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Influence of nanoscale zero-valent iron and magnetite nanoparticles on anaerobic digestion performance and macrolide, aminoglycoside, β-lactam resistance genes reduction

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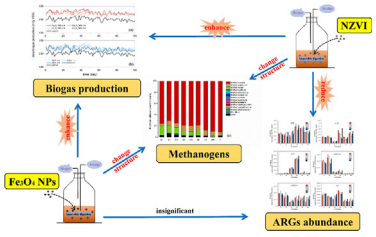
GRAPHICAL ABSTRACT



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ABSTRACT

The effect of nanoscale zero-valent iron (NZVI) and magnetite nanoparticles (Fe_3O_4 NPs) on anaerobic digestion (AD) performance was investigated through a series of 100-day semi-continuous mesophilic anaerobic digestions. The results indicated that biogas production had increased by 24.44% and 21.66% with the addition of 0.5 g/L Fe_3O_4 NPs and 1.0 g/L NZVI, respectively. Besides, the abundance of five widespread antibiotic resistance genes (ARGs) (*erm*F, *erm*A, *erm*T, *aac*(6′)-IB, *bla*OXA-1) was also studied. The decrease in abundance of *aac*(6′)-IB and *bla*OXA-1 was observed during the AD process with an average removal rate of 95.69% and 44.82%, respectively. Most of the ARGs, especially *erm*A and *erm*T, were less abundant in NZVI group compared with control group. The overall results suggested that the addition of NZVI and Fe_3O_4 NPs contributed to a better sludge anaerobic digestion performance, and NZVI was beneficial to the removal of some ARGs.

1. Introduction

With the growth of urban population and the acceleration of industrialization, the output of municipal sewage is larger than before, which is accompanied by a large amount of waste activated sludge (WAS) that could not be well treated. WAS contains considerable organic biomass and has good biodegradability. Anaerobic digestion (AD) is one of the important ways for sludge reduction, stabilization and recycling. It is a promising technology to convert organic biomass into biogas whose main content is methane, a potential renewable energy

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source. AD process can be divided into four phases according to the different metabolic activities of microorganisms (Appels et al., 2008): hydrolysis, acidogenesis, acetogenesis and methanogenesis. Among these phases, hydrolysis is the main rate-limiting phase of AD (Xu et al., 2017b). Many approaches have been carried out in order to improve the hydrolysis efficiency of AD process, such as thermophilic pretreatment (Ge et al., 2011) and microwave pretreatment (Zhang et al., 2016). Whereas, due to their high energy consumption, they have low economic efficiency. Hence, a more effective method of improving hydrolysis efficiency is urgently needed.

As one of the trace metals, iron is essential for energy transfer and material metabolism of microorganisms. It performs as an electron carrier in the process of extracellular electron transport (Newman & Kolter, 2000) and plays an important role in the synthesis of oxidases and cytochromes of methanogens (Jing et al., 2018). Nanoscale zero-valent iron (NZVI) is an active substance with strong reducibility and high surface area to volume ratio. In recent years, it has been widely used in sludge anaerobic digestion, and proved to be functional in enhancing the AD performance. Hao's study revealed that NZVI could produce hydrogen through chemical corrosion, which on the one hand can reduce the oxidation-reduction potential (ORP) to provide a better anaerobic environment for methanogens, and on the other hand can improve the efficiency of hydrogenotrophic methanogenesis (Hao et al., 2017). The addition of NZVI was also reported to be able to generate ferrous ions, which could serve as extracellular electron donors for some microorganisms involved in anaerobic digestion process (Zhang et al., 2019). Apart from NZVI, magnetite nanoparticles (Fe_3O_4 NPs) can also promote the biogas production on account of its well electrical conductivity (Abdelsalam et al., 2017). As an excellent magnetic material with electron transport capacity, Fe_3O_4 NPs is involved in interspecific electron transfer between syntrophic microorganisms, such as organic oxidizing bacteria and methanogenic archaea (Zhao et al., 2018a). It is also reported that Fe_3O_4 NPs is able to promote the enrichment of iron-reducing bacteria, which can decompose and utilize some complex organic matters to improve the hydrolysis efficiency (Peng et al., 2017).

The widespread use of antibiotics in human disease treatment and animal husbandry has led to a gradual increase of antibiotic residues in the environment (Li et al., 2020). Meanwhile, an attendant increase of antibiotic resistance genes (ARGs) has become a significant world-wide problem in recent years (Chen et al., 2019a). It is reported that WAS is an important repository of ARGs, the direct land utilization of untreated WAS will result in many potential threats to the environment (Bai et al., 2019). Previous studies have demonstrated that anaerobic digestion has the potential to reduce the abundance of ARGs in sludge (Tong et al., 2016). According to previous studies, macrolide, aminoglycoside and β-lactam resistance genes have high content in WAS (Chen et al., 2019b; Ying et al., 2013). Zhang found that the addition of NZVI could avail the reduction of *erm*B, *erm*F, *bla*CTX-M and *bla*TEM (Zhang et al., 2018). Besides, NZVI is reported to be cytotoxic, it can interact with functional proteins on cell membranes and thus destroy the integrity of microbial cells (Changha et al., 2008). And this reduces the vertical transfer efficiency of ARGs. Furthermore, Chen found that NZVI is helpful to remove some antibiotics (Chen et al., 2012). However, the role of Fe_3O_4 NPs in removing ARGs has been rarely reported. Therefore, it is necessary to study the evolution of ARGs during AD process in the presence of NZVI or Fe_3O_4 NPs.

In this study, nine digesters with different conditions were conducted to explore the effect of NZVI and Fe_3O_4 NPs on the performance of sludge anaerobic digestion, find out the optimal dosage of both NZVI and Fe_3O_4 NPs, and study the change of archaea and bacterial community under the pressure of two iron nanoparticles. In addition, the abundances of 5 ARGs (including *erm*F, *erm*T, *erm*A, *aac*(6′)-IB and *bla*OXA-1) and the class I integron (*intI*1) were also investigated to evaluate the reduction efficiency of NZVI and Fe_3O_4 NPs on ARGs during semi-continuous sludge anaerobic digestion. This study provides new theoretical support for the controlling proliferation of ARGs in sludge.

Table 1
Main parameters of seed sludge and feed sludge.

Parameters	Seed sludge	Feed sludge
pH	6.81 ± 0.1	7.54 ± 0.1
sCOD (g/L)	0.45 ± 0.1	9.47 ± 0.1
TS (g/L)	15.4 ± 0.5	81.3 ± 0.5
VS (g/L)	7.9 ± 0.5	47.7 ± 0.5
VS/TS (%)	51.3 ± 0.1	59.3 ± 0.1
NH_4^+-N (g/L)	1.16 ± 0.1	2.63 ± 0.2
Total Nitrogen (%)	2.5 ± 0.1	4.38 ± 0.1
TOC ($g\,kg^{-1}$)	200.0 ± 1.0	273.0 ± 1.0
Polysaccharides ($mg\,L^{-1}$)	160.4 ± 3.0	269.7 ± 5.0

2. Materials and methods

2.1. Preparation of substrates and feedstock materials

Dewatered sludge (DS) and WAS from the municipal waste water treatment plant (Changsha, China) were used in this experiment. Main characteristics were summarized in Table 1. The seed sludge (SS) was obtained by anaerobic reaction of WAS in a constant temperature incubator of 35 °C for a month. The feed sludge (FS) was consisted of WAS and DS with a ratio of 1:1 to achieve a total solids content of 8%. Both of nanoscale zero-valent iron (99.9% metals basis, 50 nm) and magnetite nanoparticles (II, III) (99.5%, 20 nm) were obtained from Macklin Chemistry co. Ltd, Shanghai, China.

2.2. Anaerobic batch reactor set-up and operation

Nine bottles (5.0 L) with working volume of 3.0 L were divided into three groups for anaerobic digestion experiment. The experiment was carried out using continuous stirred-tank reactors (CSTR) in a constant temperature water bath at 35 °C for 100 days with a 20 days hydraulic retention time (HRT) (Hyun Min et al., 2014). Every digester has a rubber plug with two tubes, one for gas collecting, and the other for sampling. Initially, 2.0 L FS and 1.0 L SS were mixed thoroughly and then transferred into each bottle as the digestion substrate. After sealing, the bottles were flushed with nitrogen gas for about 10 min to make sure an anaerobic condition. In general, 150 mL of digested samples were collected for further analysis daily. Meanwhile, the same volume of FS was injected into the digester to maintain the balance of reactors. During the 100-day anaerobic digestion process, for Fe_3O_4 NPs digesters (R1, R2, R3, R4) and NZVI digesters (R5, R6, R7, R8), four different concentration of Fe_3O_4 NPs or NZVI (0.5 g/L, 1.0 g/L, 2.0 g/L, 4.0 g/L) were added into digesters daily along with the FS respectively after ultrasonic treatment to avoid the unite of nanoparticles. While for the control digester (R), neither Fe_3O_4 NPs nor NZVI was added.

2.3. Chemical analysis

Soluble chemical oxygen demand (sCOD), total solids (TS), volatile solids (VS) and ammonia nitrogen (NH_4^+-N) were measured as previous study (Xu et al., 2017a). Digested samples were centrifuged at 5000 rpm for 10 min at first, and then filtered the supernatant through 0.45 μm cellulose membrane. The filtrate was analyzed for pH, sCOD, NH_4^+-N, and volatile fatty acids (VFAs). A pH-meter (PHS-3C, China) was used to determine the pH value. The VFAs were measured by a gas chromatograph (HP5890, USA) according to Xu's method (Xu et al., 2017a). The biogas production was measured daily through the water displacement method, biogas production was reported as the volume of biogas produced per gram of VS_{added} (mL per g VS_{added}). And then the hydrogen, carbon dioxide and methane contents in the biogas were

analyzed by gas chromatography (GC112A, China).

2.4. DNA extraction, real-time quantitative PCR (qPCR) and 16S rRNA amplicon sequencing

Digested sludge for real-time quantitative PCR (qPCR) of ARGs were sampled on 0, 20, 40, 60, 80, 100 day from each digester, respectively. And digested sludge samples for 16S rRNA amplicon sequencing were collected from each digester by the end of experiment. Sludge samples were freeze-dried at first, and then 0.2 g of freeze-dried samples was taken to extract DNA using E.Z.N.A. Soil DNA Kit (OMEGA, USA) according to the manufacturer's instructions. After that, the quality of DNA was determined by gel electrophoresis with 1.5% (w/v) agarose in this experiment. The qPCR reaction of three macrolide resistance genes (*erm*F, *erm*T, *erm*A), an aminoglycoside resistance genes (*aac*(6′)-IB), and a β-lactam resistance genes (*bla*OXA-1), as well as class I integron (*intI*1) was performed on iQ5 real-time PCR thermocycler (Bio-rad, USA) as previous article described (Xu et al., 2017b). Each gene was amplified in triplicates with a 20 μL reaction mixture containing 1.0 μL of template DNA, 0.4 μL both of forward and reverse primer (10 μM), 10.0 μL of 2 × Super Real PreMix Plus (SYBR Green), 0.4 μL of 50 × ROX Reference Dye (Tiangen, China) and 7.8 μL of RNase-Free ddH_2O. Besides, DNA samples were sent out for Illumina pair-ended sequencing on the MiSeq PE250 platform after PCR amplification. And the distribution and evolution of bacterial and archaeal community of different samples were analyzed according to the sequencing results. All sequencing raw datasets were deposited to NCBI with submission ID SUB5690458.

2.5. Data analysis

All tests were accomplished in triplicate. Data analysis was performed with Microsoft Excel 2016 (Microsoft, USA), and visualized through Origin 9.0 (OriginLab, USA). Circos graph and heat map showing the evolution of the microbial communities at different taxonomic levels were produced by Circos software (Krzywinski & Schein, 2009) and HemI 1.0 (Deng et al., 2014), respectively. Redundancy analysis (RDA) was completed by Canoco 4.5 (Microcomputer Power, USA) to reveal the correlation between the environmental factors and the microbial communities. The Spearman correlation between the ARGs and *intI*1 was calculated by SPSS 22.0 (IBM, USA), and $P < 0.05$ represents a statistically significant difference.

3. Results and discussion

3.1. General performance of anaerobic digestion

As a key parameter for evaluating the AD process, biogas production was followed once a day during the process. As shown in Fig. 1, both the NZVI digesters and Fe_3O_4 NPs digesters produced more biogas than the control. In the whole semi-continuous AD process, the biogas production of each digester was relatively stable. The maximum daily biogas production of the Fe_3O_4 NPs group, NZVI group and control group were 289, 283 and 242 mL g^{-1} VS_{added}, respectively. Previous studies obtained distinct results due to different addition of iron nanoparticles. Some researchers reported that the addition of NZVI had a negative influence because of a rapid release of H_2 and destruction of cell membrane, which resulted in the methanogenesis inhibition (Huang et al., 2016). While other researchers found that appropriate concentrations of iron powder could promote methane production, but excessive addition had an inhibitory effect (Feng et al., 2014). In this experiment, the daily biogas production did appear a slight decrease with the increasement of the NZVI concentration, but there was no inhibition since the biogas production was still higher than the control digester. The volumes of cumulative biogas production were 183.45, 181.20, 176.66, 179.11, 174.98, 179.35, 172.20, 168.50 and 147.42 L

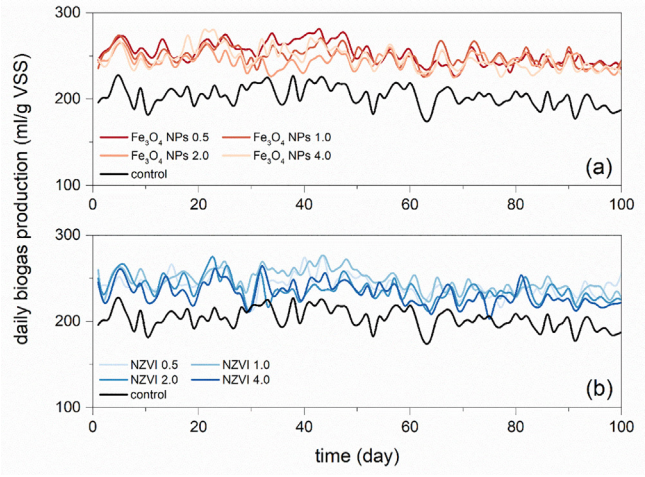

Fig. 1. Daily biogas production comparison between the NZVI group and control group (a) as well as the comparison between the Fe_3O_4 NPs group and control group (b) during 100-day AD process.

in digesters R1, R2, R3, R4, R5, R6, R7, R8 and R, respectively. All digesters outperformed the control in biogas production. Results implied that Fe_3O_4 NPs and NZVI addition could significantly increase the biogas production ($P < 0.05$). The addition of NZVI promoted hydrogen generation, and the hydrogen produced from iron corrosion reduced the ORP and provided a more favorable environment for anaerobic digestion (Jing et al., 2018). Besides, It is reported that about 30% of methane was produced by the synthesis of hydrogen and carbon dioxide (Fukuzaki et al., 1990). And slowly increased hydrogen could be used as the substrate of hydrogenotrophic methanogens and homoacetogenic bacteria to enhance methane production. Both of these two iron powders could provide iron ions in AD system, and iron ions were involved in the synthesize of some enzymes related to acidogenesis and methanogenesis (Ferry, 2010). As a magnetic material, Fe_3O_4 mediated the interspecific electron transfer between carbohydrate oxidizing bacteria and methanogenic archaea (Zhao et al., 2018b), thereby improved their metabolic activity. Besides, Fe_3O_4 could promotes the conversion of propionic acid to acetic acid (Carolina et al., 2014), which was beneficial to the subsequent methane production process.

The pH value is one of the important indexes to reveal the stability of anaerobic digestive system (Xu et al., 2018). During the experiment, pH values of each digesters were measured once a day. pH values of nine digesters were fluctuated between 7.37 ± 0.05 and 7.81 ± 0.05. In total, the pH value was relatively stable, and no sudden drop was observed. The effluent VS value of digester R (about 33.97 g/L) was much higher than others. And digester R1, added 0.5 g/L Fe_3O_4 NPs, had the highest VS removal ratio of 36.37%. The optimal removal rate of Fe_3O_4 NPs group and NZVI group were found in the digesters with concentrations of 0.5 g/L (R1) and 1.0 g/L (R6), whose VS removal ratio was 26.16% and 19.99% higher than the control, respectively. The addition of NZVI was reported to be able to produce hydrogen and iron ions, on the one hand, the released hydrogen could participate in the electron transfer process of hydrolytic fermentation bacteria, promoting the biodegradation of solid organic fractions (Zhang et al., 2011), on the other hand, the iron ions contributed to synthesize of many enzymes related to AD process (Jing et al., 2018). Besides, Fe_3O_4 NPs group performed better than NZVI group at degrading VS. In addition to the release of iron ions, Fe_3O_4 can also stimulated the enrichment of Fe (III) reducing bacteria (Carolina et al., 2014), which are able to hydrolyze refractory substrate into small molecular organic. Moreover, the effluent sCOD of both NZVI group and Fe_3O_4 NPs group were lower than that of the control. These results indicated that the biodegradability of sludge was enhanced with the addition of both two iron nanoparticles.

Table 2
Alpha-diversity indexes of bacterial and archaeal community of different digesters.

Samples	Observed species		Shannon index		Chao 1 index	
	Bacterial	Archaeal	Bacterial	Archaeal	Bacterial	Archaeal
R1	1662	673	8.40	5.07	1654.96	673.31
R2	1799	779	8.44	5.51	1672.62	786.04
R3	1924	671	8.46	5.28	2281. 23	671.20
R4	1980	641	8.38	5.06	2352.70	641.30
R5	1999	637	8.57	4.84	2409.77	637.12
R6	1799	585	8.29	4.15	2472.18	585.40
R7	1536	618	8.10	4.32	1834.96	619.81
R8	1689	606	7.85	4.36	1604.95	606.20
R	1946	418	8.58	1.88	2460.22	432.45

3.2. Alpha-diversity of AD-related microbial community

Illumina Miseq PE250 platform was used to sequence the V3-V4 variable region of microbial 16S rRNA gene. And the bacteria and archaea obtained 481,853 and 412,838 valid reads, respectively. The high-quality reads were clustered into microbial operational taxonomic units (OTUs) at 97% similarity, each OTU represented a microbial species, so we can study the microbial diversity and abundance through OTUs analysis. In order to ensure the reliability and accuracy of the analysis results, the OTUs with the richness lower than 0.001% of the total sequences were removed before the subsequent data analysis (Bokulich et al., 2013). The alpha-diversity of microbial community indexes were used to calculate microbial community richness and compare inter-species diversity in each sample (Table 2). The highest archaeal observed species number (779), Shannon index (5.51), and Chao 1 index (786.04) were all observed in R2. The average archaeal observed species of R1-R4 (691) was larger than that of R5-R8 (612), this was consistent with the results shown by Shannon index and Chao1 index, indicating that the addition of Fe_3O_4 NPs increased the archaeal community richness and diversity of the anaerobic digestion system more than NZVI. This trend was also consistent with the biogas production. As for the diversity of bacterial communities, it's different from archaea communities. The highest bacterial Shannon index was 8.58 in digest R, whose observed species (1946) and Chao 1 index (2460.22) were also fairly high. And the bacterial Shannon index of NZVI group showed a consistent decrease with the gradual increase of NZVI concentration. This indicated that the addition of iron powder had a certain negative effect on the microbial dynamics, and all the microbes found in these samples require further understanding to clarify their specific role in the different AD process.

3.3. Microbial community analysis at different taxonomy level

The presence of NZVI or Fe_3O_4 NPs exerted selective pressure on microorganisms, promoting the growth of some microorganisms and inhibiting the activity of others, thereby changing their community structure. Figs. 2 and 3 shows the species composition and distribution of archaea and bacteria communities at phylum or genus taxonomy level, respectively.

3.3.1. Variation of archaeal community

The relative abundances of bacteria and archaea at phylum and genus level were calculated based on OTUs. As shown in Fig. 2(A), *Euryarchaeota* is the only dominant archaeal phylum in each digester, since most of the methanogenic archaea are contained in the *Euryarchaeota*. Its average proportion in each digester is over 94%, and the highest is in R8, reaching 99.8%. And the relative abundances indicated archaeal phylum did not change a lot with the addition of iron nanoparticles. As for the genus taxonomy level, *Methanosarcina* (80.1%), *Methanobacterium* (8.0%) and *Methanosaeta* (7.7%) presented relative high abundances in nine digesters. As the dominant genus, the abundance of *Methanosarcina* for Fe_3O_4 NPs digesters (R1, R2, R3, R4) and NZVI digesters (R5, R6, R7, R8) were 15.9, 20.4, 16.3, 12.0% and 11.1, 12.4, 3.6, 1.4% lower than that of the control (R), respectively. *Methanosarcina* is both acetoclastic methanogen and hydrogenotrophic methanogen, it is a versatile member of methanogens who can use acetate, hydrogen, carbon dioxide, carbon monoxide, monomethylamine, dimethylamine and trimethylamine to produce methane (Wang et al., 2018a). This may be one of the reasons for its good environmental adaptability and the dominant abundance in the archaea community. *Methanosarcina* accounted for 91.0% of all archaeal genera in the control digestor, while other genera made up only a small proportion, which means that community structure of the control group was simpler than other two groups, and the addition of iron nanoparticles enriched the diversity of the archaea community. As seen in Fig. 2(B), the control digester had the lowest abundance of *Methanobacterium* (4.7%) among all the digesters. It is reported that *Methanobacterium* is one of the hydrogen-utilizing methanogenic archaeal (Shin et al., 2010), its community abundance is closely related to the concentration of hydrogen in the anaerobic digestion. Nanoscale zero-valent iron is a kind of material with strong reducibility, it can rapidly undergo chemical corrosion and generate hydrogen when it comes into contact with water in the AD reactor. And this may account for the higher *Methanobacterium* abundance in the NZVI group compared with the control group. *Methanosaeta* species is an obligate acetoclastic methanogens, and can only use acetic acid as the substrate to produce methane (Garcia et al., 2000). It has a higher affinity for acetate concentration. It is reported that the presence of iron nanoparticles could stimulated the conversion of propionate to acetate, which promoted the growth of *Methanosaeta* and improved the efficiency of acetoclastic methanogenesis (Liu et al., 2015). During this experiment, an increasement of *Methanosaeta* abundance was found in both NZVI group and Fe_3O_4 NPs group compared with control, especially in Fe_3O_4 NPs group. This can be explained that Fe_3O_4 stimulated the enrichment of Fe(III) reducing bacteria, which can hydrolyze refractory substrate into small molecular organic such as acetic acid (Carolina et al., 2014). Both *Methanosarcina* and *Methanosaeta* can use acetate to produce methane, the decrease of *Methanosarcina* and increase of *Methanosaeta* with iron nanoparticles addition showed that there was a competitive growth between these two acetoclastic methanogens. The results suggested that both NZVI and Fe_3O_4 NPs altered the structure of archaeal communities in AD process.

3.3.2. Variation of bacterial community

Compared with archaeal community, the bacterial community was more diverse. Besides, there were considerable microorganisms which could not be identified. Therefore, top 8 abundant phylum and top 30 abundant genus were selected for further analysis. *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Aminicenantes*, *Bacteroidetes*, *WS6*, *Acidobacteria* and *Chloroflexi* were the main bacterial phylum as shown in Fig. 3(A). *Proteobacteria* was the dominant phylum in digesters R1, R2, R4, R5, R8, R (counting for 25.9, 25.4, 18.3, 28.9, 30.5 and 27.9%, respectively), and *Firmicutes* was the dominant phylum in other digesters R3, R6 and R7 (counting for 27.6, 25.9 and 26.8%, respectively). Both of two types of bacterial phylum accounted for a relative high proportion in each digester. *Proteobacteria* is a kind of anaerobic, mesophilic and protein utilizing bacteria. It could convert organic matter, such as proteins, into short-chain fatty acids, which provided the necessary substrate for methane production (Hyun Min et al., 2014). Hence, it is easy to understand its dominance for anaerobic digestion of municipal sludge which is mainly composed of proteins. As can be seen in Fig. 3(B), the abundance of *Ochrobactrum* genus (phylum *Proteobacteria*) in digester R8 is particularly high compared to other digesters. It is reported that *Firmicutes* was able to degrade a wide range of organic compounds under a variety of conditions including refractory cellulose, proteins and pectin et al (Nelson et al., 2011), which greatly promoted

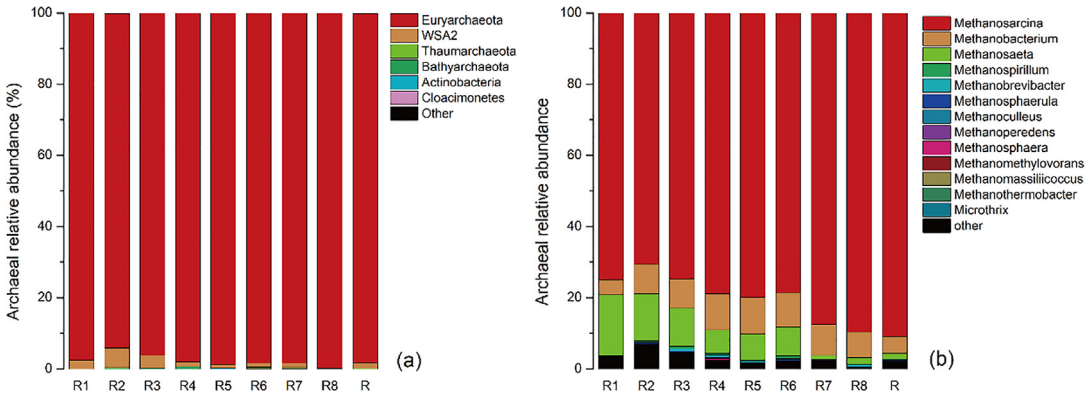

Fig. 2. Relative abundance of archaea at phylum (a) and genus (b) level in nine digesters.

the hydrolysis and fermentation of sludge. And this is one of the reasons why this bacterial phylum always carried out a major role in anaerobic digestive systems. The genus with the top three abundance were all *Firmicutes*, including *Gelria*, *Lutispora* and *Proteiniclasticum*. There was no significant fluctuation in the abundance of *Proteobacteria* and *Firmicutes* among different digesters, and both of them are acidogens and hydrolytic bacterium, which played an important role in the hydrolysis and acidification of sludge and provided sufficient substrate for the following methanogenesis. *Actinobacteria* is critical for cellulose degradation and hydrolysis, and the abundance of *Actinobacteria* for control group was about 20% higher than that of the NZVI group and Fe_3O_4 NPs group, and it was one of the largest contributors to the difference of control group and iron nanoparticles groups. *Bacteroidetes* is a type of acetogens species, which is capable of promoting the conversion of VFAs to acetate. Concerning the abundance of *Bacteroidetes*, it increased by about 68.9, 45.9, 20.3 and 40.5% for digester R5, R6, R7 and R8 as against the digester R, respectively. This may be attributed to the role of NZVI in greatly promoting the hydrolysis of organic matter in sludge, and promoting the accumulation of acetic acid (Zhen et al., 2015). *Acidobacteria* is acidophilic bacteria and played an important role in the consumption of VFAs. *Chloromonas*, mainly containing *Longilinea*, is strict anaerobic bacteria, and could degrade glucose into amino acids in anaerobic digesters (Zheng et al., 2018). As shown in Fig. 3(B), the heat map of the top 30 genus indicated that the addition of iron nanoparticles altered the dominant bacterial genus in the sludge of nine digesters.

3.4. Community correlations between dominant genus and environment factors

Redundancy analysis (RDA) was conducted to reveal the correlation between the environmental factors and the microbial communities of nine digesters, a total of 16 microbes including 13 top abundant bacteria and 3 typical archaeal at genus level, and 6 environmental factors were selected for the RDA analysis. As can be seen in Fig. 4, the first and the second axes represented 77.9% and 8.8% variation of the microbial abundance and diversity, respectively. 6 environmental factors were mainly distributed in two quadrants. As expected, VFAs, TS/VS and biogas production were clustered closely, indicating a positive correlation among them, which implied a complex relationship between substrate degradation and biogas transformation during the AD process. The increase of TS/VS value means the degradation of refractory solid organic matter in the substrate (Xu et al., 2019), and the generation of VFAs provided sufficient substrate such as acetic acid for the subsequent methanogenesis. From the results, there was no obvious positive correlation between NZVI addition and biogas production, since the NZVI addition had a critical value and did not always promote biogas production, it was also consistent with the former studies (Wang et al., 2018b). In contrast, the Fe_3O_4 NPs addition had a positive association with biogas production, this may attribute to the

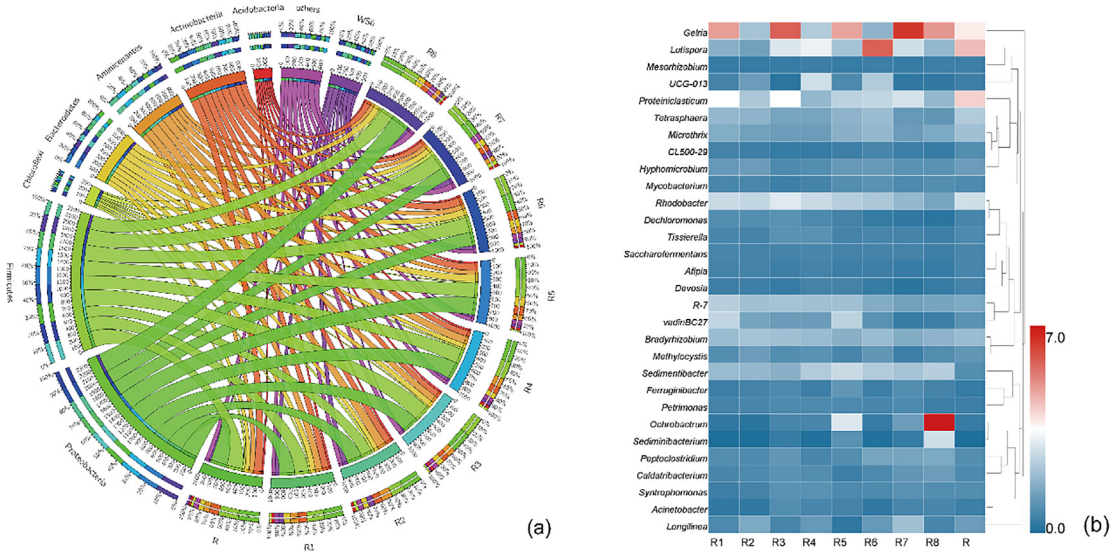

Fig. 3. The Circos graph (a) showed the distribution of bacterial community of each digester at phylum level. The heat map (b) revealed the evolution of top 30 bacterial genus in the AD process.

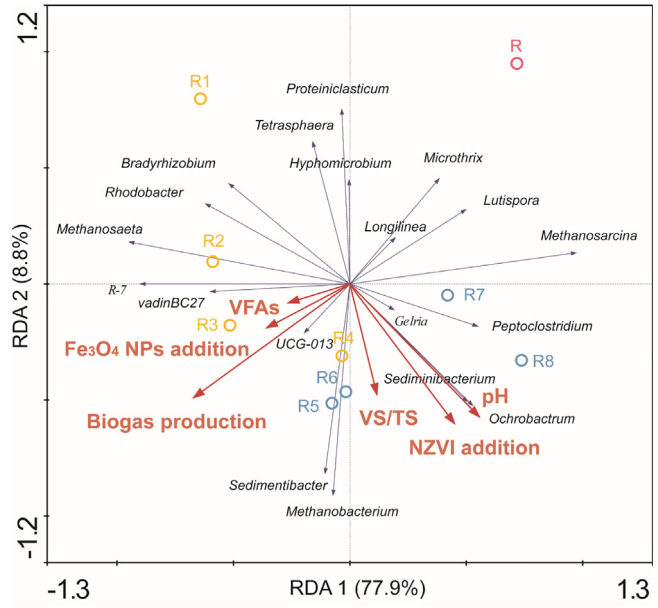

Fig. 4. Redundancy analysis (RDA) reveals the correlation between the microbial community and the environmental factors for different samples. The top 10 bacterial genus in each digester and 3 archaeal genera were selected for analysis. Different colored circles represent different groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

enhancement of biogas production by Fe_3O_4 NPs. Besides, the Fe_3O_4 NPs group had the best biogas production performance among the three groups. As shown in Fig. 4, distribution of samples appeared a significant evolution, 9 samples were well separated by group, R1, R2, R3 and R4 were clustered closely to Fe_3O_4 NPs addition, while R5, R6, R7 and R8 were distributed around NZVI addition. Additionally, sample R was separated from these two groups. The distribution of microbial communities can be also revealed in RDA results. Unlike *Methanosarcina*, *Methanosaeta* and *Methanobacterium* showed positive correlation with biogas production. As discussed above, both of them contributed a lot to the differences between two iron group and control group. Besides, as an acetoclastic methanogens, *Methanosaeta* also clustered closely to VFAs, they played an important role in converting acetic acid into methane. Interestingly, the dominant genus such as *Gelria*, *Lutispora* and *Proteiniclasticum* did not have a positive response to biogas production, while the genus of *vadinBC27* and *Ruminococcaceae_UCG-013* were located near the arrow of biogas production indicating that they had a significant effect on biogas accumulation. In addition, two genera of *Clostridia*, *Gelria* and *Peptoclostridium*, were distributed closely to NZVI addition. And *Clostridia* was reported to be able to accumulate acetic and butyric acids from the degradation of organic matter (Bao et al., 2016), indicating that NZVI could promote the hydrolysis–acidification of anaerobic digestion. The RDA analysis showed that addition of NZVI and Fe_3O_4 NPs indeed had great impacts on the clustering of microbial communities.

3.5. Changes of ARGs abundance

Untreated sludge from the wastewater treatment plants is an important repository of resistance genes, this study focused on the changes in the abundance of 5 antibiotic resistance genes (including *erm*F, *erm*T, *erm*A, *bla*OXA-1 and *aac*(6′)-IB) and the class I integron (*intI*1) with the addition of two kinds of different iron nanoparticles. Fig. 5 revealed the abundance and concentration changes of ARGs and *intI*1 during the 100-day AD process. At the beginning of the anaerobic digestion, the gene copies of *aac*(6′)-IB and *bla*OXA-1 was the highest reaching about $4.0 \pm 0.5 \times 10^{-1}$ copies/16S rRNA copies, while *erm*A had the least abundance with only $2.0 \pm 0.3 \times 10^{-5}$ copies/16S rRNA copies in the sludge. It was obvious that AD process reduced the abundance of *aac* (6′)-IB and *bla*OXA-1, whereas all three macrolide resistance genes showed an increase at different levels, including *erm*F, *erm*T and *erm*A in all digesters. The average *aac*(6′)-IB reduction rate was 96.50%, 95.83% and 94.73% in Fe_3O_4 NPs group, NZVI group and control group respectively, indicating that anaerobic digestion had a remarkable reduction function on *aac*(6′)-IB. As for the other abundant resistance gene, *bla*OXA-1 experienced an average reduction of 63.68% within 40 days, but then bounced to a higher abundance, and so did the *erm*F and *erm*A. On the contrary, the expression level of *erm*T on 60th day was more than 40 times higher than it was at the beginning, and then went down quickly to the initial level. This result indicated that sludge anaerobic digestion had different effects on the abundance of different resistance genes, it can not only promote the reduction of some specific genes, but also enrich the abundance of other genes. Obviously, the AD process stimulated the proliferation of macrolide resistance genes. Interestingly, resistance genes showed a lower abundance in the NZVI group compared with the control group at the end of the experiment, especially *erm*A and *erm*T. This suggested that the addition of NZVI had the potential to reduce the abundance of resistance genes, which may attribute to its cytotoxicity. NZVI is an active substance with strong reducibility, it can interact with key components of the cell membrane, such as functional proteins (Zhu et al., 2014), causing a damage of cell membrane and destroying the integrity of potential antibiotic resistance bacteria (ARB). This has a disadvantageous effect on the proliferation of ARGs. Besides, previous studies revealed that NZVI was functional in the removal of antibiotics, such as amoxicillin, ampicillin and metronidazole (Chen et al., 2012; Ghauch et al., 2009). And the removal of antibiotics could reduce the selection pressure of resistant genes, which contributed to the change of ARGs abundance. Differently, Fe_3O_4 NPs didn't promote the reduction of ARGs, and the abundance of *erm*T even increased. This may be because excessive electron transfer promoting the horizontal transfer of ARGs.

Generally speaking, vertical gene transfer (VGT) and horizontal gene transfer (HGT) are two main ways for the proliferation of ARGs during the anaerobic digestion process (Martinez, 2009). The VGH is always closely related to the reproduction of host microbes. And mobile integrons such as *intI*1, were the key to HGT. *intI*1 was reported to contribute to the transfer and integration of ARGs, which leading to an increased antibiotic resistance of bacterial in the environment (Didier, 2006). Ying also found that many ARGs such as *erm*B had a positive correlation with the *intI*1 (Wu et al., 2016). As can be seen in Fig. 5, although the abundance of *intI*1 had a slight increase on 40th day and then peaked at 6.25×10^{-2} copies/16S rRNA copies on 60th day, it still decreased by 51.8% on average after 100-day anaerobic digestion. Pearson correlation coefficient was calculated in order to investigate the connection of ARGs and *intI*1, While no significant correlation was observed between the five ARGs and *intI*1 in this experiment ($P > 0.05$), which showed that the horizontal gene transfer represented by the *intI*1 did not play an important role in the proliferation of ARGs compared to the vertical gene transfer way in this experiment. Hence, the addition of NZVI showed a better ARGs reduction performance, which could attribute to the destruction of host cell membrane by NZVI.

4. Conclusions

Biogas production of anaerobic digestion could be enhanced by adding NZVI or Fe_3O_4 NPs. The optimal dosage of NZVI and Fe_3O_4 NPs was 1.0 and 0.5 g/L, respectively. Antibiotic resistance gene *aac*(6′)-IB was reduced obviously (more than 94%) during the AD process in nine digesters, followed by *bla*OXA-1 decreased by 44.82% on average, but three macrolide resistance genes increased. Besides, NZVI had been shown to have the potential to remove part of ARGs from sludge. NZVI and Fe_3O_4 NPs addition can be a promising technology to enhance the

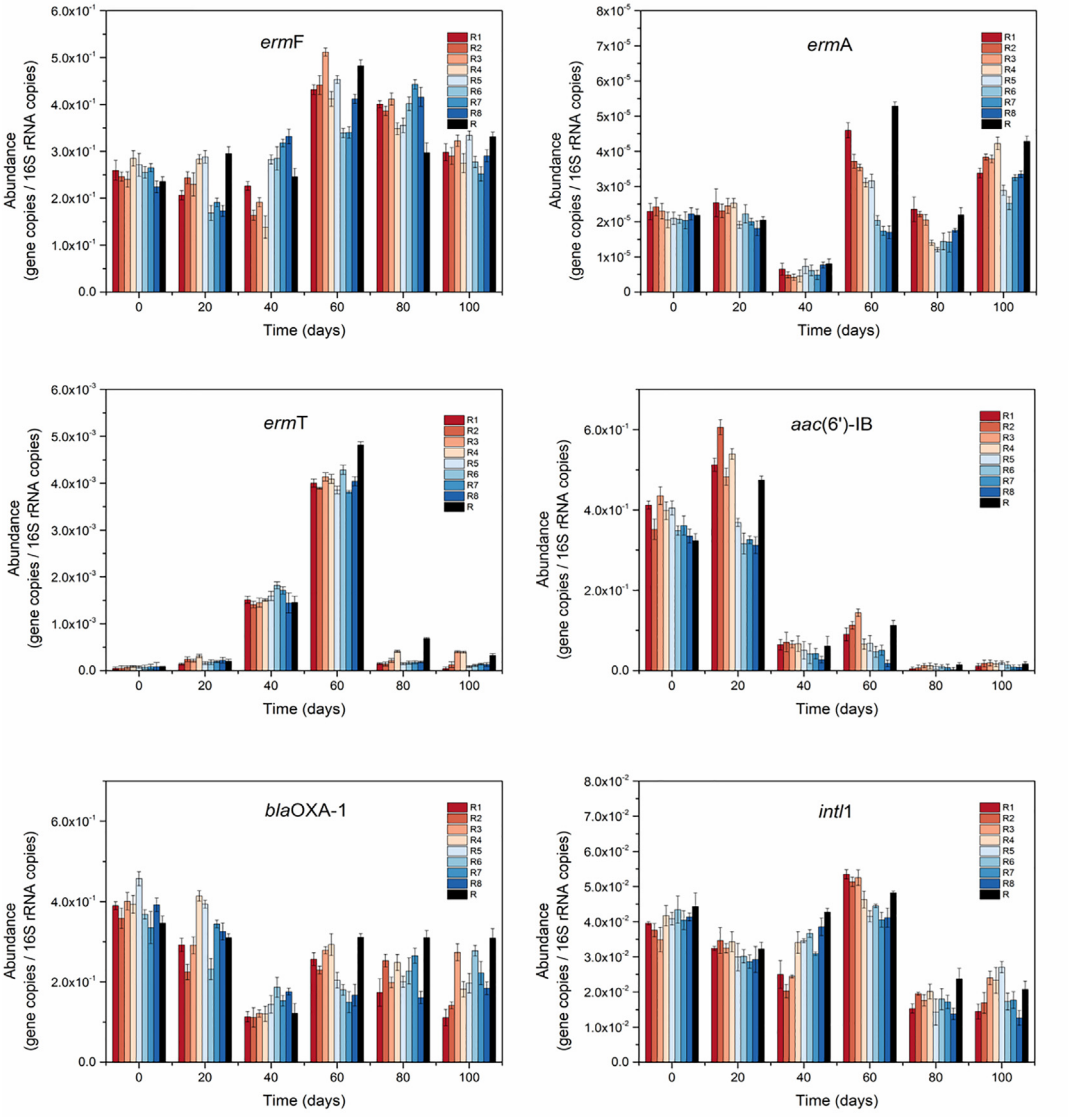

Fig. 5. Relative abundance (ARGs/16S rRNA) of macrolide resistance genes (*erm*F, *erm*T, *erm*A), aminoglycoside resistance genes (*aac*(6′)-IB), and β-lactam resistance genes (*bla*OXA-1), as well as class I integron (*intI*1) of nine digesters during the 100-day AD process.

biogas production and part of ARGs removal for sludge anaerobic digestion.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2019.122139.

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