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Effects of individual and combined zinc oxide nanoparticle, norfloxacin, and sulfamethazine contamination on sludge anaerobic digestion

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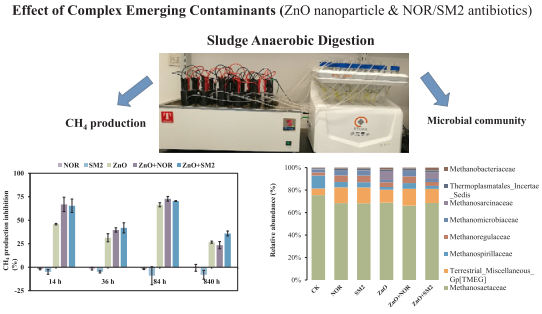
GRAPHICAL ABSTRACT



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ABSTRACT

This work investigated the individual and combined effects of zinc oxide, norfloxacin, and sulfamethazine on sludge anaerobic digestion-associated methane production, protein and carbohydrate metabolism, and microbial diversity. Norfloxacin and sulfamethazine (500 mg/kg) did not inhibit methane production, but inhibited its production rate. Zinc oxide nanoparticles with antibiotics inhibited hydrolysis, fermentation, and methanogenesis over varying digestion periods. Complex pollution had a greater impact on methane production than zinc oxide alone, with acute, synergistic toxicity to methanogenesis over short periods. Complex pollution also had varying effects on bacterial and archaeal communities during digestion. These results aid understanding of the toxicity of emerging contaminants in sludge digestion, with the potential to improve pollution removal and reduce associated risks.

1. Introduction

Two currently emerging pollutants, antibiotics and nanoparticles, have negative effects on the environment and human health (Stasinakis, 2012). Among antibiotics, sulfonamides and quinolones are frequently detected in wastewater treatment plants (WWTPs) due to their wide utilization by humans (Ivanová et al., 2018; Li et al., 2013). In WWTPs, conventional activated sludge treatment is an economical method to remove antibiotics and nanoparticles through adsorption and biodegradation by sludge (Ahmed et al., 2017; Eduok et al., 2017). In sludge samples, norfloxacin (NOR) predominates at up to 15.7 mg/kg (Cheng et al., 2014), whereas the concentration of sulfamethazine (SM2) varies from 1.0 to 69.0 μg/kg (Ashfaq et al., 2017; Chen et al., 2013; Ekpeghere et al., 2017; Li et al., 2013; Zhang and Li, 2018).

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Nanoparticles can be removed by sludge sorption and aggregation/settling, especially metal oxide nanoparticles (Li et al., 2017a), which form larger aggregates with lower negative charges than other nanoparticles (Wang et al., 2012). ZnO nanoparticles are widely used in personal care products, food additives, pigments, and biosensors (Gonzalez-Estrella et al., 2015). Choi *et al.* found a high average sorption density of ZnO onto primary sludge particulates (0.90 g/kg sludge) (Choi et al., 2018).

Treatment methods for sludge with accumulated antibiotics and nanoparticles after wastewater biological treatment are a hot research topic. Anaerobic digestion of sludge has been widely used to stabilize waste sludge and simultaneously obtain valuable products like CH_4 (Huang et al., 2017). Complex pollutants are likely to exist in the sludge anaerobic digestion system. At present, the effects of antibiotic-metal complexes in the environment can be summarized as: (1) coordination, (2) adsorption in the environment, and (3) biological impact on microbes. Bagheri *et al.* found that Pd^{2+} can form a 2:1 complex with tetracycline (Bagheri, 2015). Chlortetracycline (CTC) complexes with metals at various molar ratios: 1:1 for Mg^{2+} and Cu^{2+}, 1:0.6 for Cr^{3+}, and 1:2 for Ca^{2+} (Pulicharla et al., 2015). Wan et al. reported that tetracycline-Cd complexation increases the sorption of tetracyclines in the soil (Wan et al., 2010). At TiO_2 and ciprofloxacin concentrations of 500 and 0.5 mg/L, respectively, 53.6% of ciprofloxacin is absorbed by TiO_2 (Fries et al., 2016). Sulfamethazine-Cd complexation induces the accumulation of 6.98%–23.96% more Cd in *Phanerochaete chrysosporium* compared to the stress of Cd pollution alone (Guo et al., 2018). CTC-metal complexes are more toxic than CTC alone for Gram-positive *Bacillus thuringiensis* bacteria whereas they are similarly toxic to Gram-negative *Enterobacter aerogenes* (Pulicharla et al., 2015).

Antibiotics can inhibit cell wall production, cell membrane function, and DNA and protein synthesis (Aydin et al., 2015). Furthermore, they cause the spread of antibiotic resistance genes from WWTP facilities into the soil system due to land application (Xu et al., 2018). Nanoparticles have special characteristics due to their metal ion dissolution, nano-scale size, large surface area, and antimicrobial effects. However, the effects of the co-existence of and interactions between antibiotics and metal nanoparticles on sludge anaerobic digestion are unknown.

This study comprises the first investigation of the individual and combined effects of ZnO, NOR, and SM2 on CH_4 production, protein and carbohydrate metabolism, and bacterial and archaeal diversity during sludge anaerobic digestion. The results could aid in controlling the toxicity of complex pollution by emerging contaminants during sludge digestion, thereby improving pollution removal and reducing the risks associated with land application.

2. Materials and methods

2.1. Materials

Anaerobic sludge was collected from mesophilic anaerobic digestion reactors (35 ± 2 °C) at a full-scale sludge treatment plant in Tianjin, China. The main sludge characteristics during the sampling period were: pH 7.6; total solids, 79.4 ± 2.6 g/L; total suspended solids, 77.1 ± 2.9 g/L; volatile solids, 36.2 ± 3.1 g/L; and volatile suspended solids, 34.3 ± 1.8 g/L. Commercial NOR (> 98% purity), SM2 (> 99% purity), and ZnO nanoparticles (> 99.8% purity) were purchased from Energy Chemical (Shanghai, China), TCI Development (Shanghai, China), and Macklin Biochemical (Shanghai, China). Stock suspensions of ZnO were prepared via ultrasonication (97.5 w) with an ultrasonic JY92-IIN homogenizer (Scientz, China) for 0.5 h, resulting in an average particle size of 119.7 nm and average zeta potential of 20.7, as determined with a Malvern Zetasizer Nano ZS (Malvern Instruments, UK). All solutions were prepared using Milli-Q®-quality pure water (resistance, 18 MΩ; Elga LabWater, UK).

2.2. Digestion reactor and experiment design

Digestion was conducted in an automated methane potential testing system (RTB-BMP, RTKINS, China) with 18 channel reactors, each of which consisted primarily of a reaction flask (500 mL), mechanical mixture rotor, CO_2 absorption bottle, and CH_4 measuring system. The host computer automatically controlled the digestion temperature, mixture rate, and measurement and recording of CH_4 production with 0.1-mL accuracy. The digestion experiment was conducted according to ISO 13641-1:2003(E) with minor modifications (ISO, 2013), detailed in a previous report (Zhao et al., 2018a). In brief, the substrate contained nutrient broth (1.1 g/L peptone, 0.6 g/L NaCl, and 0.3 g/L beef powder), yeast extract (2.0 g/L), and glucose (2.0 g/L). The total solids concentration in the reaction flask was adjusted to 30.0 g/L. The efficient digestion volume was 400 mL, which was buffered using 1.0 g/L $NaHCO_3$. Nitrogen was introduced into the reaction flask for 4 min prior to initiation of the reaction to provide an anaerobic environment. The digestion temperature was maintained at 35 °C. All experiments were performed in duplicate; the detailed pollution treatment design is described in E-supplementary data.

2.3. Analytical methods

Soluble protein was analyzed by the Lowry method, using bovine serum albumin as a standard protein, and carbohydrate by the anthrone-sulfuric acid method, with glucose as the standard. The wastewater Zn^{2+} concentration was determined with an iCAP™ 7400 ICP-OES Analyzer (Thermo Fisher Scientific, USA). The volatile fatty acid (VFA) concentration was measured using a gas chromatograph (7890B, Agilent, USA) equipped with a flame ionization detector and a DB-624 column (30 m × 0.53 mm × 3.0 μm).

The modified Gompertz model (Eq. (1)) was used to simulate CH_4 production during the sludge digestion in this study.

$$P_t = P \times exp\{-exp[R_m \times e \times (\lambda - t)/p + 1]\} \quad (1)$$

Where P_t represents the cumulative CH_4 production at digestion time t (mL), P represents the predicted potential CH_4 production (mL), Rm represents the maximum CH_4 production rate, λ represents the digestion period of delay, and t represents the digestion time (h) (Zhao et al., 2018b).

2.4. Bacterial and archaeal community assessment

Total genomic DNA was extracted from sludge samples (wet weight, 300 mg) collected in the digestion reactors (March 15, 2018) using a PowerSoil® DNA Isolation Kit (Mo Bo Labs, Solana Beach, CA, USA). The V3–V4 variable regions of bacterial and archaeal 16S rRNA genes were amplified using the primer pairs 338F (5′- ACTCCTACGGGAGG CAGCA-3′)/806R (5′- GGACTACHVGGGTWTCTAAT-3′) and 349F (5′-GYGCASCAGKCGMGAAW-3′)/806R (5′-GGACTACVSGGGTATCT-AAT-′3), respectively. High-throughput sequencing was conducted by Biomarker Tech (Beijing, China) using an Illumina HiSeq 2500 (Illumina, CA, USA). After sequencing, paired-end reads were assembled with a minimum overlap of 10 bp using FLASH (version 1.2.11). Sequences with an average quality score < 20 over a 50-bp sliding window were truncated using Trimmomatic (version 0.33). Chimeras were identified and removed using UCHIME. Effective sequences were clustered into operational taxonomic units with a similarity cutoff of 97% using USEARCH (version 10.0). Finally, the taxonomy of sequences was analyzed using an RDP classifier (version 2.2) against Silva databases (Release 128). Community and adversity comparisons were primarily determined using the absolute and relative abundances of microbes at different levels through alpha diversity, beta diversity, and mean difference analysis.

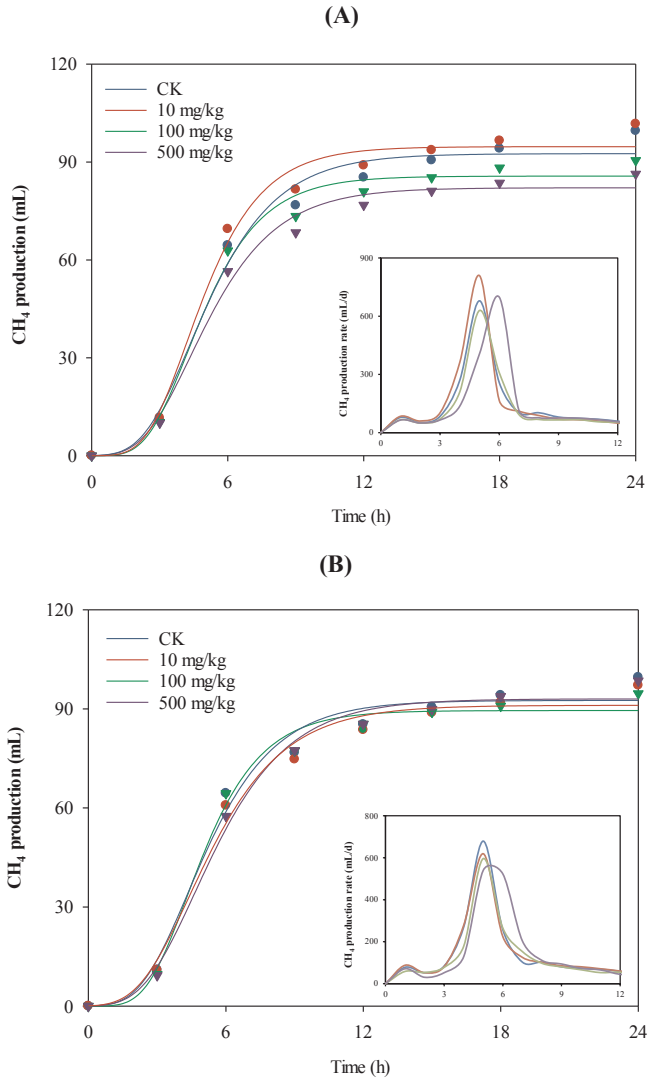

Fig. 1. Effects of NOR (A) and SM2 (B) concentrations on CH_4 production and rates (insert) during sludge digestion. CK means control treatment.

3. Results and discussion

3.1. Effects of individual and complex contaminants on methane production during sludge digestion

3.1.1. Effects of norfloxacin or sulfamethazine

The individual effects of NOR and SM2 on CH_4 production were first examined during sludge anaerobic digestion (Fig. 1). CH_4 production in the presence of NOR did not significantly differ from that in the control group after 24 h of anaerobic digestion ($p > 0.05$). On the other hand, the CH_4 production rate was inhibited at the higher concentrations of NOR. The highest concentration of NOR (500 mg/kg) delayed the peak CH_4 production rate by 1 h; however, the peak in the high concentration group was similar to that in the low concentration group. Three concentrations of SM2 showed similar trends. No obvious effects of SM2 were observed on total CH_4 production, but the highest concentration (500 mg/kg) inhibited its production rate.

In the previous study, ZnO nanoparticles (30 mg/g dry weight of sludge) delayed the production of CH_4 until 12–15 h after the initiation of the reaction and reduced peak production (Zhao et al., 2018a). Li *et al.* studied the effects of the fluoroquinolone antibiotics ofloxacin, NOR, ciprofloxacin, and lomefloxacin (2–100 mg/L) on anaerobic sludge digestion and found that CH_4 production was slightly inhibited

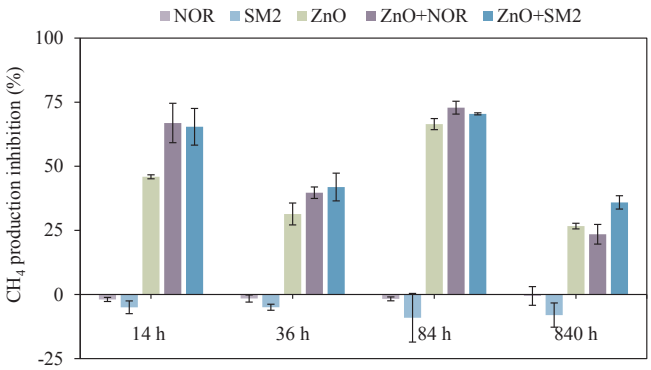

Fig. 2. Inhibition of CH_4 production in sludge digestion by individual and complex pollution compared to control conditions.

by the higher concentrations of antibiotics (Li et al., 2017b). Mitchell *et al.* found no evidence of changes in CH_4 production in the presence of SM2 (0.28–280 mg/L) during anaerobic digestion of cattle manure (Mitchell et al., 2013). In this study, the addition of NOR and SM2 antibiotics (up to 15 mg/L) did not significantly inhibit ($p > 0.05$) CH_4 production, which was consistent with the previous literature. However, significant acute inhibition by NOR and SM2 was observed on the CH_4 production rate ($p < 0.05$).

3.1.2. Pollutant complexes

The rate of the inhibition of CH_4 production in the presence of individual and complex pollutants was investigated over different lengths of digestion (Fig. 2). NOR did not significantly affect CH_4 production ($p > 0.05$), whereas SM2 slightly promoted production by 5.0%–9.1% ($p > 0.05$) over the course of the digestion. At 14 h, ZnO inhibited production by 45.9%. As the sludge microbes adapted to the toxic environment, their methanogenic capacity continued to recover and the inhibition rate of ZnO decreased to 31.4% at 36 h. After 84 h, the inhibition rate rose to 74.9% due to the continuous inhibition of CH_4 production in the ZnO treatment group and the increasing CH_4 production rate in the control group. After 35 days, the final inhibition rate in the presence of ZnO was only 26.7%.

At 14 h, the CH_4 inhibition rates in the ZnO + NOR and ZnO + SM2 groups were 66.9% and 65.4%, respectively, which exceeded the sums of the rates in the individual ZnO and antibiotic groups by 22.9% and 26.0%, respectively. At 36 h, the ZnO + NOR and ZnO + SM2 inhibition rates dropped to 39.7% and 41.9%, respectively. The decreasing effects of the complex pollutants were likely mainly due to the partial recovery of methanogenic activity in the reactions. There were no significant effects of either individual antibiotic after 84 h, but ZnO + SM2 inhibited CH_4 production by 17.3% more than the sum of individual ZnO and SM2 after 35 days.

Comparison of the inhibition rates of the complexes with those of the individual pollutants revealed that complex pollution with ZnO + NOR or ZnO + SM2 synergistically inhibited CH_4 production in the short term, although the effect was continuously reduced with the extension of digestion time. Coordination, adsorption, and competition for binding sites form the main complex mechanism for the complex pollution in the environment. In this study, the effects of the complexes may be related to the interactions of ZnO with the antibiotics via coordination. As described in the E-supplementary data, at 14 h, the Zn^{2+} concentrations in the ZnO + NOR and ZnO + SM2 groups increased by 22.7% and 22.3%, respectively, compared to that in the individual ZnO group. The increases in Zn^{2+} concentration could explain the significantly higher toxicities of ZnO + NOR and ZnO + SM2 compared to ZnO. The findings indicated that Zn^{2+} could be rapidly dissolved from ZnO in the ZnO + NOR and ZnO + SM2 conditions, probably due to Zn^{2+} and NOR or SM2 coordination reaction equilibrium. Thus, toxicity was accordingly strengthened by the increasing

Table 1
Changes in protein and carbohydrate degradation inhibition rates during sludge digestion compared with control condition (%, mean ± SD).

Substrate	Digestion time (h)	NOR	SM2	ZnO	ZnO + NOR	ZnO + SM2
Protein	14	0.5 ± 0.5	1.9 ± 0.9	28.3 ± 0.8	26.0 ± 2.7	34.3 ± 4.3
	36	−3.4 ± 1.1	−2.7 ± 0.0	7.7 ± 0.5	7.5 ± 1.1	6.1 ± 1.1
	84	3.7 ± 1.2	−0.2 ± 0.4	1.9 ± 0.6	2.5 ± 0.8	−1.3 ± 0.2
	840	0.4 ± 0.1	0.9 ± 0.5	1.7 ± 0.6	0.9 ± 0.1	2.9 ± 0.2
Carbohydrate	14	−0.3 ± 0.0	−0.1 ± 0.0	−0.3 ± 0.1	4.6 ± 0.3	6.3 ± 0.1
	36	−2.8 ± 0.1	−2.0 ± 0.2	−2.6 ± 0.2	−2.0 ± 0.1	−2.0 ± 0.1
	84	0.1 ± 0.0	0.1 ± 0.0	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
	840	0.8 ± 0.1	−0.1 ± 0.4	0.3 ± 0.5	0.3 ± 0.4	0.5 ± 0.4

Zn^{2+} and coordination product concentrations in the presence of NOR or SM2.

Chlortetracycline (CTC) complexes with the metals Mg^{2+}, Cu^{2+}, and Cr^{3+}, which are more toxic than CTC alone for Gram-positive *Bacillus thuringiensis* bacteria, due to the uptake of the metal complexes (Pulicharla et al., 2015). Complexes of antibiotics and Ag nanoparticles increased by 0.1–5.3 times (ciprofloxacin) and 3.0–6.1 times (trimethoprim) antimicrobial activity against *Bacillus* spp., due to the interactions by chelation between active groups, like hydroxyl and amino groups in antibiotics with Ag nanoparticles (Naqvi et al., 2013).

3.2. Effects of emerging contaminants on metabolism during sludge anaerobic digestion

3.2.1. Protein and carbohydrate degradation

During sludge anaerobic digestion, microorganisms mainly use carbohydrates and proteins as substrates for metabolic activities; they decompose organic matter into small molecules such as volatile organic acids, which are converted into CH_4 by methanogens and participate in hydrolysis, fermentation, acetogenesis, and methanogenesis (Chen et al., 2014; Li et al., 2018). Table 1 shows the protein degradation inhibition rates of the different pollutant treatments. Within 840 h, neither individual NOR nor SM2 significantly affected protein degradation. The inhibition rate of individual ZnO decreased with digestion time; it was highest (28.3%) at 14 h and decreased to 7.7% after 36 h. After 84 h, ZnO did not significantly inhibit protein degradation. The protein inhibition rates of ZnO + NOR and ZnO + SM2 did not significantly differ from that of individual ZnO at 14 h ($p > 0.05$). The inhibition caused by the complex pollutants rapidly decreased to 7.5% and 6.1% for ZnO + NOR and ZnO + SM2, respectively, by 36 h. Compared with proteins, carbohydrates are more easily decomposed by bacteria via hydrolysis and fermentation (Ahring et al., 2015). At 14 h, reactions in the presence of individual NOR, SM2, and ZnO showed similar rates of carbohydrate degradation as the control reaction, but those in the presence of complex pollutants had significantly lower rates of carbohydrate degradation (by 4.6% and 6.3% for ZnO + NOR and ZnO + SM2, respectively; $p < 0.01$). After 36 h, no inhibitory effects were observed for any of the treatments. These results showed that neither individual NOR nor SM2 significantly affected protein and carbohydrate degradation. However, ZnO and its complexes with the antibiotics significantly inhibited protein and carbohydrate degradation. Complex pollution showed significant additional inhibition of carbohydrate degradation compared to ZnO pollution alone.

3.2.2. Volatile fatty acids

The main products of protein and carbohydrate hydrolysis, VFAs, including acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid and isovaleric acid, are important products and intermediates that affect anaerobic digestion (Lu et al., 2014). The VFA concentrations were measured after various lengths of digestion (Fig. 3). The control reaction and those in the presence of individual antibiotics rapidly produced VFAs within 14 h, whereas the reactions in the presence of individual ZnO and complex pollutants formed significantly lower

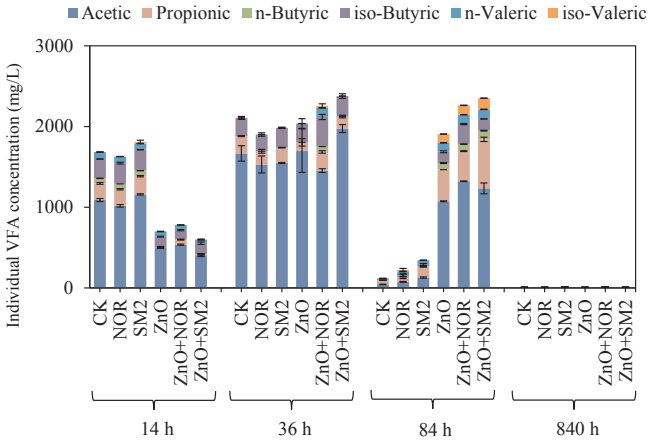

Fig. 3. Effects of different pollution treatments on individual VFA concentrations in sludge digestion.

concentrations of VFAs. At 14 h, the inhibition rates of ZnO + NOR or ZnO + SM2 did not significantly differ from that of individual ZnO ($p > 0.05$). This finding indicated the complex pollutants did not have significantly greater effects on bacterial hydrolysis and fermentation than individual ZnO, although they had toxic synergistic effects on methanogenesis. By 36 h, VFA generation by bacterial hydrolysis and fermentation was restored in all treatment groups, whereas bacterial methanogenesis was still inhibited by individual ZnO and complex pollution. At 84 h, the VFAs in the control and individual antibiotic treatment groups had been largely consumed to produce CH_4, resulting in much lower concentrations of VFAs. However, the VFA concentrations in the individual ZnO and complex pollution groups were maintained due to the continuous inhibition of methanogenic bacteria. In addition, the VFA levels in the complex pollution groups were significantly higher than that in the individual ZnO group ($p < 0.01$), which was consistent with their greater inhibition of CH_4 production. After 35 days, all VFAs were consumed and the VFA concentrations returned to their initial levels. Stone *et al.* previously found that the inhibition of CH_4 production by CTC was mainly due to hindering the ability of hydrogenotrophic or acetoclastic methanogens to use VFAs, rather than the conversion of proteins and carbohydrates to VFAs by hydrolytic-fermentative bacteria (Stone et al., 2009).

3.3. Microbial communities

3.3.1. Bacteria

45 bacterial phyla were detected in the sludge digestion samples after 35 days. *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, WS6, and *Chloroflexi* were the most highly represented phyla in all sludge samples (Fig. 4A). Similarly high abundances of *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* were found in anaerobic swine manure digestions (Song et al., 2017). These 3 bacterial groups are usually found in anaerobic digesters due to their capacity to decompose a wide range of substrates

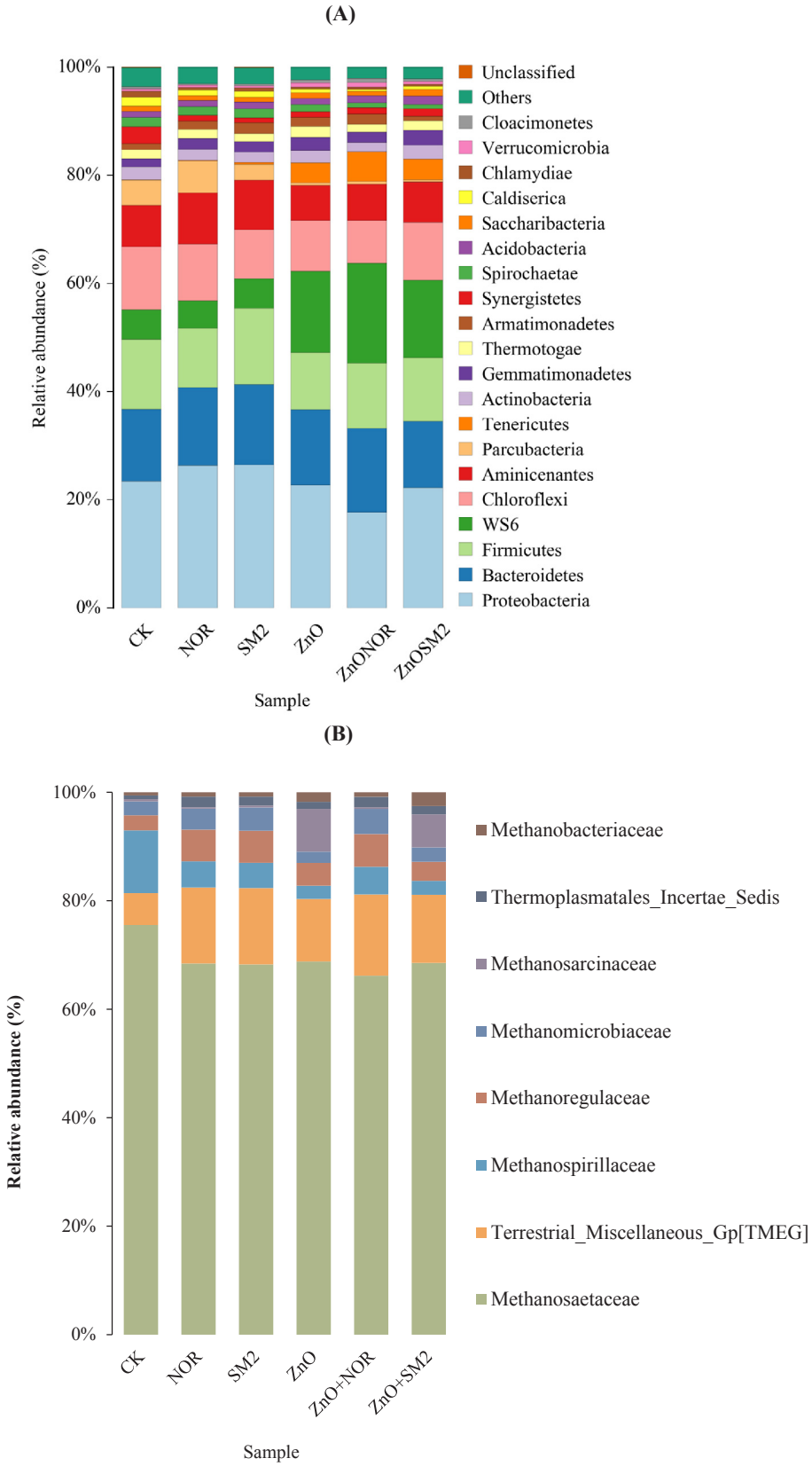

Fig. 4. Relative abundance (A: bacteria at phylum level; B: archaea at family level) of microbial community in digested sludge after 35 days.

(Lim et al., 2018). The proportions of these groups in the ZnO individual and complex treatments were lower than in the control and individual antibiotic treatment groups, demonstrating the inhibition of the growth of hydrolytic and fermentative bacteria. Compared with the control group, the ZnO, ZnO + NOR, and ZnO + SM2 groups had altered proportions of *Parcubacteria* (−90.0%, −88.6%, and −90.8%), *WS6* (+176.4%, +237.8%, and +162.0%, respectively), and *Tenericutes* (+2813.1%, +4213.7%, and + 2902.8%, respectively)

Table 2
Inhibition (−) and Promotion (+) ratios (%) of different treatments on the relative abundance of selected bacterial and archaeal in sludge digestion compared to control conditions.

Phylum	Class	Order	Family	Pollution treatments				
				NOR	SM2	ZnO	ZnO + NOR	ZnO + SM2
Proteobacteria	*Betaproteobacteria*	*Hydrogenophilales*	*Hydrogenophilaceae*	+34.5	−5.0	−50.7	−64.1	−57.8
		Desulfovibrionales	*Desulfomicrobiaceae*	+25.6	+45.9	−81.4	−78.8	−84.6
	Deltaproteobacteria	*Syntrophobacterales*	*Syntrophaceae*	+16.9	+36.6	−47.1	−41.1	−50.4
	Gammaproteobacteria	*Xanthomonadales*	*Xanthomonadaceae*	+7.1	−0.9	−7.0	−35.8	−23.9
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	+6.2	+19.8	+168.1	+188.3	+397.0
	Sphingobacteriia	*Sphingobacteriales*	*Lentimicrobiaceae*	−11.5	−5.7	−86.2	−71.5	−93.0
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	−32.6	−0.3	+105.8	+128.7	+138.1
	Negativicutes	*Selenomonadales*	*uncultured_bacterium_o_Selenomonadales*	−75.8	−66.4	+220.6	+236.1	+55.0
WS6				−6.8	−0.7	+176.4	+237.8	+162.0
Chloroflexi	*Anaerolineae*	*Anaerolineales*	*Anaerolineaceae*	+3.8	−16.3	−13.7	−26.0	−2.4
Parcubacteria				+28.0	−35.5	−90.0	−88.6	−90.8
Tenericutes				−6.8	+132.9	+2813.1	+4213.7	+2902.8
Euryarchaeota	*Methanobacteria*	*Methanobacteriales*	*Methanoregulaceae*	+109.9	+112.8	+50.8	+116.4	+26.1
	Methanomicrobia	*Methanomicrobiales*	*Methanobacteriaceae*	+36.0	+38.1	+203.2	+43.3	+337.2
			Methanospirillaceae	−58.3	−59.9	−79.0	−55.9	−77.7
			Methanomicrobiaceae	+52.0	+68.8	−18.0	+83.1	+4.1
		Methanosarcinales	*Methanosaetaceae*	−9.4	−9.6	−8.9	−12.4	−9.3
			Methanosarcinaceae	−39.9	−19.0	+1693.2	−31.8	+1287.8
	Thermoplasmata	*Thermoplasmatales*	*Terrestrial_Miscellaneou_Gp[TMEG]*	+138.0	+139.5	+96.2	+154.6	+113.6
			Thermoplasmatales_Incertae_Sedis	+191.3	+141.8	+91.6	+183.9	+124.7

($p < 0.05$). At the phylum level, ZnO + NOR showed strong synergistic toxicity to *Proteobacteria* and *Chloroflexi*, whereas ZnO + SM2 showed strong synergistic toxicity to *Bacteroidetes*.

The analysis of the different levels of bacteria is shown in Table 2. The dominant classes in all samples were *Betaproteobacteria*, *Bacteroidia*, *Clostridia*, and *Anaerolineae*, which belong to the phyla *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Chloroflexi*. The important VFA consumers *Betaproteobacteria*, *Deltaproteobacteria*, and *Gammaproteobacteria* were the main *Proteobacteria* classes (Ping et al., 2018). At the family level, ZnO inhibited *Desulfomicrobiaceae* and *Syntrophaceae* by 81.4% and 47.1%, respectively, compared to the control conditions, whereas the complex pollutants synergistically inhibited the families ($p < 0.01$).

As fermentative bacteria, *Bacteroidetes* play important roles in the degradation of carbohydrates and proteins into acetate and NH_3 (Tang et al., 2016). However, at the family level, *Porphyromonadaceae* was significantly more abundant in the individual ZnO and complex treatment groups than in the control ($p < 0.01$).

Firmicutes are hydrolytic bacteria; *Clostridia* and *Negativicutes* belong to *Firmicutes* and are important microbes for the degradation of organic matter (Ping et al., 2018). Both *Ruminococcaceae* and *unculture-d_bacterium_o_ Selenomonadales* were more abundant (by 55.0%–236.1%) in the individual ZnO and complex pollutants groups than in the control. They may have become tolerant to the anaerobic environment and even proliferated after long-term incubation (Eduok et al., 2017).

In *Chloroflexi*, *Anaerolineae* is a gas-forming bacteria that is responsible for the hydrolysis and fermentation of organic matter (Zhao et al., 2018c). It was less abundant in the ZnO + NOR group than in the other treatment groups.

These results showed that individual ZnO and complex pollutants had significant effects on functional bacteria, including hydrolytic, fermentative, and VFA-consuming bacteria. However, ZnO, ZnO + NOR, and ZnO + SM2 had different additional effects on the inhibition or promotion of the bacteria in the digestion system.

3.3.2. Archaea

The archaeal phylum *Euryarchaeota*, a well-known microorganism involved in biogas production, was identified in the digestions (Fig. 4B). It has been previously identified as the predominant core microbe in digesters (Fitamo et al., 2017). *Methanosaetaceae*, which belong to the acetoclastic methanogens of order *Methanosarcinales*, were dominant at the family level (accounting for 66%–76% of total archaea in different treatments). All pollutant treatments had lower proportions of *Methanosaetaceae* (NOR: −9.4%, SM2: −9.6%, ZnO: −8.9%, ZnO + NOR: −12.4%, and ZnO + SM2: −9.3%) than the control condition, contributing to the significant inhibition of CH_4 production. Another acetoclastic methanogen, *Methanosarcinaceae*, was significantly more abundant in the individual ZnO (+1693.2%) and ZnO + SM2 (+1287.8%) groups than in the control, but was significantly less abundant in the ZnO + NOR (−31.8%) group.

Most hydrogenotrophic methanogens, such as *Terrestrial_Miscellaneous_Gp[TMEG]* and *Methanoregulaceae* (the second most abundant archaea at 15.7%–21.0%) were more abundant in the individual and complex pollution conditions than in the control; however, ZnO + SM2 strongly inhibited those families compared to ZnO + NOR. The family *Methanospirillaceae* (the third most abundant archaea at 2.4%–5.1%) was inhibited in all pollution treatment groups, but was significantly stronger in the ZnO and ZnO + SM2 groups.

3.3.3. Principal coordinates analysis

The differences in bacterial and archaeal communities under the different treatments were revealed by principal coordinates analysis (Fig. 5). All bacterial samples (Fig. 5A) could be divided into 3 groups along the principal coordinate 1 and principal coordinate 2 vectors: (1) control, NOR, and SM2; (2) ZnO + NOR; and (3) ZnO and ZnO + SM2. The communities in the control, NOR, and SM2 treatment groups were similar, which was consistent with the non-significant differences in CH_4 production among the them (Fig. 1). The ZnO + NOR community differed greatly from the ZnO and ZnO + SM2 communities, suggesting strong changes in microbial community structure upon complex pollution exposure, which was consistent with the changes in bacterial richness (Table 2).

Archaea were more sensitive to exposure to these emerging contaminants than bacteria (Fig. 5B). The control archaeal community significantly differed from the communities of the pollution treatment groups, which could be divided into 3 groups: (1) control; (2) NOR, SM2, and ZnO + NOR; and (3) ZnO and ZnO + SM2. This finding that indicated (1) significant effects of these 2 types of pollutants on archaeal communities; (2) that ZnO + NOR showed similar toxicity to NOR, whereas ZnO + SM2 showed similar toxicity to ZnO; and (3) that

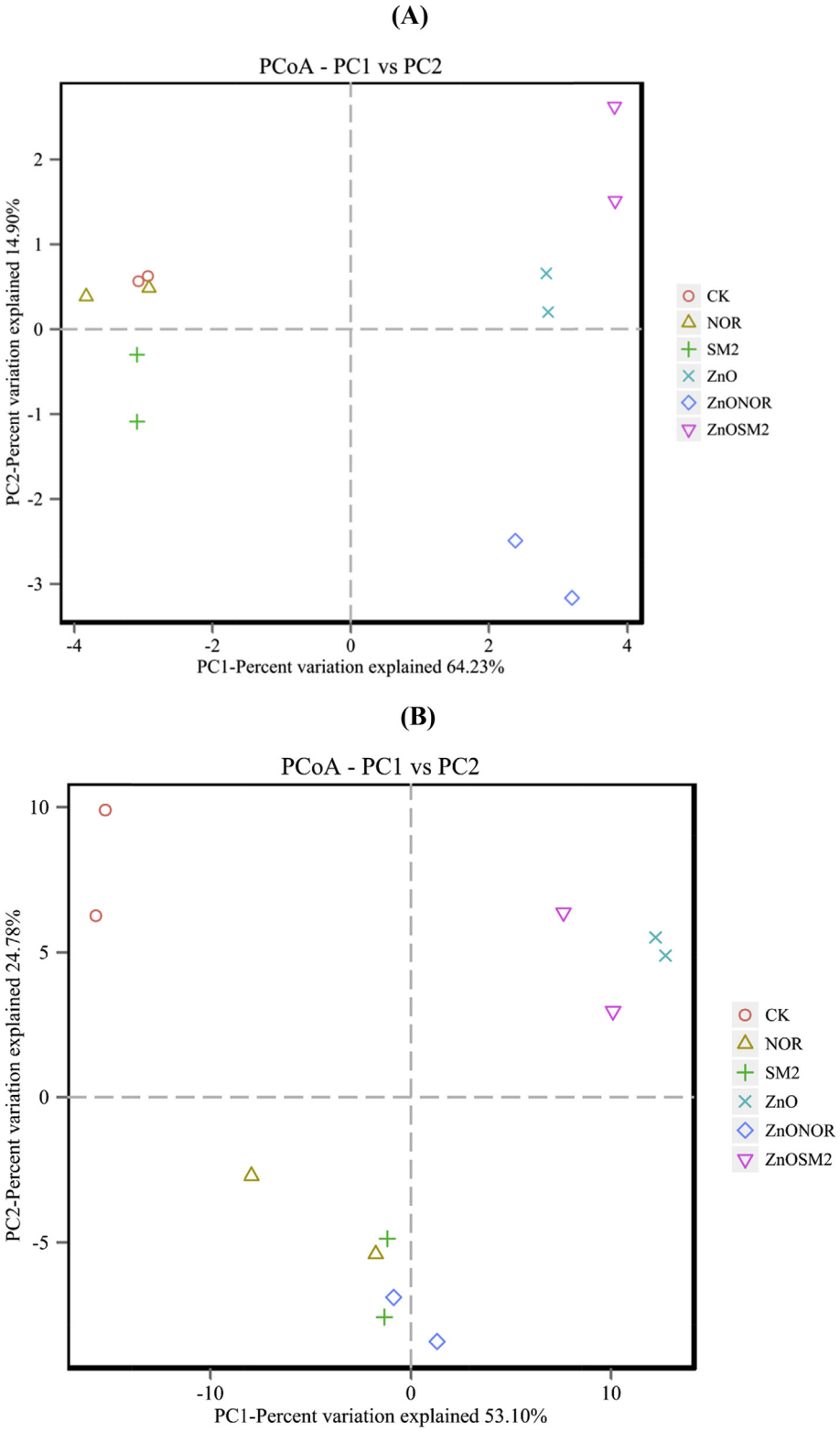

Fig. 5. Principal coordinates analysis (PCoA) analysis based on OTU abundance for microbial in digested sludge under different treatments after 35 days.(A: bacteria; B: archaea).

ZnO + NOR showed different complex effects from ZnO + SM2. These observations were consistent with the differences in CH_4 production among the groups (Fig. 2) and the effects of both complex pollutants on the inhibition or promotion of archaeal communities (Section 3.3.2).

These results showed that complex pollution causes varied additional effects on bacterial and archaeal communities. ZnO + NOR had greater effects on the inhibition or promotion on bacteria compared to individual ZnO, whereas it had lesser effects on the inhibition or promotion of archaea compared to individual ZnO. ZnO + SM2 showed similar effects to individual ZnO on both types of microbes.

4. Conclusions

NOR and SM2 did not inhibit overall CH_4 production, but high concentrations inhibited its production rate. ZnO + SM2 and ZnO + NOR showed synergistic toxicity to CH_4 production at early time. Complex pollutants more persistently inhibited methanogenesis than hydrolysis, fermentation, or acetogenesis during digestion. ZnO + NOR and ZnO + SM2 had differential effects on bacteria and archaea in the digestions. Complex effects were likely related to the interactions of ZnO with the antibiotics, such as coordination and promotion of Zn^{2+} dissolution. These results could aid in developing toxicity controls for complex pollution by emerging contaminants in sludge digestion, thereby improving pollution removal and reducing pollution-related risks.

Conflicts of interest

The authors declare no financial/commercial conflicts of interest.

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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.2018.11.049.

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