes in two reactors (Fig. 4). In total, 27 genes encoding function pathways were assigned against KEGG database (level 2), mainly classified into Metabolism group (12/27, level 1). The assigned genes encoding Membrane Transport (11.6%), Amino Acid Metabolism (10.4%) and Carbohydrate Metabolism (10.3%) were the most abundant function genes in this study. It should be noted that a significant transformation of metagenomes occurred on 80 d in both SW and OS, which implied OS achieved similar metabolic pathway with SW under thermophilic condition. On the other hand, thermophilic digestion samples showed a higher relative abundance of the KEGG level 2 categories related to Cell Growth and Death (belong to Cellular Processes), Translation (belong to Genetic Information Processing), Energy Metabolism (belong to Metabolism), Nucleotide Metabolism (belong to Metabolism).

3.4. Removal of ARGs in two reactors

Digesters were fed untreated sludge from a full-scale WWTP. The concentrations of selected ARGs (6 tetracycline resistance genes and 2 sulfonamides resistance genes) in the feed sludge were presented in Table 1. The effects of temperature change on the reduction of ARGs as well as integrase gene of class 1 integrons were investigated (Fig. 5). RT-qPCR is a reliable approach to detect the concentrations of gene fragment from the complex environment samples (e.g. sludge in this study) (Table 3). Seed sludge exhibited high ARGs relative abundance, such as *sul*I and *tet*X. With the increase of digestion temperature, concentrations of *sul*I, *sul*II, *tet*A, *tet*O, *tet*X, as well as *int*I1 almost reduced by an order of magnitude in two reactors, and even lower than 2.0×10^{-3}

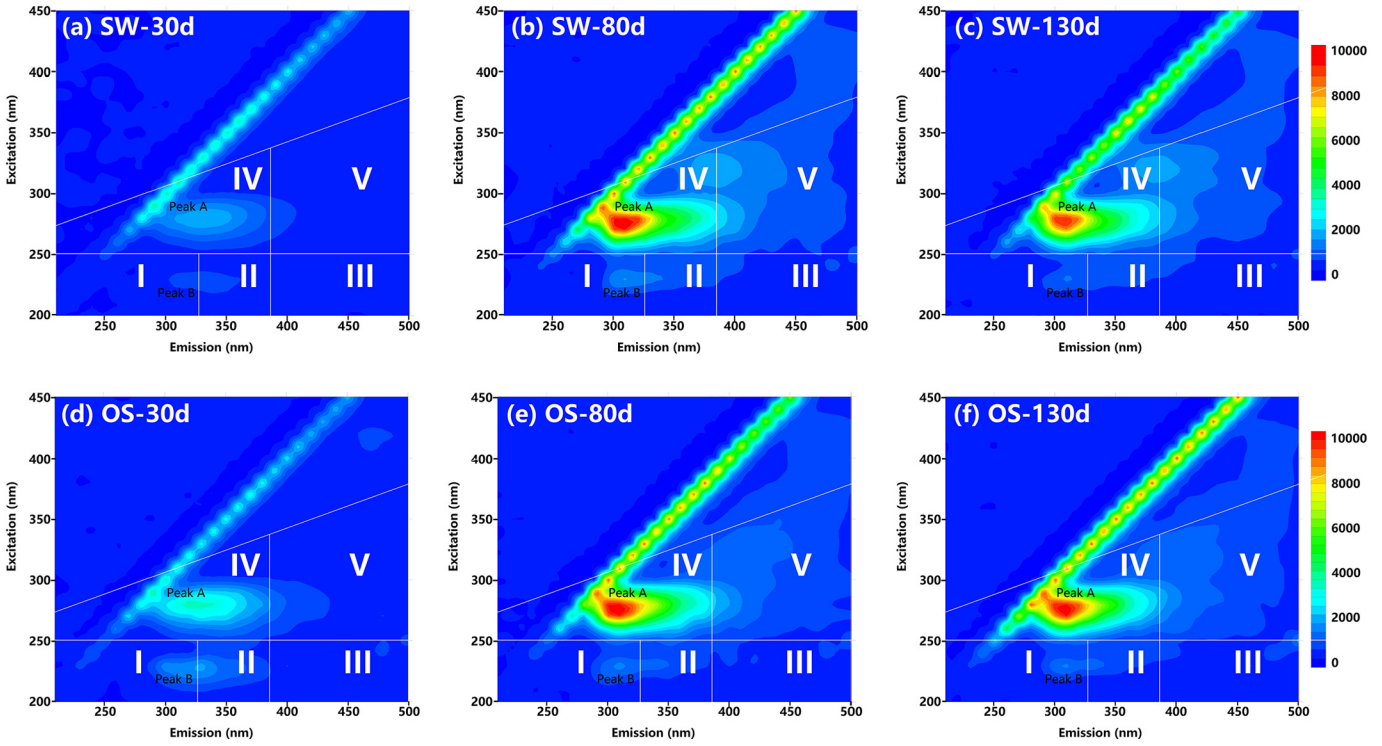

Fig. 2. 3D-EEM fluorescence spectroscopy for the supernatant phase of digested sludge on 30 d, 80 d and 130 d: (a–c) from SW reactor, (d–f) from OS reactor.

copies/16S rDNA copies at the end of thermophilic digestion. Concentrations of *sul*I, *tet*A, *tet*O, and *tet*X displayed a positive correlation with *int*I1 ($p < 0.05$). Reduction rates of *sul*I, *tet*A, *tet*O, and *tet*X were 95.2–99.3%, 92.7–99.3%, 86.0–93.2%, and 81.7–93.7%, respectively. In contrast, *tet*M was enriched at thermophilic condition and even reached over 5.0×10^{-2} copies/16S rDNA copies in both two reactors. Besides, concentration of *tet*L and *tet*W gene showed no statistical rules during the transformation from mesophilic to thermophilic condition, which might imply that anaerobic digestion was unable to remove these ARGs. Generally, OS was more effective in removing the most ARGs at stable stage of thermophilic digestion (130 d), including *sul*I, *sul*II, *tet*A, *tet*W, and *tet*X. Results suggested that thermophilic digestion can be used to control the proliferation of ARGs from sewage sludge.

3.5. Network co-occurrence between ARG and potential hosts

Many researches showed the wide-spread of ARGs in sludge (Diehl and Lapara, 2010; Tian et al., 2016; Zhang et al., 2015), but few studies investigated how different ARGs dynamics associated with potential hosts under different operating conditions. In this study, temperature-raising affected the abundance of both microbial population and ARGs' concentrations in two reactors (Figs. 3 and 5). To discern the potential hosts for selected ARGs, we hypothesized that the similar trends in microbial population were due to some specific members carrying some specific ARGs (Ju et al., 2016; Tian et al., 2016; Zhang et al., 2015). Co-occurrence network helps to present the relationships between selected ARGs and potential hosts (Fig. 6). 127 genera (belong to 14 phyla) were identified as the potential hosts for 8 targeted ARGs and 1 related mobile element. The arrow (from potential host to gene) represented a statistical correlation ($p < 0.05$, r-value > 0.8) between each node. All 138 nodes were clearly divided into 4 major modules. In module 1, most bacteria were associated with *sul*I, *tet*A, *tet*O, *tet*X, and *int*I1. Module 2 and 3 consisted of potential hosts related to *tet*M and *sul*II, respectively. Notably, module 4 showed that 2 selected ARGs (*tet*L and *tet*W) had no correlation with any potential hosts in this study. The phylum *Proteobacteria*, *Firmicutes* and *Bacteroidetes* were found to be the most frequent hosts for selected ARGs.

4. Discussion

4.1. Rapid startup of thermophilic digester

This study proposed a rapid startup mode to achieve effective thermophilic digestion for ARGs reduction. Rapid startup of a thermophilic digester is of important meaning for engineering application. Many thermophilic digesters were started up using the gradual temperature-raising mode, mainly due to the sensitivity of methanogens groups to different temperature (Tian et al., 2015). The present study found that it will be required 50 days to transform a stable mesophilic digester to thermophilic system (31–80 d) by adopting the step-wise mode. Required time for the successful transformation even has been reported to be up to 70 days (Bousková et al., 2005). This study suggested the possibility of rapid establishment of effective thermophilic digesters using one-step temperature-raising mode saved at least 20 days in temperature accelerating process (31–50 d). What calls for special attention is that the startup time might be also influenced by many other AD parameters, such as HRT, properties of feeding sludge, organic loading rates, VFAs concentrations, etc. For example, Bousková et al. (2005) also applied one-step strategy to startup thermophilic digesters within 30 days, but observed significant accumulation of VFAs with temperature-raising. This study also overserved the accumulation of VFAs when temperature increasing, which is the main obstacle of thermophilic digestion.

Change of temperature affects AD performance via regulating microbial community, metabolism activity and their interactions. Temperature also alters the biochemical conversion pathways and thermodynamic equilibrium of the biochemical reactions for methane production (Wilson et al., 2008). In this study, the rapid achievement of thermophilic digestion can be largely attributed to the successful establishment of thermophilic microbial community which are responsible for substrates degradation (Zinder and Mah, 1979). Archaeal and

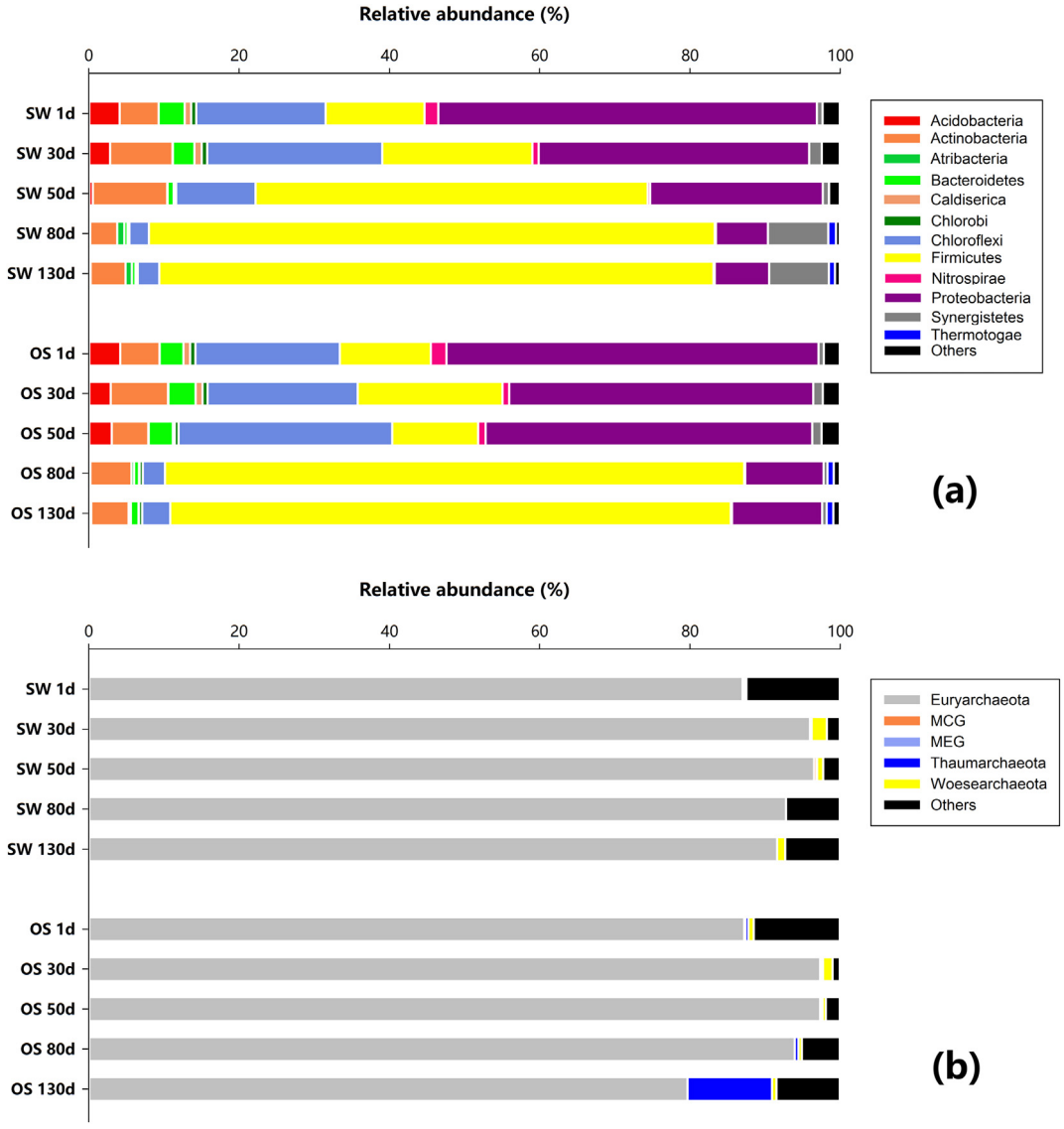

Fig. 3. Dynamic change of dominant phyla in SW and OS reactor: (a) *Bacteria* kingdom and (b) *Archaea* kingdom.

bacterial population formed steady communities at mesophilic condition (day 1–30). On day 80, the dominant thermophilic methanogenic community was successfully established in SW. During this process, the variable conditions (from 35 to 55 °C) are not favorable for the thermophilic groups. Methane daily production and VFAs concentration also fluctuated during this period (Fig. 1 and Table 2). Variation of AD performance suggested the gradual change of microbial population in reactors. On the contrary, using one-step temperature-raising strategy, the similar thermophilic groups were quickly established on 80 d, due to the growth under their favorite condition from the domestication (Tian et al., 2015). Furthermore, the predictive KEGG metagenomic functional profiles of two reactors were investigated using PICRUSt based on the 16S rDNA sequences. The results of metagenome prediction were summarized at various KO levels (Fig. 4). The main metabolic gene groups were assigned with the Metabolism, Genetic Information Processing, Environmental Information Processing. The PICRUSt analysis generated 2 assumptions: (1) On 80 d, a stable thermophilic digester was established by OS mode, because OS and SW showed no significant difference in the functional metagenome. (2) High-temperature increased the metabolic activity for substrate degradation and microbial proliferation, because many genes encoding Cell Growth and Death, Energy Metabolism, Nucleotide Metabolism were enriched along with the increase of digestion temperature from 80 d, which was also implied in Figs. 1 and 2.

4.2. Remove of ARGs from sludge

This study also showed that a large amount of ARGs presented in the untreated sludge from a full-scale WWTP. Different ARGs responded to different AD temperature substantially. In China, over 30-million-tons sewage sludge was generated every year. These unsterilized waste solids were usually applied for further land-use, like the soil conditioner or fertilizer. It is very important to control the spread of ARGs from sludge to downstream environment. Generally, this study observed the advantages of thermophilic digestion, which lead to lower concentrations of most selected ARGs, including *sul*I, *sul*II, *tet*A, *tet*O, *tet*X as well as *int*I1 (Fig. 5). Effective reduction of most tetracycline resistance genes in thermophilic digestion was also reported (Tian et al., 2016). Diehl et al. found that the reduction of these gene quantities with temperature-raising fit well to a first-order kinetic model, implying that higher temperatures resulted in significant reduction of ARGs (Diehl and Lapara, 2010). Notably, it is reported that thermophilic digester could rapidly reduce tetracycline ARGs (including *tet*M and *tet*L) (Diehl and Lapara, 2010; Ma et al., 2011b; Zhang et al., 2015).

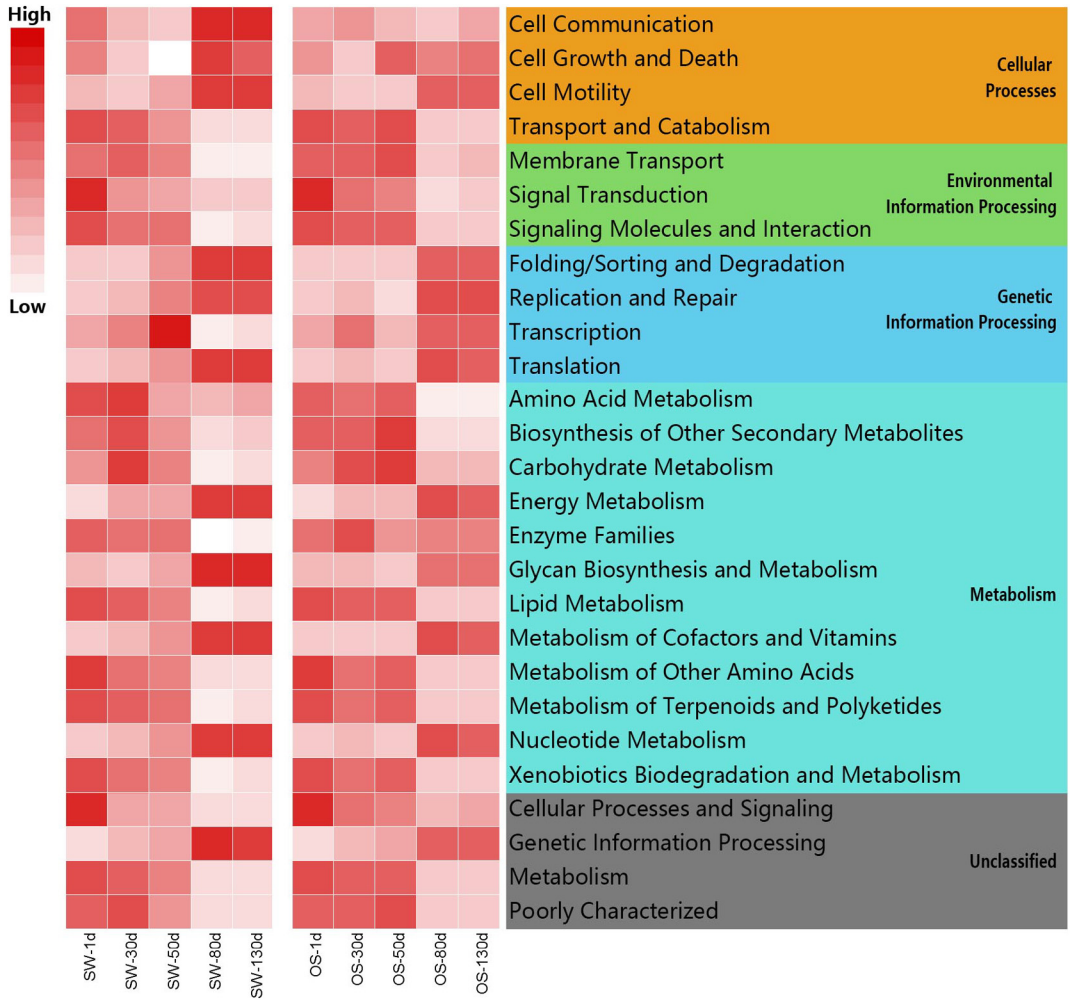

Fig. 4. Relative abundance of predicted function genes during the transformation from mesophilic to thermophilic condition in SW and OS reactor.

But *tet*M was found to be enriched in this study. Moreover, *tet*L could not be removed in neither thermophilic nor mesophilic digestion. One possible reason is that these genes encoded for different resistance mechanisms. For example, *tet*A and *tet*L are reported to encode for tetracycline efflux, *tet*O and *tet*W for ribosomal protection and *tet*X for tetracycline transformation (Chopra and Roberts, 2001). Moreover, these targeted genes may share resistance via the same mechanism but for different bacteria (Diehl and Lapara, 2010), e.g. *tet*A and *tet*L are specific for Gram-negative and Gram-positive bacteria respectively. This can be confirmed by the most detected phylum *Proteobacteria* (Gram-negative) was correlated with *tet*A (Fig. 6). Above results indicated that the mechanisms of resistance of different antibiotics were quite distinctive. Because microbial populations carrying ARGs of different resistance mechanisms responded to different digestion conditions (e.g. feeding substrates or operational temperature) (Zhang et al., 2015). Further research on the fate of these ARGs during AD processes would be of interest.

4.3. Mechanisms of ARGs' reduction

During sludge treatment process, bacteria might share ARGs via horizontal/vertical gene transfer pathway (Tian et al., 2016). Elimination of ARGs spread was supposed to be related with the cutoff of their horizontal gene transfer (HGT) among different hosts (Diehl and Lapara, 2010; Ma et al., 2011b). Mobile genetic elements like intI1 usually represented the HGT. This study also demonstrated that thermophilic AD is particularly effective in removing *int*I1. Integrons are believed to be important genetic items for gene recombination, which allow microorganism to merge exogenous gene cassettes and regulate their expression. Although integrons can be transfer among microbes alone, they were reported to correlated with other mobile genetic elements which can, e.g. insertion sequences, transposons and conjugative plasmids (Berglund, 2015). Therefore, integrons are suggested to contribute to the exchange and incorporation of ARGs, resulting in proliferation of bacterial antibiotic resistance in environment (Mazel, 2006). Municipal wastewater treatment plant is recognized as an important reservoir of ARB and ARGs. Because the treatment process makes it to biomass compression, which is a prerequisite for the proliferation of ARGs (Ghosh et al., 2009a). As one of the most abundant integron in environmental microbe (Stalder et al., 2014), *int*I1 has been found to co-exist with *tet*A in conjugative plasmids (Agersø and Sandvang, 2005; L'Abée-Lund and Sørum, 2001) and they are readily transferred into recipients together, which was regarded as a carrier for ARGs. This research also observed the co-occurrence between *int*I1 and many targeted ARGs, such as *tet*A, *tet*X, *tet*O and *sul*I (Fig. 6). Therefore, *int*I1 was suggested as the indicator ARGs pollution. This study proved that high-temperature is critical to control the horizontal gene transfer possibility among bacteria, because the quantities of *int*I1 and most potential host was declined >80% using thermophilic AD, which is much lower than mesophilic one (Figs. 5 and 7). On the other hand, increased digestion temperature showed the greatest impact on the microbial population, which may influence the vertical gene transfer (VGT)

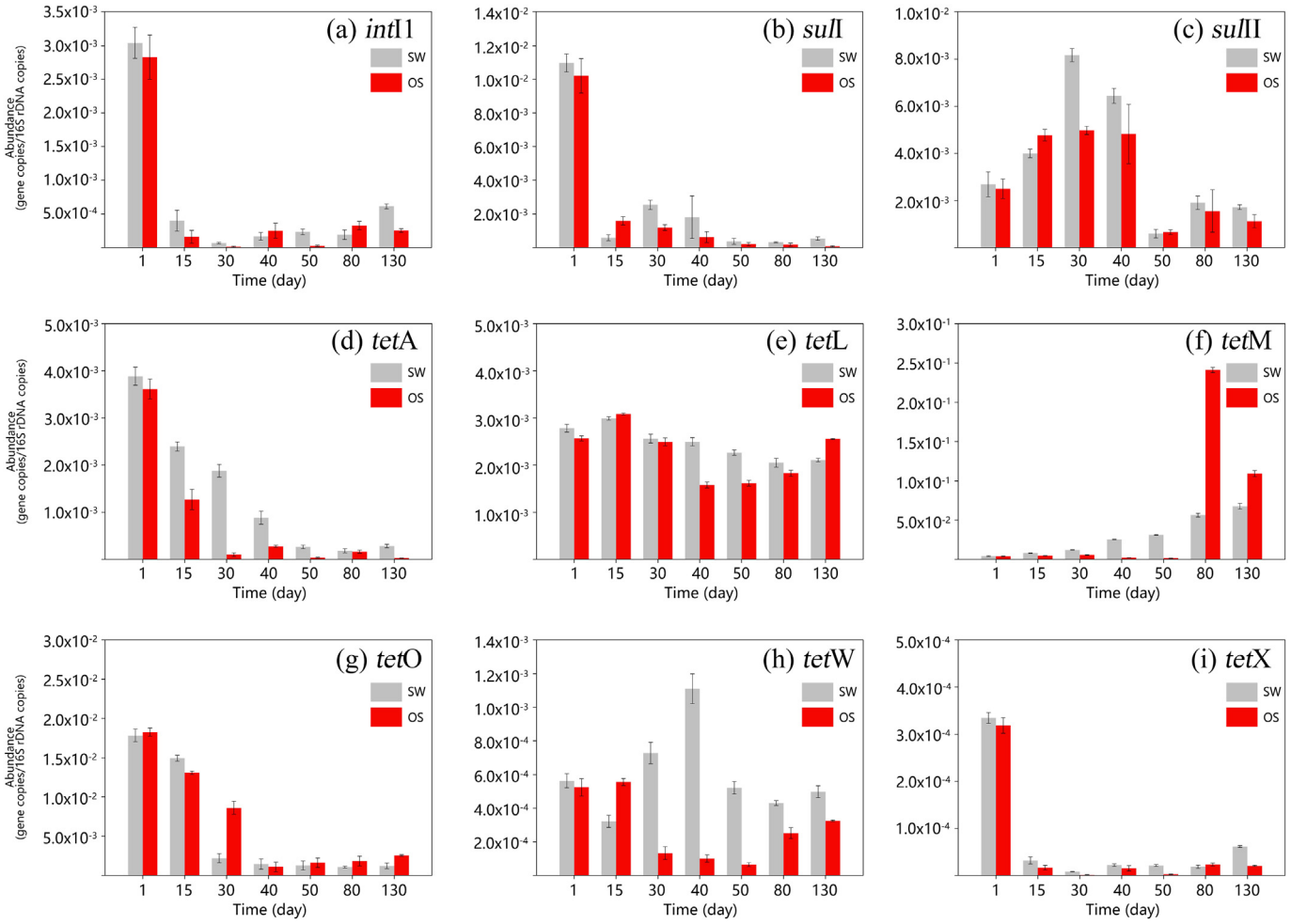

Fig. 5. Relative abundance of class 1 integrons (*intI*1), sulfonamides resistance genes (*sulI*, *sulII*) and tetracycline resistance genes (*tetA*, *tetL*, *tetM*, *tetO*, *tetW*, *tetX*) in two reactors. Quantity of targeted genes was normalized by the 16S rDNA gene numbers (bacterial + archaeal) to minimized the basis caused by different microbial abundance.

directly. Previous study suggested that network analysis could be applied to provide new insights into ARGs and their potential hosts in the complex environment (Ju et al., 2016). As showed in Fig. 6, a total of 127 genera were identified as the potential hosts for targeted ARGs through network analysis. Host diversity of *sulI*, *tetA*, *tetO*, and *tetX* is much higher than *sulII* and *tetM*. ARGs targeted in this study were strongly correlated with various species. Tetracycline and sulfonamides resistance genes were mainly carried by *Proteobacteria*, *Firmicutes* and *Bacteroidetes*. Among the 127 hosts, the relative abundance of 106 genera (>80%) declined apparently after temperature-raising (Fig. 7),

Table 3
Standard curves and amplification efficiency of targeted genes by RT-qPCR approach.

Gene	Standard curves	R^2	Efficiency (%)
tetA	y = −3.335 lgx + 35.897	0.9936	96.8
tetL	y = −3.112 lgx + 34.723	0.9917	98.4
tetM	y = −3.462 lgx + 39.473	0.9950	101.1
tetO	y = −2.791 lgx + 29.925	0.9948	96.6
tetW	y = −3.446 lgx + 35.230	0.9903	95.1
tetX	y = −3.197 lgx + 33.839	0.9948	99.4
sulI	y = −3.206 lgx + 30.280	0.9897	99.4
sulII	y = −3.446 lgx + 32.363	0.9936	94.2
intI1	y = −3.018 lgx + 33.129	0.9936	100.6
Bac-16S[a]	y = −3.332 lgx + 32.612	0.9978	99.8
Arc-16S[a]	y = −3.328 lgx + 31.447	0.9905	97.2

[a] Bacterial 16S rDNA; Archaeal 16S rDNA.

which suggested that the lower capacity of vertical gene transfer is the main reduction mechanism of ARGs. For example, the potential bacterial hosts of *tetX* were mainly confirmed as *Bacteroides* (Bartha et al., 2011), which was characterized as the dominant fermentative bacteria in mesophilic digesters (Tian et al., 2015). Relative abundance of *Bacteroides* declined rapidly after temperature increasing (Fig. 3). Meanwhile, *tetX* has not been verified to be transferred to other groups through horizontal pathway (Ghosh et al., 2009b). Consequently, the high removal rate of *tetX* was attributed to the reduction of *Bacteroides* due to the effective inactivating pathogenic bacteria during high-temperature treatment (Ghosh et al., 2009a). Therefore, ARGs are believed to persist in AD systems if related bacterial hosts survive under specific conditions. Notably, the potential hosts analyzed by network method depended on its functional connection, which may be not real antibiotic resistance bacteria. Nevertheless, these assigned hosts are most likely to become the antibiotic resistance bacteria through HGT or VGT due to their function connection. Furthermore, many studies suggested that the quantity of ARGs can be reduced when their hosts were eliminated (Tian et al., 2016). However, most potential hosts were also identified as core members for AD process, such as *Proteobacteria*, *Firmicutes* and *Bacteroidetes* in this study (Figs. 3 and 6) (Rivière et al., 2009; Tian et al., 2015; Yang et al., 2016). More than 10% species were shared between methane production-related groups and potential hosts groups (Fig. A.1). The strategy for reducing these host members to eliminate ARGs might also restrain digestion performance.

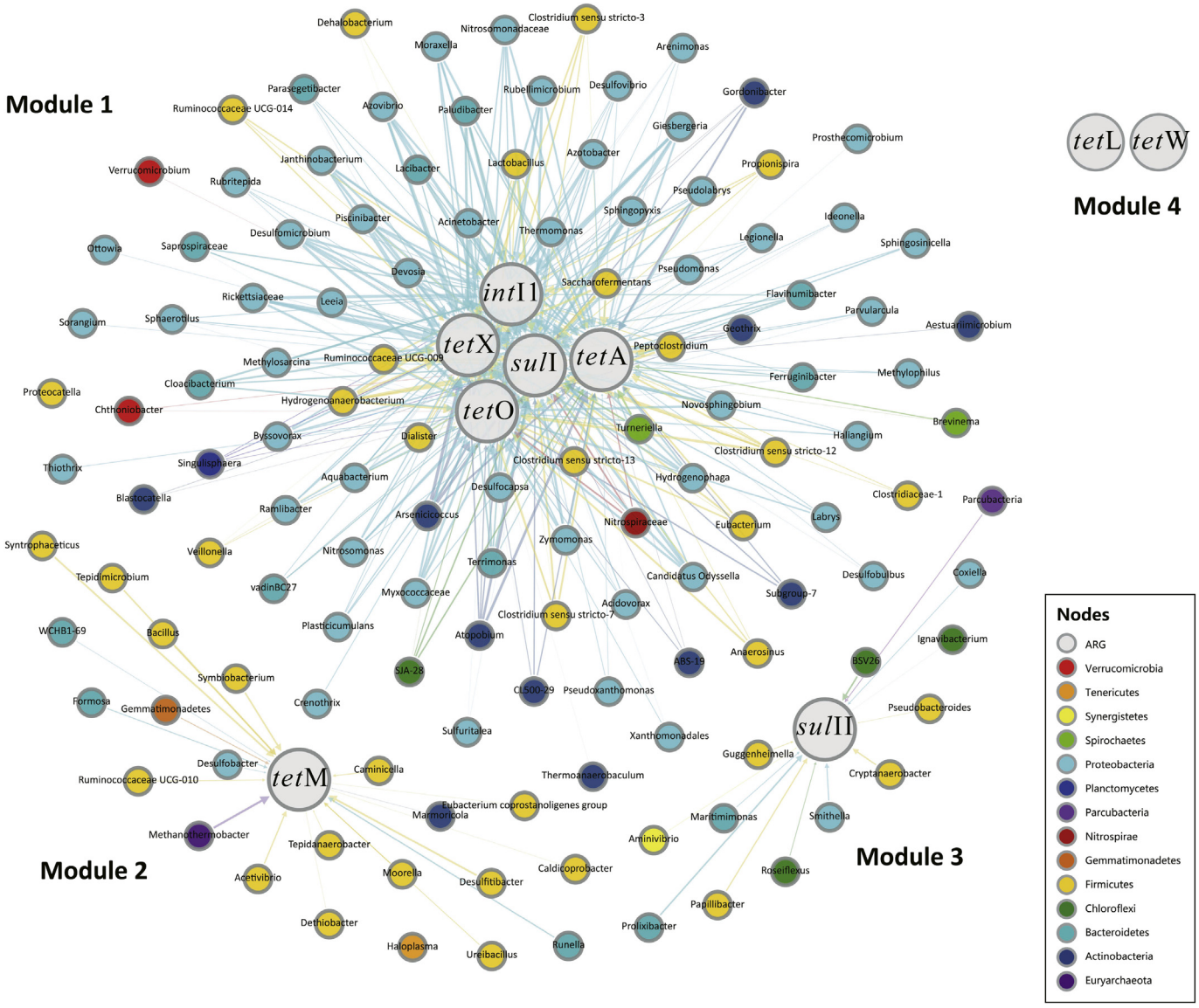

Fig. 6. Network analysis revealing the co-occurrence patterns between targeted ARGs and potential hosts during the digestion process. An arrow indicates a strong correlation ($r > 0.8$, p-value < 0.05). The thickness of each connection is proportional to the Pearson's correlation coefficient, ranging from 0.8 to 1.0. Grey nodes and colorful nodes represented the ARG types and hosts, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4.4. Perspective

Lastly, application of untreated sewage sludge might increase the risk of ARGs spread from sludge to soil environment, and even result in the development of antibiotic resistance in human pathogens (Ashbolt et al., 2013; Munir et al., 2011; Pruden et al., 2006; Riesenfeld et al., 2004). Study of ARGs controlling during sludge treatment processes is an urgent issue to eliminate their spread. RT-qPCR method has its unique advantage in evaluating the change of targeted ARGs during AD process (Diehl and Lapara, 2010). Nevertheless, it is impractical to select the targeted ARGs, which are known to encode for antibiotic resistance. Also, RT-qPCR characterizes the presence of gene fragments, which cannot discern ARGs from dead/live cells and regardless of whether the host is capable to express them. Besides, although *int*I1 was recognized as an main indicator of horizontal ARGs transferability (Ma et al., 2011a), it does not integrate many typical ARGs such as tetracycline resistance genes (Toleman et al., 2006). Consequently, additional horizontal mobile items (e.g. transposons or plasmids) should be considered together to verify this speculation. To guide the selection of RT-qPCR approach for targeted ARGs, metagenomics approach may be jointly used in the future to give a wide-spectrum detection of ARG/ARG subtypes beforehand.

5. Conclusions

The rapid thermophilic anaerobic digester can be established using one-step temperature-raising mode. Growth conditions favorable for the colonization of core thermophilic members can be saved at least 20 days. Genes related to microbial metabolism were predicted by PICRUSt, including Cell growth and Death, Energy Metabolism Nucleotide Metabolism, suggesting high-temperature increased microbial metabolism activity. In addition, the thermophilic digestion outperformed mesophilic one in removing most tetracycline and sulfonamides resistance genes from untreated sludge. Reduction of *int*I1 and potential genetic hosts implied the remove of ARGs were mainly attributed to the lower level of horizontal and vertical transferability. This study proposed that rapid startup of thermophilic AD is a promising technology for sludge treatment to prevent the spread of various ARGs into downstream environment along with the further land-use.

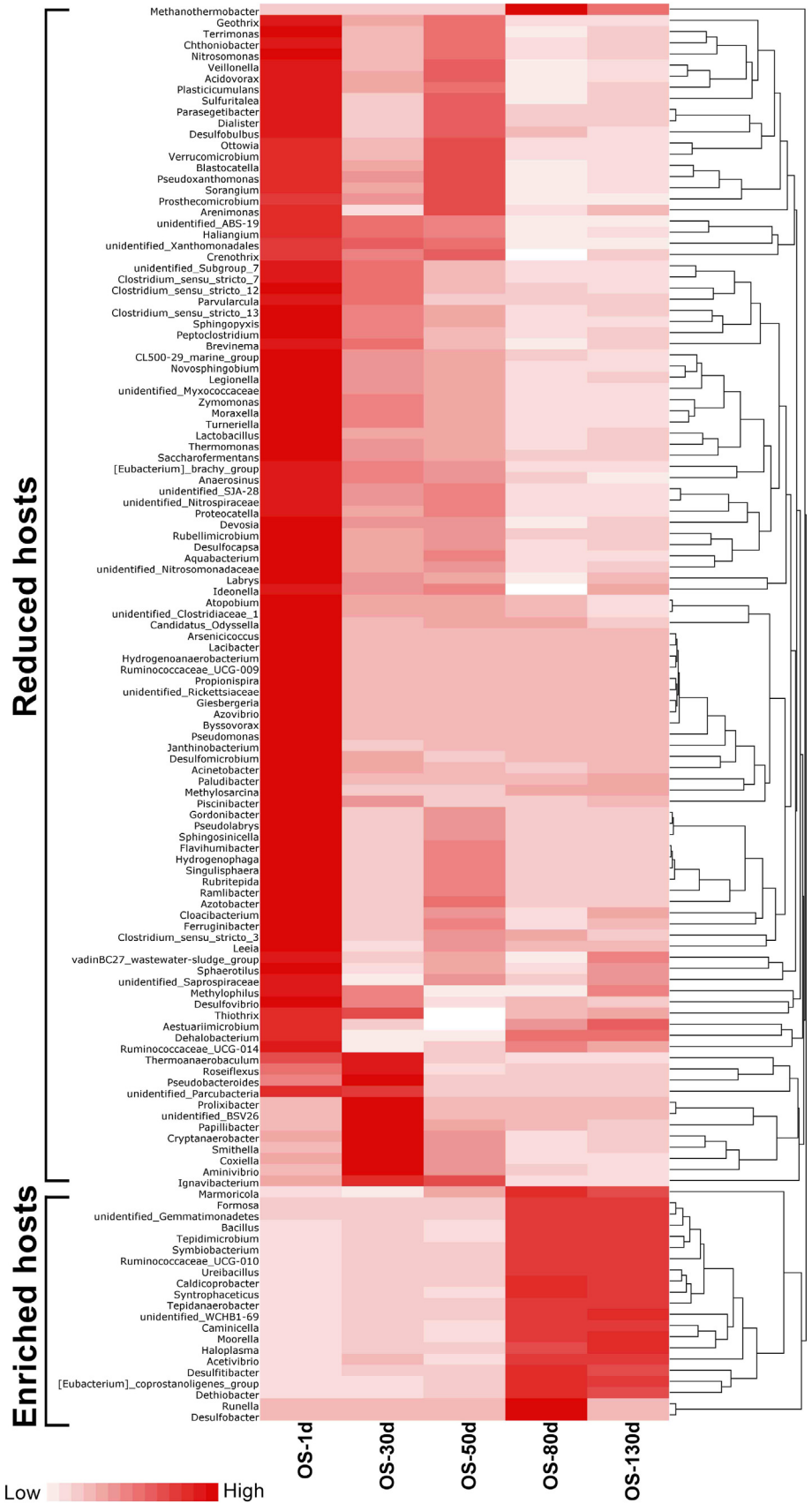

Fig. 7. Heatmap revealing the reduction of ARGs-related hosts in OS reactor, from mesophilic to thermophilic condition.

Acknowledgments

This work was supported by National Natural Science Foundation of China (NSFC, Grant No. 51378189, 51578223 and 51521006).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.08.295.

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