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Effects of individual and complex ciprofloxacin, fullerene C_{60}, and ZnO nanoparticles on sludge digestion: Methane production, metabolism, and microbial community

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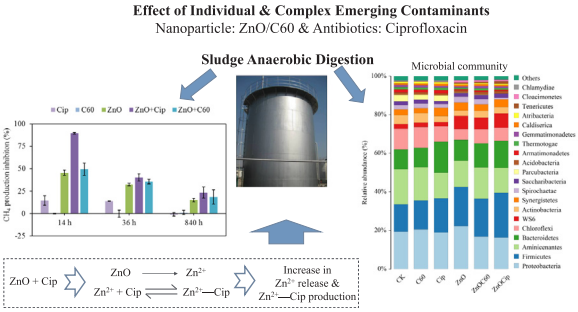
GRAPHICAL ABSTRACT



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ABSTRACT

Antibiotics and nanoparticles, emerging contaminants, present great environmental risks and human health concerns. Sludge adsorption, a biological wastewater treatment removal mechanism, targets ciprofloxacin (Cip) antibiotics, C_{60}, and ZnO, leaving complex pollution in sludge anaerobic digestion. This study investigated the mechanisms through which individual and combined ZnO, Cip, and C_{60} affect sludge anaerobic digestion by studying their effects on CH_4 production, metabolism, and microbial community. ZnO was generally more toxic to CH_4 production than Cip. The ZnO + Cip complex was more influential (> 29%) than ZnO or ZnO + C_{60}, with short-lasting acute and additive toxicity effects on methanogenesis and degradation of protein and carbohydrate. ZnO + C_{60} and ZnO + Cip exerted apparent additional complex effects on *Firmicutes*, *Aminicenantes*, *Chloroflexi*, and *Parcubacteria*. These results would potentially aid toxicity control related to complex pollution, and improve energy production and reduce pollution risks when used in land applications.

1. Introduction

Antibiotics and nanoparticles (NPs), two types of contaminants that have emerged in recent years, are causing great environmental risk and human health concerns (Ivanová et al., 2018). Fluoroquinolones are a class of broad-spectrum antibiotics used in human and veterinary medicine and farming, and ciprofloxacin (Cip) is a second-generation fluoroquinolone with very strong and long-term antimicrobial activity.

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Increased antibiotic usage may create an abundance of antibiotic-resistant genes in the environment, increasing bacterial resistance (Luo et al., 2010). Calero-Cáceres et al. detected quinolone (*qnr*A, *qnr*S) resistance genes in the water and sediment of the Mediterranean river (Calero-Cáceres et al., 2017).

Unlike antibiotics, NPs toxicity in microorganisms is primarily caused by the release of metal ions, production of reactive oxygen species (ROS), and direct cell damage. One widely used carbon-based nanoparticle, C_{60}, could cause an increase in superoxide dismutase (SOD) activity in *Bacillus* and subsequently inhibit growth and reduce the respiration rates of *Bacillus subtilis* and *Escherichia coli* (Huang et al., 2014). The potential elution of Zn^{2+} from ZnO, a metal oxide NP, may induce the production of ROS in microorganisms. Beyond Zn^{2+} elution and ROS production, ZnO NPs can directly damage cell walls, increasing cell membrane permeability and resulting in the inhibition of bacteria growth (Kumar et al., 2011).

Such emerging antibiotic and NP contaminants have been widely detected in wastewater and sludge (Demirel, 2016; Yang et al., 2016). Sludge adsorption is a major mechanism for removing antibiotics and nanoparticles during commonplace biological wastewater treatments (Kunhikrishnan et al., 2015). Pollutants demonstrate concentration levels of up to 31.0 mg/L, up to 20 μg/L, and 0.1–8.6 mg/L in terms of Cip (Guo et al., 2018; Rodriguez-Mozaz et al., 2015), C_{60} (Emke et al., 2015; Farré et al., 2010), and ZnO (Choi et al., 2017; USEPA 2009) within municipal and industrial wastewaters. In municipal sludge, Cip concentrations could reach up to ~13.8 mg/kg with a > 95% removal primarily due to sludge adsorption (Ivanová et al., 2017). Furthermore, at sludge concentrations of 2000 mg/L, 74% of C_{60} nanoparticles can be removed via adsorption (Yang et al., 2013). Choi et al. found ~80% of ZnO NPs were removed via primary and secondary sludge adsorption in conventional municipal biological treatment processes (Choi et al., 2017).

Once these pollutants have accumulated in the sludge at wastewater treatment plants (WWTP), excess sludge treatment becomes highly important. Anaerobic sludge digestion is a widely used, harmless sludge pretreatment process used in land applications to control the discharge of related pollutants into the environment and energy production (Feng et al., 2015). With regards to anaerobic digestion sludge treatment, a treatment capacity of 120–600 ton dry weight (DW) sludge/day corresponds to an operational cost of approximately $ 80–120 per ton of DW sludge. At the same time, the treatment could produce a benefit of $ 52–139 per ton of DW sludge from the electricity produced from methane and save transport costs of the order of $ 40–93 per ton of DW sludge owing to sludge-volume reduction (Yang et al. 2015a).

Since Cip and NPs are primarily removed by sludge adsorption during wastewater treatment, complex pollution from them surely exists in the sludge anaerobic digestion system. Some previous studies have attempted to illuminate the complex effects of antibiotics and metal ions on the environment. Their findings can be summarized into three core discoveries: (1) coordination between pollutants. Bagheri et al. found that Pd^{2+} can form a 2:1 complex with tetracycline (Bagheri, 2015), (2) interactive effects on biodegradation due to the toxicity towards microorganisms. Lu et al. found the root number of *Eichhornia crassipes* was reduced by 21% with tetracycline single stress; however, the root number decreased by 39% under complex stresses from Cu^{2+} and tetracycline, showing significant enhancements in toxic effect (Lu et al., 2014). During the wastewater treatment process, complex pollution from chlortetracycline and metal ions (Ca^{2+}, Mg^{2+}, Cu^{2+}) could increase the toxicity of gram-positive bacteria in the sludge, but failed to significantly affect gram-negative bacteria (Pulicharla et al., 2015), and (3) interactive influencing of adsorption on environmental media via hydrophobic and electrostatic forces. Cd^{2+}, Cu^{2+}, and Pb^{2+} can significantly enhance the adsorption of tetracycline in soil, correlating with their coordination (Zhao et al., 2013)

Since NPs are characterized by nanoparticle sizes, large surface areas, metal ions releasing, and antimicrobial effects, they have great potential to create strong complex effects with antibiotics through coordination, adsorption, degradation and other means. However, the method by which these individual and complex processes affect sludge digestion remains unclear. This study, considering concentrations occurring in activated sludge, took ZnO, C_{60}, and Cip as model metal NPs, carbon NPs, and antibiotics. The aim was to investigate the mechanisms through which individual and combined ZnO, Cip, and C_{60} affected sludge anaerobic digestion by studying effects on the CH_4 production, metabolism, and microbial community. To our best understanding, this represents the first attempt to study the complex effects of these nanoparticles and Cip antibiotics on sludge digestion. These results could thus aid toxicity control for complex pollution created by emerging contaminants in sludge digestion, improving energy production and reducing pollution risks once used in land applications.

2. Materials and methods

2.1. Anaerobic sludge

Anaerobic sludge was collected from mesophilic anaerobic digestion reactors running at a treatment capacity of 800 ton/day, SRT ~ 20 day, and 35 ± 2 °C located in a full-scale sludge treatment plant in Tianjin city, China. The main sludge characteristics during the sampling period were: pH 7.58, total solid 80.57 ± 8.85 g/L, total suspended solid 75.69 ± 5.56 g/L, volatile solids 39.94 ± 8.51 g/L, and volatile suspended solid 31.62 ± 5.46 g/L.

2.2. Preparation of ZnO NPs, C_{60} NPs, and Cip solution

Commercial antibiotic Cip (> 98% purity), ZnO NPs (> 99.8% purity) and C_{60} (> 99.9% purity) were purchased from Macklin biochemical Co., Ltd, China. Stock suspensions of ZnO and C_{60} were prepared via ultra-sonication (97.5 w) using an ultrasonic homogenizer JY92-IIN (Scientz, China) for 0.5 h and 20 h, respectively. The average particle sizes of ZnO and C_{60} in the stock suspensions were measured as 119.7 and 129.5 nm, respectively. Their zeta potentials were 20.7 and −16.7 mV using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK). All solutions were prepared using Milli-Q quality pure water (18 MΩ resistance, Elga Ultrapure, UK).

2.3. Digestion reactor and exposure experiments

The digestion process was conducted using an automated methane potential testing system (RTK-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 amounts with 0.1 ml accuracy.

The digestion experiment was conducted according to ISO 13641–1, 2003 (E) with minor modifications (ISO, 2013). The substrate contained nutrient broth, yeast extract, and glucose, each at 2.0 g/L concentration. A 1.0 g/L $NaHCO_3$ buffer was added to the reactors, preventing abrupt pH changes during the sludge process. The total solids concentration in the reaction flask was adjusted to 30.0 g/L. Nitrogen was introduced into the reaction flask for 4 min before the reaction began to create an anaerobic environment. The digestion temperature was maintained at 35 °C using the above system. The amount of spiked model pollutants varied across a wide range, covering their concentrations common in municipal and industry sludges. All experiments were performed in duplicate in this study.

2.4. Analytical methods

pH was measured with a pH meter (Mettler-Toledo, Switzerland).

Soluble protein and carbohydrate concentrations were analyzed with the Lowry method using bovine serum albumin as a standard protein and the anthrone-sulfuric method with glucose as standard. The volatile fatty acid (VFA) concentration was measured using a gas chromatograph (Agilent, 7890B) equipped with a flame ionization detector and a DB-624 column (30 m × 0.53 mm × 3.0 μm). The wastewater Zn^{2+} concentration was determined via ICP-OES (Thermo, iCAP 7400, USA). Concentrations of CIP and ZnO were directly determined by weight once the solution or suspension was prepared. With regards to the C_{60} suspension, a 1.0-μm-filtration process was performed after sonication. And C_{60} concentration in this aqueous suspension was determined by extracting C_{60} into toluene and quantifying it at a wavelength of 332 nm using a DR-6000 UV/visible spectrometer (Hach, Colorado, USA), as described in detail in our previous work (Yang et al., 2013).

To analyze the microbial community structure, duplicated digested sludge samples were collected from all reactors after 35 days. The total genomic DNA was extracted from 300 mg wet-weight sludge samples using a Power Soil DNA isolation Kit (MO BIO Labs, Solana Beach, CA, USA). DNA quality and quantity were assessed using 1% agarose gel electrophoresis and spectrophotometry (260 nm/280 nm ratio). The V3–V4 variable regions of the microbial 16S rRNA gene were targeted using primer pairs: PCR primers 338F (5′- ACTCCTACGGGAGGCA GCA-3′) and 806R (5′- GGACTACHVGGGTWTCTAAT-3′) combined with adapter sequences and barcode sequences for bacterial and archaeal community analysis. High-throughput sequencing was conducted by Biomarker Tech, Ltd (Beijing, China) using an Illumina HiSeq 2500 (Illumina Lnc., CA, USA). After sequencing, paired-end reads were assembled with a minimum overlap of 10 bp using FLASH (version 1.2.11). The assembled tags were compared against primers, and tags corresponding to more than 6 mismatches were discarded using the FASTX-Toolkit. 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 a RDP classifier (version 2.2) against Silva databases (Release 128). Community and adversity comparisons were primarily determined using the absolute and relative abundance of different level microbes and principal component analysis (PCA).

Mean statistical differences in experimental data were analyzed using one-way analysis of variance (ANOVA) in SPSS (IBM SPSS Statistics, version 20).

3. Results

3.1. Effect of individual and complex emerging contaminants on CH_4 production in sludge digestion

3.1.1. ZnO, Cip, or C_{60}

Fig. 1 shows the individual effects of isolated ZnO, Cip, and C_{60} on CH_4 production in anaerobic sludge digestion. Low ZnO concentrations (0.015, 0.300, and 3.000 mg/g DW of sludge) did not significantly ($p > 0.05$) alter CH_4 production from the control (CK) value (Fig. 1A), and the CH_4 production rate peaked almost simultaneously with the control, at ~6 h. ZnO concentrations of 15.000 mg/g DW of sludge wielded significant negative influence over CH_4 production amounts and rates ($p < 0.01$), reducing production to 76.8% of the control after 24 h and delaying the peak production rate to 9 h. At ZnO concentrations of 30.000 mg/g DW of sludge, CH_4 production reached 71.4% of the control with an initial production time delayed more significantly to 12–15 h and a notably reduced peak value relative to other concentrations ($p < 0.01$). Nguyen et al. also found similar CH_4 production inhibition by ZnO (Nguyen et al., 2015). This study found low ZnO concentrations did not significantly inhibit the anaerobic digestion process, potentially explained by the Zn^{2+} release from ZnO NPs into solution where it could combine with an extracellular polymeric substance (EPS) to reduce toxicity (Mu et al., 2012). High ZnO concentrations, however, significantly increased toxicity to exceed the combination capability of EPS and react with enzymes or sulfydryl and amidogen in other microorganism proteins or nucleic acids, impeding the synthesis of peptidoglycan, destroying their activity, and thus

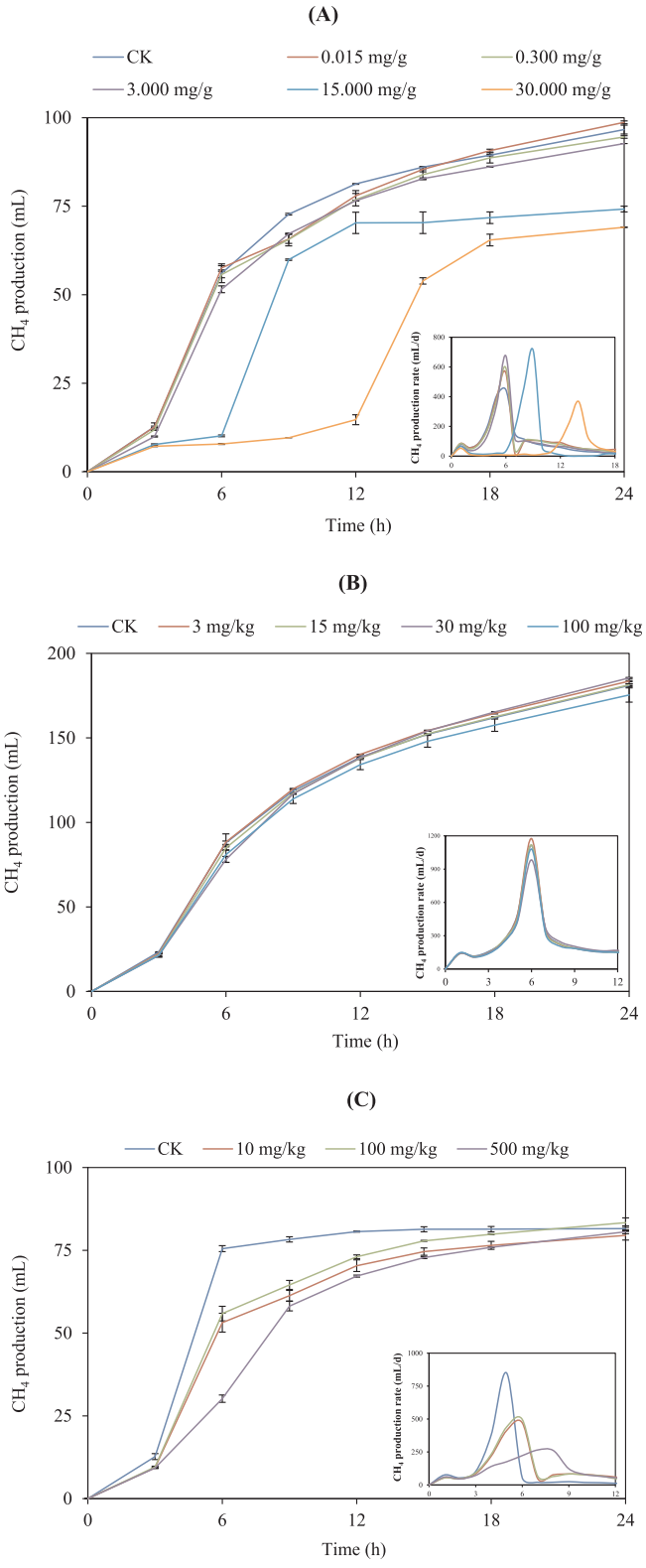

Fig. 1. Effects of ZnO (A), C_{60} (B) and Cip (C) concentrations on CH_4 production and rates (intert) during sludge digestion.

causing irreversible damage to the sludge microorganisms (Mu and Chen, 2011).

C_{60} concentrations up to 100 mg/kg DW of sludge failed to significantly alter CH_4 production amounts or rates from those of control after 24 h ($p > 0.05$) (Fig. 1B). Cip concentrations of 10, 100, and 500 mg/kg DW of sludge, did not significantly alter CH_4 production from the control after 24 h (Fig. 1C) ($p > 0.05$); CH_4 production rate, however, was significantly inhibited and the peak reaching time was delayed. Comparing the control and low concentration Cip (10,100 mg/kg DW of sludge), Cip delayed the peak CH4 production rate by 1 h with a 42.9% lower peak value; similarly, high concentration Cip (500 mg/kg DW of sludge) delayed the peak CH_4 production rate by 2 h and the peak value by 68.7%. Yin et al. found two antibiotics (chlonetracycline and oxytetracycline) have low toxicity in the hydrolytic acidification process, and do not influence metabolic activity in CH_4 production (Yin et al., 2018). Feng et al. found four antibiotics (clarithromycin, erythromycin, sulfamethoxazole and trimethoprim) had no obvious effect on the anaerobic digestion of pig manure (Feng et al., 2017). Mai et al. however, recently found that added Cip significantly inhibited organic removal and methanogenic activity as well as increasing VFA accumulation (Mai et al., 2018).

3.1.2. Complex

Though isolated Cip inhibited CH_4 production by ~14% at 36 h, its presence failed to significantly influence CH_4 production after 35 days ($p > 0.05$) (Fig. 2). Furthermore, averaging over the first 35 days, C_{60} showed no significant influence on the CH_4 production ($p > 0.05$). Individual ZnO inhibited CH_4 production by 49.5% at 14 h but only 15% after 35 days. Complex pollutants had varied effects after 14 h: Cip + ZnO inhibited the CH_4 production rate by up to 89.7% (a 29.8% increase relative to the sum of the individual pollutant inhibition rates) whereas ZnO + C_{60} showed an inhibition increase of only 3.9% relative to the sum of its individual pollutants. At 36 h, the inhibition rates of individual pollutants C_{60} and Cip were not significantly changed ($p > 0.05$); however, but that of ZnO was reduced to 32.3%. Compared with the 14 h inhibition rate, the complex inhibition effect of ZnO + Cip was significantly lower at 36 h relative to its 14 h rate ($p < 0.01$), potentially due to the fact that CH_4 production activity recovered partially from the early period of complete inhibition under the effects of ZnO + Cip. After 35 days, the inhibition effects of individual Cip and C_{60} were reduced almost to zero, that of individual ZnO was reduced to 15.3%. The complex inhibition rates of two-pollutant ZnO + Cip and ZnO + C_{60} were reduced to 23.3% and 18.5%, respectively. Complex contamination from ZnO + Cip or ZnO + C_{60}, thus, displayed synergistic effects; however, these effects continuously decrease over time. Furthermore, the complex effect of ZnO + Cip was observed to be significantly stronger compared to that of the ZnO + C_{60} ($p < 0.05$), possibly because ZnO + Cip may demonstrate

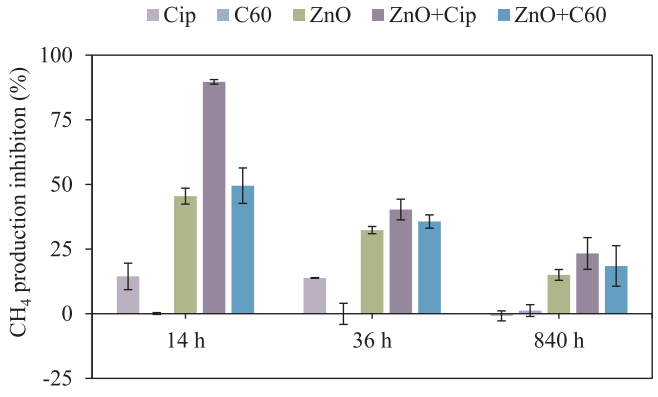

Fig. 2. Inhibition of CH_4 production in sludge digestion by individual and complex pollution compared to control conditions.

coordination and adsorption interactions.

The existing complex mechanism in environmental research consists primarily of mutual competition for binding sites, coordination, adsorption, and precipitation, thus affecting enzyme activity, changing biological cell structures and functions, and disrupting normal biological physiological processes (Huang et al., 2009; Pulicharla et al., 2015). At 14 h the Zn^{2+} concentration from ZnO + Cip was 2.3× that of the individual ZnO treatment, whereas this value was not significantly changed with ZnO + C_{60} ($p > 0.05$). This could explain the significantly higher toxicity of ZnO + Cip relative to ZnO + C_{60} and ZnO. After 36 h, the Zn^{2+} concentration in each treatment decreased rapidly; however, the maximum concentration (0.46 mg/L) was also observed for ZnO + Cip. After 35 days, the Zn^{2+} concentration of all treatments had decreased to ~0.045 mg/L, indicating that Zn^{2+} could be further rapidly dissolved out from ZnO under ZnO + Cip complex treatments, probably related to the Zn^{2+} and Cip coordination reaction equilibrium. Toxicity is accordingly strengthened. Cuprys et al. also found that Cip - metal ion (Al^{3+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}) are more toxic to gram-negative bacteria (*Enterobacter aeruginosa*) (Cuprys et al., 2018). ZnO nanoparticles were observed to enhance antibacterial activity of chloramphenicol and ampicillin on *Escherichia coli*. by > 30% owing to non-lethal bacterial-membrane damage (Applerot et al., 2012). Combinations of CIP and Ag nanoparticle resulted in an increase of the order of 0.1–5.3 times in terms of antibacterial activity against 8 bacterial isolates. This was might related to the interaction between active groups present in CIP with Ag nanoparticle by chelation (Naqvi et al., 2013).

3.2. Effect of emerging contaminants on metabolism in anaerobic sludge digestion

3.2.1. Protein and carbohydrate degradation

The anaerobic sludge digestion process is primarily divided into hydrolysis, fermentation, acetogenesis, and methanogenesis, and microorganisms in the sludge depend primarily on carbohydrates and proteins as substrates for metabolic activities in the anaerobic digestion process (Yang et al. 2015a). Comparing protein degradation inhibition rates for different treatments (Table 1) shows that after 14 h individual Cip and C_{60} did not significantly inhibit protein decomposition ($p > 0.05$), whereas the inhibition rate of individual ZnO was 28.5%. The protein degradation inhibition rate of the ZnO + Cip complex was greater (43.9%) than that of individual ZnO; however, the inhibition rate of the ZnO + C_{60} complex failed to demonstrate significant effects ($p > 0.05$). After 36 h, the inhibition rate of individual Cip increased to 9.0%, but the inhibition rates of individual C_{60} and ZnO did not change significantly ($p > 0.05$). The inhibition rate of the ZnO + Cip complex was 23.7%, a reduction (17.3%) from the 14 h value, and that of ZnO + C_{60} was 15.8%, significantly lower than the individual inhibition effect of ZnO (27.5%) ($p < 0.05$) and thus demonstrating a detoxification effect. Individual ZnO can exert significant, relatively stable, long-lasting inhibition on the hydrolytic acidification process of sludge (Mu et al., 2011). Conversely, ZnO + Cip demonstrates short-lasting acute and additive toxicity effects on protein degradation: after 35 days, anaerobic digestion tends to stop, but the protein degradation rates of various treatment groups were ~80%. Compared with CK, some treatment groups promoted protein degradation to varying extents, possibly due to the stimulation of fermentative and acid forming bacterial activities, and the additional carbon provided by Cip and C_{60} for biological metabolism (Stone et al., 2009).

In Table 1, carbohydrates were more easily decomposed by hydrolyzation and fermentation bacteria than proteins (Yang et al. 2015b). Furthermore, the inhibition of carbohydrate degradation rate containments decreased rapidly over time: at 14 h, Cip and C_{60} demonstrated minimal effects; the individual inhibition rate of ZnO was 7.2%; the rate for ZnO + Cip increased by 70.9%; and ZnO + C_{60} showed no obvious additional effects. After 36 h, individual containment no longer

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

Substrate	Digestion time (h)	Cip	C_{60}	ZnO	ZnO + Cip	ZnO + C_{60}
Protein	14	2.4 ± 1.4	4.8 ± 1.0	28.5 ± 5.1	41.0 ± 12.2	29.8 ± 1.0
	36	9.0 ± 4.9	−0.6 ± 1.9	27.5 ± 0.8	23.7 ± 0.0	15.8 ± 1.1
	840	−7.5 ± 0.9	−15.4 ± 4.4	−4.4 ± 2.2	−8.2 ± 2.2	−11.3 ± 0.3
Carbohydrate	14	0.5 ± 0.3	0.4 ± 0.1	7.2 ± 0.6	78.1 ± 4.5	8.0 ± 0.6
	36	−0.6 ± 0.3	0.2 ± 0.5	0.0 ± 0.6	9.5 ± 0.4	4.2 ± 0.2
	840	0.2 ± 0.4	0.7 ± 0.0	−0.2 ± 0.0	0.5 ± 0.1	0.5 ± 0.2

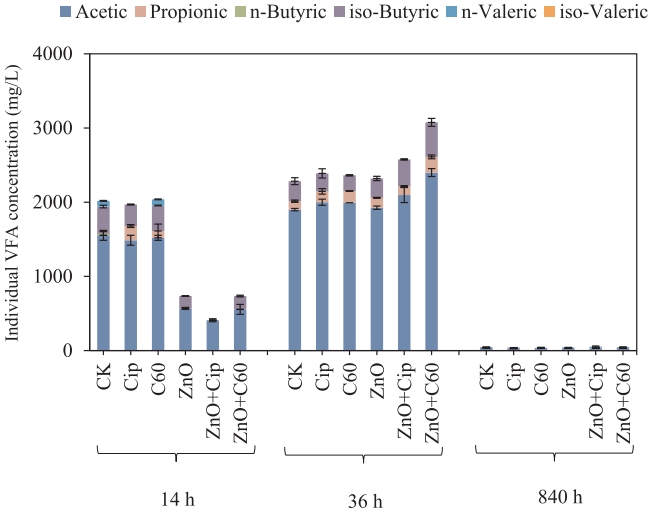

Fig. 3. Effects of different pollution treatments on individual VFA concentrations in sludge digestion.

influenced degradation, and the inhibition rates of ZnO + Cip and ZnO + C_{60} were reduced by 68.6% and 3.8%, relatively, compared with their 14 h rates.

3.2.2. Volatile fatty acids

VFAs, the main hydrolysis products of glucose and protein, are important digestion products which can greatly influence the anaerobic digestion including acetic, propionic, butyric, and valeric acids. As shown in Fig. 3, VFAs within the digestion system mainly comprised acetic, propionic, and iso-butyric acids and rapid VFAs generation within the first 14 h increases their concentration from 90.5 to ~2000 mg/L under individual CK, C_{60} and Cip treatments. However, VFAs are significantly inhibited (63.6%–79.7%) under individual ZnO and complex treatments, especially in the case of acetic acid ($p < 0.01$). Stone et al. found the accumulation of VFAs under the effect of chlortetracycline, suggesting their generation was efficient, but the utilization of acetate by either homoacetogenic bacteria or aceticlastic methanogens was inhibited (Stone et al., 2009). ZnO significantly inhibited the hydrolysis and acid-forming bacteria decomposing nutrient substances into micromolecule acids, with VFAs occupying only 735 mg/L ($p < 0.01$). After Cip was added, such toxicity was intensified, especially in the case of iso-Butyric acid production, and the corresponding VFAs amount measured only 55.6% of that under the individual ZnO. However, adding C_{60} had no significant additional effect ($p > 0.05$). At 36 h, VFAs concentrations of each treatment group measured approximately 2300 mg/L, and the activities of hydrolysis and acid-forming bacteria had greatly been recovered, especially with regards to acetic and propionic acids. Under complex treatment VFAs increased more significantly, potentially related to the reduced inhibition of hydrolysis and acid-forming bacterial activities produced by complex toxicity and the continuous inhibition of methanogens microbe activity. After 35 days, VFAs had been almost completely consumed, and the VFAs concentrations had recovered to their initial levels.

The pH values of different treatments were measured during anaerobic digestion. At 14 h, the pHs of individual CK, Cip, and C_{60} were all ~6.7; however, those of individual ZnO and the complexes were > 7.0, indicating great inhibition of hydrolysis and acid-forming bacteria (consistent with the VFAs concentrations, Fig. 3). After 36 h, although the microbial activities recovered and VFAs concentrations had increased under complex treatments (Fig. 3), the methanogens (especially acetoclastic methanogens) could not consume such VFAs quantities in time, resulting in lower pH values (< 7.0). After 35 days, the pH values had recovered to their initial levels indicating a reduction in toxicity and recovery of microbial activities in the sludge digestion process.

3.3. Microbial community

Overall, 52–54 bacterial and 1 archaeal phyla were selected for digestion sludge samples. Fig. 4A shows phyla level for the bacteria, indicating that *Proteobacteria, Firmicutes*, *Aminicenantes*, *Bacteriodetes*, and *Chloroflexi* were the most dominant in all digesters. These strains have previously been shown to play important roles in hydrolysis and fermentation (Tian et al., 2017; Wang et al., 2018). Overall, compared to the CK, at the phyla level, ZnO, ZnO + C_{60}, and ZnO + Cip exerted obvious effects on *Firmicutes* (+43.4%, +39.6%, and + 64.1%), *Aminicenantes* (−25.9%, −11.0%, and −28.7%), *Chloroflexi* (−47.3%, −31.1%, and −37.0%), and *Parcubacteria* (−95.7%, −96.0%, and

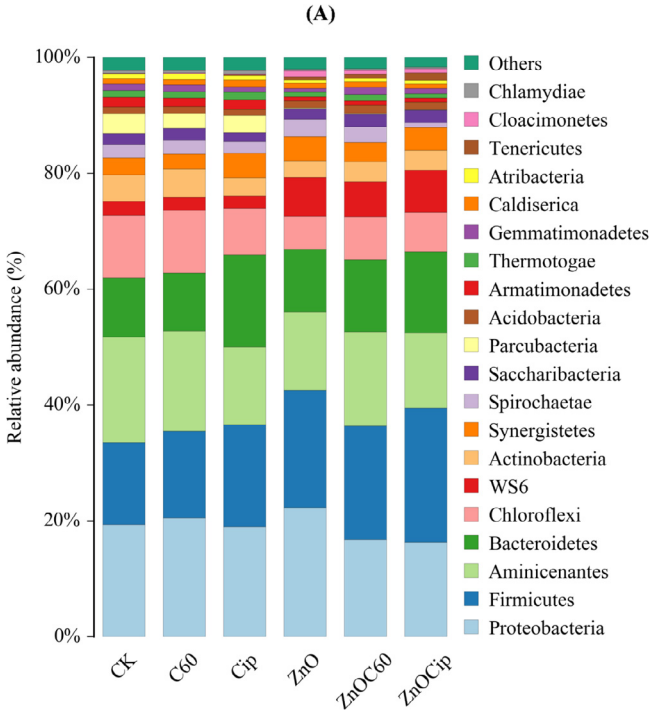

Fig. 4. Relative abundance (A: phylum level) and heat map (B: family level) of microbial community in digested sludge after 35 days.

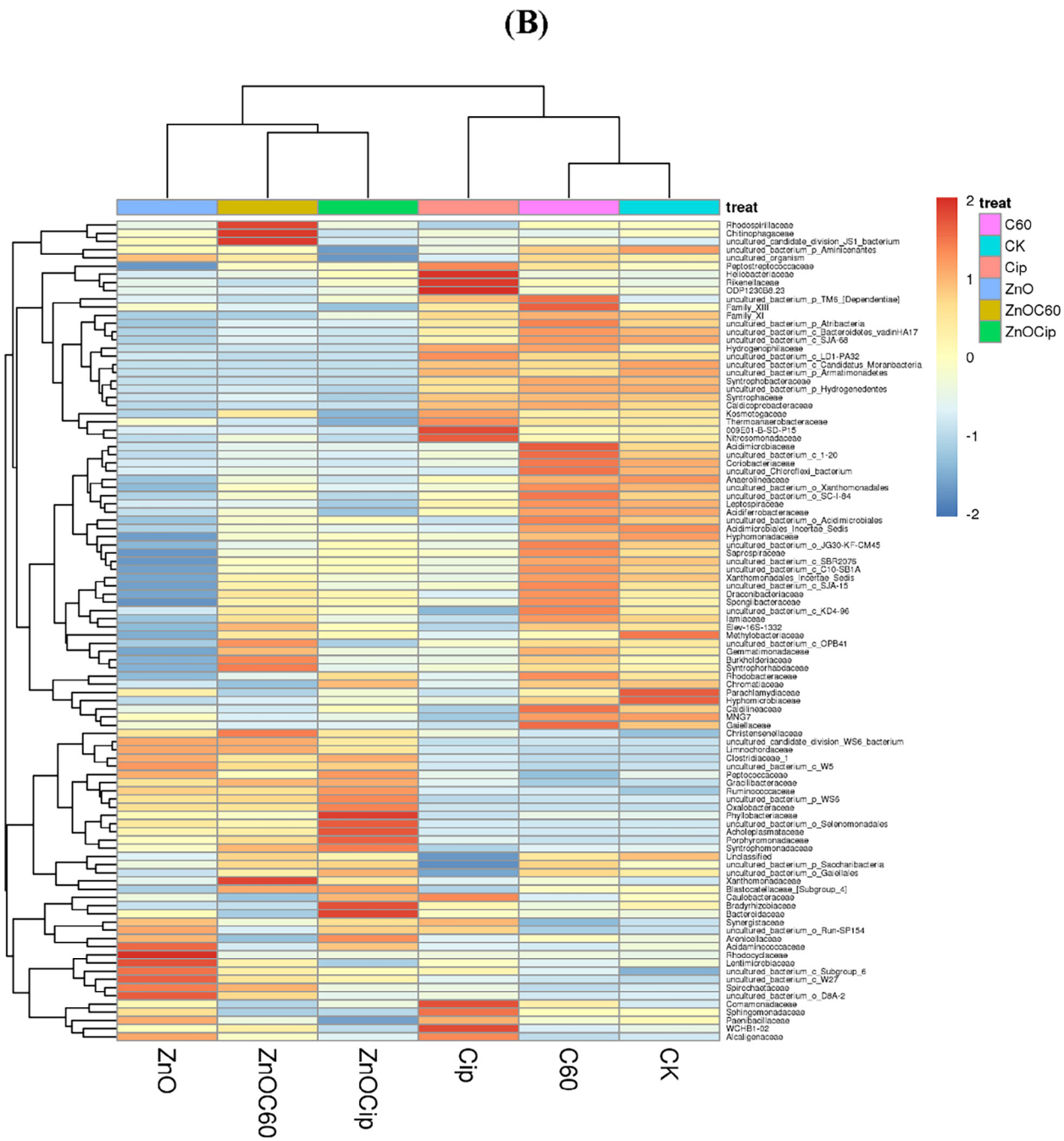

Fig. 4. (*continued*)

−97.1%) bacteria. Cip alone showed slight effects on *Firmicutes* (+25.3%), *Aminicenantes* (−26.3%), and *Chloroflexi* (−25.5%), but individual C_{60} demonstrated almost no significant effects ($p > 0.05$).

Further analysis (Fig. 4B and Table 2) investigated the inhibition various pollution treatments exerted on the following microbes. *Proteobacteria*: at a family level, ZnO treatment decreased *Hydrogenophilaceae*, *Desulfomicrobiaceae*, and *Syntrophaceae* by 63.9%, 78.6%, and 64.0%, respectively, whereas complexes containing C_{60} and Cip showed slight alleviating effects. These bacteria were generally reported to utilize VFAs highly (Yang et al., 2017). *Chloroflexi*: at the class level, *Dehalococcoidia* decreased by 35.1% and 74.6% under ZnO and ZnO + Cip treatments, indicating certain synergistic effects in the complex. *Chloroflexi* primarily utilize various carbohydrates, including hexose and pentose sugars, offering a competitive advantage for methanogens to thrive in the digesters (Yin et al., 2018). *Actinobacteria*: at the class level, individual Cip and ZnO treatments inhibited *Acidimicrobiia* and *Actinobacteria* (29.6% and 32.3% vs 36.3% and 43.9%, respectively), whereas complexes containing C_{60} and Cip showed some antagonistic effects. Although C_{60} and Cip mildly inhibited (< 25%) *Candidatus_Moranbacteria*, individual and complex ZnO treatments demonstrated almost 100% inhibition.

Certain microbes were promoted by pollution treatments. In *Firmicutes*, *Clostridiaceae_1*, *Gracilibacteraceae*, and *Ruminococcaceae* increased by 0.8–54.2% under C_{60} and Cip treatments, whereas ZnO presence (alone and in conjunction with other pollutants) increased these microbes by 149.2–469.7%. ZnO additions increased *Selenomonadales* by 3.8 × compared to the control, and ZnO + Cip further increased this by 2.1 × . A similar effect was observed *Porphyromonadaceae*, in *Bacteroidetes*, and *Spirochaetaceae*, in *Spirochaetae*. These microbes are capable of transforming various

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

Phylum	Class	Order	Family	Pollution treatments				
				C_{60}	Cip	ZnO	ZnO + C_{60}	ZnO + Cip
Proteobacteria	*Betaproteobacteria*	*Hydrogenophilales*	*Hydrogenophilaceae*	+47.9	+45.4	−63.9	−59.1	−62.3
	Deltaproteobacteria	*Desulfovibrionales*	*Desulfomicrobiaceae*	+2.9	+15.0	−78.6	−76.1	−65.5
	Deltaproteobacteria	*Syntrophobacterales*	*Syntrophaceae*	+2.6	+0.2	−64.0	−56.3	−72.9
Chloroflexi	*Anaerolineae*			−5.3	−28.1	−57.2	−36.2	−47.8
	Dehalococcoidia			−15.5	+11.5	−35.1	−53.8	−74.6
	Thermomicrobia			+1.9	−25.3	−46.9	−40.8	−36.3
Actinobacteria	*Acidimicrobiia*			+8.7	−29.6	−36.3	−19.4	−23.0
	Actinobacteria			+7.7	−32.3	− 43.9	−34.9	−30.2
Parcubacteria,	*Candidatus_Moranbacteria*	*uncultured_bacterium_c_Candidatus_Moranbacteria*		−24.5	−13.0	−99.8	−99.8	−100.0
Firmicutes	*Clostridia*	*Clostridiales*	*Caldicoprobacteraceae*	+11.8	+6.6	−50.1	−46.4	−46.4
	Clostridia	*Clostridiales*	*Clostridiaceae_1*	+0.8	+46.7	+467.1	+340.5	+469.7
	Clostridia	*Clostridiales*	*Gracilibacteraceae*	+24.0	+30.2	+149.2	+193.4	+203.1
	Clostridia	*Clostridiales*	*Ruminococcaceae*	+47.4	+54.2	+217.1	+195.1	+266.5
	Limnochordia	*Limnochordales*	*Limnochordaceae*	−32.2	+149.7	+2297.8	+2226.2	+1563.4
	Negativicutes	*Selenomonadales*	*uncultured_bacterium_o_Selenomonadales*	−22.0	+52.0	+3787.7	+2973.1	+7606.4
Bacteroidetes	*Bacteroidetes_vadinHA17 –*	*uncultured_bacterium_c_Bacteroidetes_vadinHA17*		+9.7	−23.7	−75.6	−61.4	−66.2
	Bacteroidia	*BClostridiales*	*Porphyromonadaceae*	−30.7	+118.9	+395.9	+725.4	+1224.1
WS6				−8.6	−12.5	+176.3	+148.2	+200.7
Spirochaetae	*Spirochaetes*	*Spirochaetales*	*Leptospiraceae*	+10.6	−35.5	−78.8	−70.1	−96.6
	Spirochaetes	*Spirochaetales*	*Spirochaetaceae*	−46.2	+74.6	+515.1	+415.9	+87.8

Fig. 5. Principal component analysis (PCA) analysis based on OTU abundance for microbial in digested sludge under different treatments after 35 days.

carbohydrates and proteins into simple compound (Song and Zhang, 2015). These results again indicate that organisms were inhibited upon initial exposure, but became tolerant and even proliferated long-term in the digesters (Eduok et al., 2017).

A single-phylum Archaeal population (*Euryarchaeota*) was present at low concentrations in all samples (0.4%), approximately consistent with previous studies (Hanreich et al., 2013; Yang et al., 2017). No significant variations were observed between samples for this population ($p > 0.05$), possibly related to the significant microbial activity recovery after 35 days. The most abundant order microbe was *Methanosarcinales* (0.4%), followed by *Methanomicrobiales* (0.02%) –important acetoclastic and hydrogenotrophic methanogens, respectively (Peng et al., 2018; Venkiteshwaran et al., 2017). The larger *Methanosarcinales* concentration indicated the acetoclastic pathway dominated, as *Methanospirillum* and *Methanosaeta* species competed for feed in this study (Wang et al., 2018; Zhang et al., 2016).

The observed differences in the bacterial communities were supported by PCA (Fig. 5). Samples were clustered in three groups along the PC1 vector (accounting for 57% variation): CK, C_{60}, and Cip; ZnO; and ZnO-based complex. PCA analysis exhibited similarities in CK, C_{60}, and Cip bacterial communities, whereas the complex treatment showed the similarities compared to individual ZnO, indicating the impact of complex pollution and pollutants type on microbial community structure.

4. Conclusions

The ZnO + Cip complex was significantly more influential on CH_4 production than individual ZnO or ZnO + C_{60}, primarily acting through acute and additive toxicity on methanogenesis and degradation of protein and carbohydrate. The complex exerted obvious effects on *Firmicutes*, *Aminicenantes*, *Chloroflexi*, and *Parcubacteria* bacteria in digestion system. One proposed complex mechanism is that ZnO + Cip presence allows greater, Zn^{2+} dissolution from ZnO, related to the Zn^{2+} and Cip coordination reaction. These results could help in toxicity control of the complex pollution as well as co-treatment of emerging antibiotics and nanoparticles during waste sludge digestion.

5. Conflicts of interest

The authors declare no financial/commercial conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.biortech.2018.07.024.

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