Environmental Engineering and Management Journal December 2018, Vol.17, No. 12, 3013-3024
http://www.eemj.icpm.tuiasi.ro/; http://www.eemj.eu

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EFFECTS OF CHLORTETRACYCLINE AND COOPER ON SWINE MANURE ANAEROBIC DIGESTION

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Abstract

Chlortetracycline (CTC) and copper (Cu) are simultaneously used as feed additives to improve the growth performance and to prevent infection in pig farming. After ingestion by animals, most of these compounds are excreted and coexist in swine manure. They might have an influence on anaerobic digestion when the swine manure is used as substrate in a biogas plant. The purpose of this study was to investigate the effects of both CTC and Cu on batch swine manure anaerobic digestion in order to better estimate and control the risk from antibiotics and heavy metals to anaerobic digestion. The results showed that high concentrations of CTC (32.86 mg/L) had no significant influence ($P > 0.05$) on the cumulative methane production, but seriously delayed the daily methane production peaks. The effect of both CTC and Cu spiked together on methane production was much greater than either of them spiked separately. There was significant difference ($P < 0.05$) in daily methane production when CTC and Cu spiked concentrations were up to 4.14 and 98.42 mg/L during the swine manure anaerobic digestion. Furthermore, methane production was inhibited completely when CTC and Cu spiked concentrations were up to 11.55 and 144.83 mg/L. The removal rate of CTC was up to 85% after anaerobic digestion. The transformation of Cu from Cu-exchangeable, Cu-carbonate, Cu-bound to Fe/Mn forms to more stable forms of Cu-organic and Cu-residual occurred during the swine manure anaerobic digestion, resulting in 80% of the total Cu being bound as stable Cu-organic after anaerobic digestion. These results indicated that anaerobic digestion had a positive influence on antibiotics removal and heavy metals transformation in swine manure.

Key words: anaerobic digestion, chlortetracycline, copper, swine manure

Received: September, 2014; Revised final: February, 2015; Accepted: March, 2015; Published in final edited form: November 2018

1. Introduction

Veterinary antibiotics (VAs) are widely used as feed additives to promote animal growth and control diseases in concentrated livestock and poultry industry all over the world (Kemper, 2008; Liu et al., 2016; Sarmah et al., 2006). In 2000, more than 2.27×10^8 kg of antibiotics was produced in the United States and more than 40% of them were used as feed supplements to enhance animal feeds (Sarmah et al., 2006). In China, 2.1×10^8 kg of antibiotics is produced every year, of which 48% is applied in agriculture and livestock industry (Luo et al., 2010). Tetracyclines (TCs) such as tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC) are among the most common antibiotics used in animal husbandry

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for growth promotion and prevention of bacterial disease (Kumar et al., 2005; Sarmah et al., 2006). It is estimated that as high as 3.0×10^6 kg of TCs were produced in 2003 for both farm and companion animals in the United States. According to Sarmah et al. (2006), CTC, tylosin and bacitracin were the three most frequently used antibiotics in swine productions identified in 1996 in the US. However, these antibiotics are poorly absorbed in the digestive tract of animal, and 50-80% is excreted through feces and urine as unmetabolized parent compound (Sarmah et al., 2006).

Literature data of seven provinces in China (Zhang et al., 2005) indicated that TCs residues in swine manure were higher than in poultry manure, and concentrations of OTC and CTC residues were higher than that of TC residues, i.e., the average concentrations of OTC, CTC and TC were 20.94, 15.26, 12.20 mg/kg DW (dry weight), respectively. These compounds are strongly adsorbed in manure samples because they create complexes with metal ions, humic acids, proteins, particles and organic matter in the manure matrix (Loke et al., 2003). Due to high level of these antibiotics remained in animal manure, it may result in serious problems for the environment and public health safety, such as antibiotics resistant bacteria and antibiotics resistant genes (Chee-Sanford et al., 2001; Kemper, 2008; Sarmah et al., 2006; Wang and Wei, 2013). Additionally, residues of antibiotics or their metabolites in animal manure can have negative effects on treatment systems such as anaerobic digesters (Arikan et al., 2006; Poels et al., 1984) and nitrifying systems (Campos et al., 2001).

Copper (Cu) concentration in some animal wastes remained in a high level because copper salts, by spreading of copper sulfate and copper chloride, are frequently overused in animal feed to promote growth and control diseases (Nicholson et al., 1999; Wang and Wei, 2013). Nicholson et al. (1999) found that Cu concentrations ranged from 18 to 217 mg/kg DW in swine feeds and concentrated up to 360 mg/kg DW in swine manure. Zhang et al. (2005) investigated Cu residues in animal manure ranged from 0 to 2016 mg/kg DW in seven provinces of China. The results from Li et al. (2007) showed that the concentrations of copper in swine feed and swine manure ranged from 6.9 to 395 mg/kg DW and 50 to 2017 mg/kg DW, respectively, in Beijing and Fuxin, China. In addition to as a feed additive, Cu in form of $CuSO_4$ solution is occasionally used as a footbath or disinfectant to prevent spread of infectious diseases on swine farms (Guo et al., 2012).

Anaerobic digestion is an established and proven technology for treatment and resource recovery of animal manure (Arikan et al., 2006; Yang et al., 2016). It involves biodegradation and stabilization of organic materials under anaerobic conditions by microbial organisms and leads to the formation of biogas and microbial biomass (Chen et al., 2008; Kelleher et al., 2002; Vasmara and Marchetti, 2016). However, high level antibiotics and heavy metals residues in animal manure have a significant influence on methane production during anaerobic digestion (Guo et al., 2012; Sanz et al., 1996). For instance, Sanz et al. (1996) found that CTC was very powerful protein synthesis inhibitor of anaerobic digestion which IC_{50} level (the 50% inhibiting concentration) was 40 mg/L. Álvarez et al. (2010) demonstrated that methane production was reduced by 56%, 60% and 62% at OTC and CTC concentrations of 10, 50 and 100 mg/L, respectively, which the IC_{50} level was estimated to be around 9 mg/L. Zilles et al. (2005) found CTC in swine manure holding pond samples at above known minimum inhibitory concentrations (>4.8 µg/L) and the antibiotic was suspected to impact manure degradation. Stone et al. (2009) reported that the presence of CTC enhanced hydrolysis reaction rates in anaerobic batch reactors degrading swine manure but inhibited both methane and carbon dioxide production.

In addition, some studies demonstrated that severe Cu inhibition concentration on anaerobic digestion of sewage sludge has been found in the range of 2-90 mg Cu /L dosed in the form of $CuCl_2$ (Ahring and Westermann, 1983; Hickey et al., 1989; Jin et al., 1998; Karri et al., 2006; Lin, 1992) and 200-800 mg Cu /L dosed in form of $CuSO_4$ (Hobson and Shaw, 1976; Wong and Cheung, 1995). Ahring and Westermann (1983) indicated that the complete inhibition of methane production by copper (dosed as $CuCl_2$) was 300 mg/L with sewage sludge during the thermophilic semi-continuous anaerobic digestion. And Karri et al. (2006) reported that the IC_{50} of Cu (II) to acetoclastic and hydrogenotrophic methanogens were 20.7 and 8.9 mg/L, respectively.

Generally, the inhibition of heavy metals on anaerobic digestion largely depends on their concentrations, chemical forms, temperature, and pH value. For instance, heavy metals in the precipitated form have little toxic effect on the biological system (Ahring and Westermann, 1983; Hayes and Theis, 1978; Lawrence and McCarty, 1965; Mosey and Hughes, 1975). The relative sensitivity of acidogenesis and methanogenesis to heavy metals is Cu > Zn > Cr > Cd > Ni > Pb and Cd > Cu > Cr > Zn > Pb > Ni, respectively (Chen et al., 2008).

Although a number of researchers have investigated the effect of antibiotics and heavy metals on anaerobic digestion, respectively, there is still information gap regarding the combined effect of both TCs and heavy metals on swine manure anaerobic digestion, such as threshold value of TCs, Cu and their combination inhibiting anaerobic digestion. Thus, the purpose of this study was to investigate effects of both CTC and Cu on swine manure anaerobic digestion in batch experiments to better control the risk from antibiotics and heavy metals in animal manure spreading to the environment.

2. Materials and methods

2.1. Chemicals and standards

The three parent TCs including tetracycline hydrochloride (TC, 97.5% purity, CAS No. 64-75-5), oxytetracycline hydrochloride (OTC, 98.5% purity, CAS No. 2058-46-0) and chlortetracycline hydrochloride (CTC, 99.0% purity, CAS No.64-72-2) were purchased from Dr. Ehrensdorfer Co. (Germany). Metabolites of these three parent TCs with high purity (>97%) including 4-epitetracycline (ETC), 4-epioxytetracycline (EOTC), β-Apo-oxytetracycline (β-AOTC), 4-epichlortetracycline (ECTC), demeclocycline (DMCTC) were obtained from Acros Organics (Geel, Belgium). Acetonitrile, methanol and ethyl acetate from J.T. Baker Co. (USA) were all of HPLC-grade. The HPLC-grade formic acid (88% purity, CAS No. 64-16-6) was purchased from Mallinckrodt Baker Inc. (USA). All the following chemicals, including disodium hydrogen phosphate dodecahydrate, citric acid monohydrate and disodium ethylenediamine tetraacetic acid (Na_2EDTA), copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), magnesium chloride ($MgCl_2$), sodium acetate (NaOAc), acetic acid (HOAc), nitric acid (HNO_3), hydrogen peroxide (H_2O_2), perchloric acid ($HClO_4$), hydrofluoric acid (HF), hydroxylamine hydrochloride ($NH_2OH \cdot HCl$) were of analytical pure grade. The ultra-pure water was supplied by a Millipore Milli-Q system.

All the stock solutions of these standards (1 g/L) above were dissolved in methanol and stored at -20 ºC in the darkness.

2.2. Anaerobic digestion setup

Swine manure was collected from a concentrated swine farm located in eastern Beijing, China. Inoculum sludge was collected from an upflow anaerobic sludge blanket (UASB) reactor used to treat pig waste slurry of the same swine farm. The biochemical methane potential (BMP) is a measure of substrate biodegradability determined by monitoring cumulative methane production from a sample which is anaerobically incubated in a chemically defined medium (Owen et al., 1979). The BMP assays determining effects of different concentrations of CTC and Cu on the swine manure anaerobic digestion were carried out by an Automatic Methane Potential Test System II (Bioprocess Control, Sweden). This system allows determining the biochemical methane potential and dynamic degradation profile of any substrate. The Automatic Methane Potential Test System II includes sample incubation unit, CO_2 fixing unit (using 3M NaOH uptaking CO_2 and H_2S from anaerobic digestion, only allowing CH_4 to pass through this unit to methane monitoring unit) and methane monitoring unit. In the methane monitoring unit, the volume of CH_4 released from CO_2 fixing unit is measured using a wet gas flow measuring device according to the principle of liquid displacement and buoyancy that can monitor ultra-low gas flows, a digital pulse is generated when a defined volume (2 mL) of gas flows through the device. The BMP batch assays were carried out in a series of 12 × 500 mL serum bottles as reactors equipped with plastic caps including agitators and rubber stoppers. 200 mL of swine manure slurry and 200 mL inoculum were transferred into each reactor while different concentrations of CTC and Cu were spiked as inhibitors (Table 1). Each batch including control and 3 levels inhibition assays was carried out triplicates. After sealing each reactor, the headspaces were flushed with nitrogen gas for 2 minutes to remove traces of oxygen. Then the reactors were incubated in a water bath sink and the temperature was controlled at 37±1ºC. The termination of BMP tests was judged by no obvious gas producing from the reactors (daily gas production < 2 mL). Durations of the three BMP batch tests were 42 days, 65 days and 46 days for CTC, Cu and CTC + Cu treatments, respectively.

$CuSO_4$ was spiked directly as Cu additives to the reactors; CTC was dissolved into methanol for concentrated stock solutions (10g/L) firstly, and then spiked in the reactors. Methanol volume introduced in each anaerobic digestion system was showed in table 1. In order to determine the effect of methanol on the swine manure anaerobic digestion, inoculum and methanol were experimented with the reaction system mentioned above.

Table 1. BMP tests of the swine manure anaerobic digestion.

Batches	**groups**	***Methanol introduced volumes***	***CTC initial concentrations after spiking***	***Cu initial concentrations after spiking***
		mL	***mg/L (mg/kg DW)***	***mg/L (mg/kg DW)***
CTC	**C-1**	2.0	4.98 (199.24)	20.65 (826.00)
	1	0.3	8.13 (325.34)	18.20 (727.90)
	2	1.0	19.04 (761.57)	18.72 (749.09)
	3	2.0	32.86 (1314.62)	19.19 (767.59)
Cu	**C-2**	0.0	1.13 (85.02)	14.24 (1071.33)
	1	0.0	1.35 (102.03)	49.03 (3686.81)
	2	0.0	1.07 (80.50)	107.30 (8067.62)
	3	0.0	0.93 (70.02)	150.03 (11325.62)
CTC+Cu	**C-3**	2.0	0.23 (20.02)	12.35 (1064.74)
	1	0.3	1.67 (144.13)	53.42 (4605.82)
	2	1.0	4.14 (356.97)	98.42 (8484.31)
	3	2.0	11.55 (995.50)	144.83 (12485.60)

The results showed that methanol was depleted rapidly to produce methane in the 5-8th day.

2.3. Analytical procedures

Parameters such as total solids (TS), volatile solids (VS), pH, moisture content, alkalinity and electric conductivity (EC) were measured according to methods described by Bao (2000). Chemical oxygen demand (COD) in the supernatant of anaerobic digestion was analyzed by Hach methods 8000 with a DR 2800 spectrometer.

Concentrations of water-soluble and solid-extractable TCs in all the samples were determined in duplicate. The slurry samples from the BMP tests were firstly centrifuged and the supernatant and solid were used for determining water-soluble and solid-extractable TCs respectively. The solid from the slurry centrifuging was firstly freeze-dried (ALPHA1-2LD PLUS, Christ, Germany) and then sieved by nylon screen with mesh size of 100 (150μm), and finally determined concentrations of TCs and their metabolic products by using an improved method described by Wu et al. (2011). Each sample at 0.5 g was extracted with 20 mL McIlvaine-Na_2EDTA buffer (pH=4.0) prepared by mixing 0.2 mol/L citric acid monohydrate solution with 0.2 mol/L disodium hydrogen phosphate dodecahydrate solution at the ratio of 1.6:1 (v:v), adding 0.1 mol/L disodium ethylenediamine tetraacetic acid (Na_2EDTA) (adding 37.2 g Na_2EDTA to 1 liter mixture solution of citric acid monohydrate and disodium hydrogen phosphate dodecahydrate). After intensively shaking for 30 min, each sample was centrifuged at 15000 rpm/min of 4 ºC for 10 min. The same extraction procedure was repeated three times. Then all of the supernatant was combined and transferred into a new flask and diluted to 100 mL with the ultra-pure water.

For sample purification and pre-concentration, a vacuum system (SUPELCO VISIPREP™DL, USA) was applied. Firstly, the solid phase extraction (SPE) cartridges (Oasis HLB, 150 mg, 6 mL) were conditioned with 5 mL methanol followed by 5 mL McIlvaine-Na_2EDTA buffer. After the conditioning step, the extract of samples was percolated through the cartridges at a flow rate of around 1.0 mL/min. Then the cartridges were sequentially rinsed with 5 mL water and 5 mL 5% methanol aqueous solution. Finally, the cartridges were eluted with 9 mL methanol/ethyl acetate solution (1:9, v/v).

The elute was collected and concentrated to dryness under a stream of nitrogen and then reconstituted with 1.0 mL acetonitrile containing 0.1% formic acid (1:9, v/v) followed by filtration with 0.22 μm nylon membrane before determined by an ultra-performance liquid chromatography (UPLC) with tandem mass spectrometry (MS) (Waters Corp., USA). Additionally, 10 mL supernatant from the slurry centrifuging was used directly for sample purification and pre-concentration with the SPE cartridges (Oasis HLB, 150 mg, 6 mL) and the same and methane yield was 382.25 mL CH_4/ mL methanol. This part of methane was subtracted in the results.
analysis procedure of solid-extractable TCs was applied. Separation of TCs and their metabolites was achieved with an Acquity UPLC™ C18 column (i.d. 2.1 mm × 50 mm, 1.7 μm. Waters, USA). The column was maintained at 35 ºC while the sample room was kept at 10 ºC and the injection volume was 10 μl. The flow rate was 0.30 mL/min and the gradient of mobile phase increased from 5% A to 17% A in 5 min, and then continued to go up to 30% A over 1 min followed by an increase to 85% during the next 4 min. Finally the gradient was back to the initial 5% A and held for 1 min to maintain equilibration (A = acetonitrile and B = 0.1% aqueous formic acid). The total run time was 11 min. The tandem mass spectrometry (MS) was performed using a Waters Micromass Quattro Premier XE triple quadrupole mass spectrometer equipped with an electrospray ionization source that operated in the positive ionization mode (ESI^+). The operation conditions were optimized as follows: source temperature 120 ºC, dissolution temperature 350 ºC, capillary voltage 3.0 kV, dissolution gas flow 600 L/h, cone gas flow 50 L/h. Acquisition was done in the multiple reaction monitoring mode (MRM). The parent and daughter ions for compound identification and quantitation were 445/427 for TC and ETC; 461/426 for OTC and EOTC; 479/444 for CTC and ECTC. Peak integration and quantification were processed by MassLynx V 4.1 software using the external standard calibration method.

The sensitivity of the method was evaluated by estimating the limit of detection (LOD) and limit of quantification (LOQ) at a signal to noise ratio of 3 and 10 respectively. The LOD was 0.017 mg/kg DW and LOQ was 0.054 mg/kg DW. In order to determine the extraction efficiency of the method mentioned above, 0.5 g aged samples were spiked with 50 mg/L standard mixture solutions of TC, ETC, OTC, EOTC, β-AOTC, CTC, ECTC, DMCTC at three levels of 0.2, 1.0 and 4.0 mg/kg DW, respectively. The recoveries of these tetracyclines and their degradation products showed in table 2 below.

Table 2. Recovery ± RSD (%, n=3) of three tetracyclines and their degradation products.

Compounds	***Spiked level (mg/kg DW)***		
	0.2	***1.0***	***4.0***
TC	71 ± 5.4	89 ± 9.2	88 ± 2.8
ETC	51 ± 11.4	37 ± 15.7	36 ± 7.9
OTC	66 ± 25.7	94 ± 17.1	73 ± 7.2
EOTC	32 ± 28.8	35 ± 1.3	46 ± 2.2
β-AOTC	50 ±6.7	43 ± 10.0	24 ± 14.5
CTC	84 ± 1.9	83 ± 7.4	66 ± 4.1
ECTC	124 ± 0.1	102 ± 4.8	48 ± 2.1
DMCTC	53 ± 6.9	64 ± 6.1	52 ± 3.7

The sequential chemical extraction of Cu in all the samples was applied according to method described by Tessier et al. (1979) determining different Cu fractions including exchangeable (1M

$MgCl_2$, pH=7), bound to carbonates (1M NaOAc, pH=5), bound to Fe-Mn oxides (0.04 M $NH_2OH \cdot HCl$ in 25% (v/v) HOAc), bound to organic matter (0.02 M HNO_3 + 30% H_2O_2, pH=2,), residual (digested with HF-$HClO_4$). All of the copper extraction solutions were stored at 4 ºC and their concentrations were determined by Varian AA240FS Fast Sequential Atomic Absorption Spectrophotometer (Varian, USA).

To determine the total copper concentrations, including residual solid and liquid samples, 1.0 g sample was digested with 5 mL HNO_3 (69%) and 5 mL $HClO_4$ for 24 hours and filtered through 0.45 µm polyethersulfone membrane filters for Varian AA240FS Fast Sequential Atomic Absorption Spectrophotometer (Varian, USA) analysis. Characteristics of raw materials for anaerobic digestion are shown in Table 3.

2.4. Statistical analysis

Batch tests were performed in triplicate. Average and standard deviation values from BMP tests were calculated according to standard procedures, and the results were analyzed by t-test using SPSS statistical software, version 15.0 (SPSS. Chicago, IL). Significant differences were determined at the $P < 0.05$ level of significant.

3. Results and discussion

3.1. Effect of CTC, Cu and CTC + Cu concentrations on methane production

Fig. 1 a-c showed that though the cumulative CH_4 production of the control (C-1) was higher in the first 38 days than that of CTC spiked assays, the cumulative CH_4 production of CTC-3 exceeded that of the control assays since the 39th day till the end of the BMP test. Daily methane production peaks could better reflect the CTC effect on anaerobic digestion (Fig. 1c). In this study, there were two major daily methane production peaks in which the first one was produced from easily degradable organics (around the 14th day) such as protein and carbohydrate, and the second one was from refractory organics (around the 25th day) such as cellulose.

Interestingly, there was one sharp peak in the beginning (about 5 days) of anaerobic digestion in CTC and CTC + Cu assays respectively, it was assumed that the added methanol in the stock solution of CTC contributed to this peak. As showed in Fig. 1c, the first daily methane production peak (around 14th day) of the control was higher than that of CTC-1, CTC-2, CTC-3. However, the second daily methane production peak (around 25^{th} day) of the control was lower than that of CTC spiked assays. These results indicated that the spiked CTC inhibited anaerobic digestion of swine manure in the early stage. After functional bacteria of anaerobic digestion acclimatized to the environment with spiked CTC, the daily methane production was gradually increased even higher than that of the control. Especially, the second daily methane production peak of CTC-3 appeared 8 days later in comparison with others in the same batch. This result implied that high level of CTC (CTC-3) had greater influence on methane production and need more time to adapt. These results showed that adding CTC at three levels of 8.13, 19.04, 32.86 mg/L had no significant reduction of the total methane production during the BMP tests (Fig. 1b), e.g., total methane production (subtracted methane generating from methanol) at the CTC dosage of 8.13, 19.04, 32.86 mg/L was 4.95%, 3.15%, 9.83% more than that of the control (Fig. 1b). Stone et al. (2010) reported that CTC could enhance initial hydrolysis reactions through volatile suspended solids, while inhibited methane and carbon dioxide production. Fig. 1d-f showed the effects of spiked Cu at different levels on the BMP tests.

Table 3. Characteristics of raw materials in swine manure anaerobic digestion BMP tests.

Parameters	*Materials (n=3)*	
	Swine slurry	*Inoculum*
pH	6.65 ± 0.21	7.60 ± 0.16
Moisture (%)	97.64 ± 0.51	99.63 ± 0.07
VS (%)	1.80 ± 0.37	0.14 ± 0.04
TS (%)	2.39 ± 0.46	0.36 ± 0.06
COD (mg/L)	18641.00 ± 722.00	5651.00 ± 215.00
TC (mg/kg DW)	9.08 ± 0.47	0.43 ± 0.03
ETC (mg/kg DW)	3.23 ± 0.19	0.00 ± 0.00
OTC (mg/kg DW)	1.07 ± 0.05	25.28 ± 1.43
EOTC (mg/kg DW)	0.04 ± 0.00	0.00 ± 0.00
CTC (mg/kg DW)	39.60 ± 2.15	0.06 ± 0.00
ECTC (mg/kg DW)	106.47 ± 7.32	3.05 ± 0.17
β-AOTC (mg/kg DW)	0.16 ± 0.01	0.42 ± 0.03
DMCTC (mg/kg DW)	0.00 ± 0.00	0.03 ± 0.00
Total Cu (mg/kg DW)	538.75 ± 29.93	2280.44 ± 109.02
Exchangeable Cu (mg/kg DW)	9.12 ± 0.56	3.52 ± 0.18
Carbonates Cu (mg/kg DW)	1.24 ± 0.05	4.34 ± 0.25
Fe/Mn bound to Cu (mg/kg DW)	252.99 ± 11.65	74.50 ± 3.90
Organic Cu (mg/kg DW)	167.33 ± 10.56	2265.98 ± 123.97
Residual Cu (mg/kg DW)	3.74 ± 0.11	274.80 ± 17.87

The cumulative methane production and daily methane production peaks of Cu-1 and Cu-2 varied in accordance with that of the control (C-2) and there was no significant difference ($P > 0.05$) (Fig. 1d). Though the total methane production of Cu-1 (Fig. 1e) was little higher than that of the control assay (+8.93%), those of Cu-2 and Cu-3 were reduced by 10.42%, 11.06% compared to the control assay ($P > 0.05$) (Fig. 1e). The 65 days of Cu-3 assay was extremely longer than the others at around 45 days.

The daily methane production peak of Cu-3 was extraordinary different comparing with others ($P < 0.05$) (Fig. 1f), e.g., relatively lower (less than 30 mL/day) in the first 30 days and then increasing in the following days while the other assays almost finished. As reported by Ahring and Westermann (1983), the added Cu at 100 mg/L caused a largely increase in VFA concentrations from 130 to 450 mg/L, in which the methane production could be severely inhibited by the excess VFA and methanogens in the digesters needed some time to adapt. This might well explain why the daily methane production peak of the Cu-3 assay showed severe delay throughout the BMP tests.

Obviously, the inhibition of both CTC and Cu on methane production was much greater than either of them spiked separately (Fig. 1g-i production of CTC+Cu-1, CTC+Cu-2 and CTC+Cu-3 assays were decreased by 4.76% ($P > 0.05$), 22.81% ($P > 0.05$) and 100% ($P < 0.05$), respectively (Fig. 1h). It was evident that the swine manure anaerobic digestion with CTC+Cu-3 was completely inhibited and there was no methane produced throughout the BMP test. The tendency of daily methane production peak with CTC+Cu-1 was similar to the control. The situation in CTC+Cu-2 assay was obviously different ($P < 0.05$) and irregularly (Fig. 1 i). These results indicated that both CTC and Cu severely inhibited methane production throughout anaerobic digestion. However, the mechanism of the combined effect of CTC and Cu was unclear yet and needs further study in the future.

Inhibition is usually indicated by a decrease of the steady-state rate of methane gas production and accumulation of organic acids (Kroeker et al., 1979). The delay in daily methane production peaks of assays spiked with CTC/Cu/CTC+Cu clearly reflected their inhibition on anaerobic digestion of swine manure. In general, methanogens are commonly considered the most sensitive to toxicity of the microorganisms in anaerobic digestion.

However, the process can acclimatize, and higher concentrations of the chemicals can be tolerated after a period of adaptation (Angelidaki et al., 2003). In this study, the situations in CTC-1, CTC-2, Cu-1 and Cu-2 might be the result of the acclimation experienced by their inoculum because the inoculum was collected from an UASB digester treating swine wastes containing antibiotics and heavy metals. When the CTC concentration was up to 32.86 mg/L, it did have a great impact on daily methane production peak resulting in almost 8 days retard on the second peak compared with others.

The results of Cu-3 were similar but more complicated. Due to high level copper residue in inoculum (2280.44 mg/kg DW, Table 3), it was assumed that methanogens had already acclimatized themselves to this substrate, the inhibited levels of copper were therefore set as 1071.33, 3686.81, and 8067.62 mg/kg DW in this study. The daily methane production in the tests of Cu-3 showed an extremely unstable trend in the first 30 days (Fig. 1f), and began to climb up and reached the daily methane production peaks during day 40 ~50 after long period for adaptation. Actually, such retard in methane production would no doubt affect anaerobic digestion and then prolong solid retention time, which would increase the operational costs in practical applications.

It is obvious that the inhibition of anaerobic digestion was aggravated when CTC and Cu were spiked together, e.g., the obvious reduction of methane production was clearly observed in the tests of CTC+Cu-2 and CTC+Cu-3 (Fig. 1h). Furthermore, methane production was completely inhibited by the test of CTC+Cu-3. In other words, high level of these compounds spiked together absolutely made anaerobic digestion of swine manure upset or failure.

3.2. Batch assay performance

As far as CTC, Cu and CTC + Cu assays were concerned, all the pH values of these assays were around 7 in the beginning (Table 4), and then increased to approximately 8 at the end of the tests which attributed to ammonia produced from protein biodegradation and other compounds such as urea during the swine manure anaerobic digestion. Alkalinity values were around 3000 mg/L $CaCO_3$ in the beginning, almost all of them increased to 4000 ~ 5000 mg/L $CaCO_3$ at the end of anaerobic digestion. The variation trend of EC was similar to pH and alkalinity during the swine manure anaerobic digestion. However, the situations in CTC+Cu-3 were totally different. The pH and alkalinity values were opposite to the other tests. The pH value was around 7 in the beginning and decreased to 5.7 at the end of the test, and the initial alkalinity was around 3000 mg/L $CaCO_3$ and it decreased to 1785 mg/L $CaCO_3$ at the end of anaerobic digestion.

These results indicated that a great amount of alkalinity in manure was depleted and a great quantity of organic acids accumulated and could not be used effectively throughout anaerobic digestion. Each of the microbial groups involved in anaerobic digestion has a specific pH optimum range. The methanogens and acetogens have pH optimum at approximately 7, while acidogens have lower pH optimum around 6. Methanogens at pH lower than 6.6 grow very slowly (Angelidaki et al., 2003). During anaerobic digestion process, instability will be often caused by VFA accumulation, which can lead to a drop in pH (acidification).

However, accumulation of VFA will not always be expressed as a pH drop due to the buffer capacity of some waste types. In manure there is a surplus of alkalinity, which means that the VFA accumulation shall exceed a certain point before this can be detected as a significant change in pH (Angelidaki et al., 2003). Sanz et al. (1996) reported that the majority of the antibiotics tested lacked activity against acetoclastic methanogens, being active only on the acetogenic bacteria. However, chloramphenicol and chlortetracycline could cause the complete inhibition of the acetoclastic methanogenic archaea. In this study, the inhibitory effects of CTC were mainly on the early stage of anaerobic digestion. Obviously, the effect of CTC and Cu spiked together was much greater than any of them spiked separately on pH and alkalinity because it caused organic acid accumulation severely which totally destroyed methanogens survival environment. Kohn (1961) reported that several biological and biochemical effects of the tetracycline antibiotics depend on the presence of divalent metal ions. However, the interaction of CTC and Cu needed further study. As shown in Fig. 2, though the removal rates of TS and VS in the CTC-3, Cu-3, CTC+Cu-3, respectively, were 16%, 22%, 27% and 25%, 28%, 27%, respectively, TS reduction rates were ranged from around 30% to 50% and VS reduction rates were ranged from around 40% to 60% throughout anaerobic digestion of the other assays.

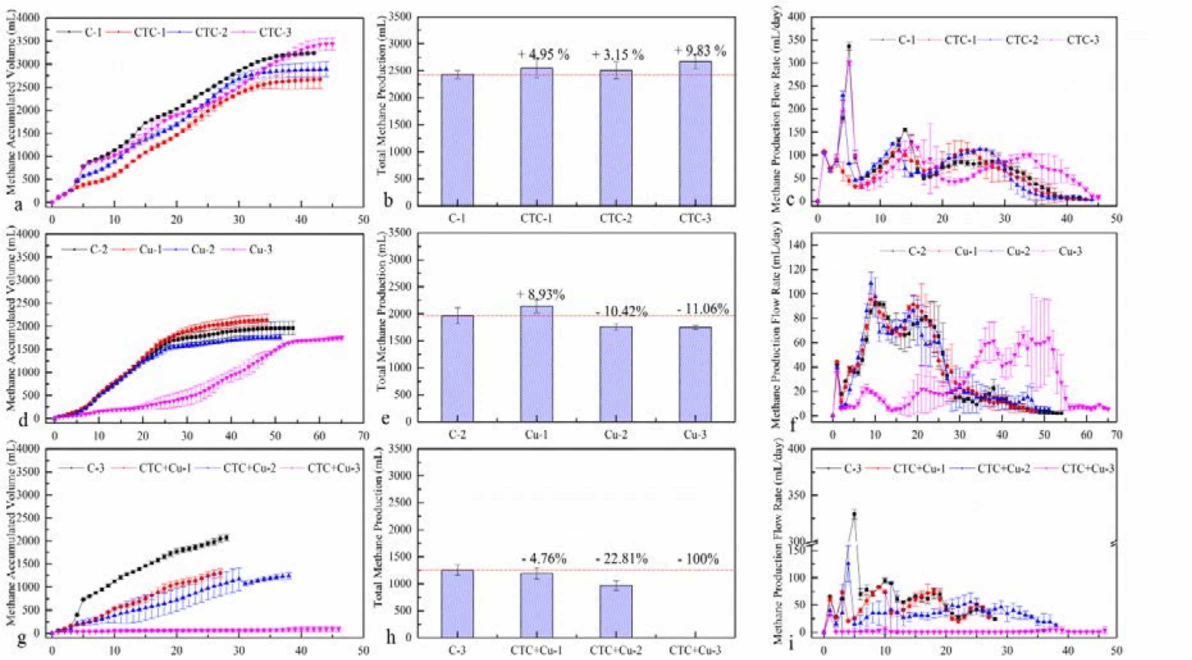

Fig. 1. Methane accumulated volume, total methane production and methane daily production during the swine manure anaerobic digestion BMP tests with CTC (a, b, c), Cu (d, e, f) and CTC + Cu (g, h, i) treatments

Table 4. The changes of initial and final pH, alkalinity and EC values among three batches of the swine manure anaerobic digestion BMP assays

	Initial pH	***Final pH***	***Initial alkalinity mg/L as $CaCO_3$***	***Final alkalinity mg/L as $CaCO_3$***	***Initial EC ms***	***Final EC ms***
C-1	6.98±0.01	8.20±0.03	3353.35±25.02	5805.80±25.02	10.35±0.01	12.27±0.10
CTC-1	6.96±0.01	8.20±0.03	3603.60±50.05	5822.48±38.22	10.45±0.01	12.47±0.09
CTC-2	6.94±0.01	8.10±0.20	3436.76±52.09	6006.00±25.02	10.47±0.01	12.76±0.24
CTC-3	6.95±0.01	8.23±0.04	3053.05±25.02	6006.00±50.05	10.45±0.01	12.85±0.12
C-2	7.28±0.02	7.87±0.07	4020.68±28.89	4913.24±52.09	9.73±0.00	10.79±0.03
Cu-1	7.25±0.01	8.16±0.09	3661.99±52.09	4929.92±25.02	9.68±0.04	10.28±0.17
Cu-2	7.24±0.01	8.04±0.18	4045.70±72.24	4938.26±14.44	9.63±0.00	10.71±0.04
Cu-3	7.20±0.01	7.78±0.04	3595.25±38.22	4796.45±38.22	9.53±0.00	10.52±0.02
C-3	7.14±0.03	8.07±0.14	3069.73±38.22	3820.48±28.89	14.88±0.21	8.48±0.25
CTC+Cu-1	7.52±0.01	8.10±0.11	3144.80±14.44	3920.58±38.22	14.89±0.01	8.58±0.10
CTC+Cu-2	7.06±0.01	8.07±0.10	2519.18±38.22	3845.50±52.09	14.15±0.01	8.38±0.12
CTC+Cu-3	7.19±0.00	5.76±0.42	2719.38±14.44	1785.11±80.44	7.08±0.20	8.83±0.26

These results demonstrated that high concentrations of CTC/Cu/CTC+Cu spiked have an influence on degradation of organic matter during the swine manure anaerobic digestion. In addition, COD removal rates were around 80% at the end of anaerobic digestion for almost all the assays (Fig. 2) except for CTC+Cu-3 (35%), which indicated that high concentrations of CTC and Cu spiked together had a greater impact on COD reduction than either of them spiked separately.

3.3. Reduction of TCs and their metabolites during swine manure anaerobic digestion

The concentrations of TCs and their metabolic products were ranged from 0 to158.4 μg/L in the supernatant of anaerobic digestion (Fig. 3), which was much lower than that in the solid ranging from 0 to 969.18 mg/kg DW (Fig. 4) during these three BMP tests. These demonstrated that residuals TCs in the swine manure were easily concentrated in the solid. The removal rates of CTC and ECTC ranging from 64.05% ~ 98.67% (Fig. 4c, f, i) were much higher than those of TC and OTC. Due to significant transformations of the parent compounds, especially CTC, occurred in spiking these compounds in the BMP tests, the percentage of CTC (25.87%) was thus much lower than that of ECTC (73.70%) throughout anaerobic digestion. It was observed that there was a slightly increase on total CTC (including CTC, ECTC and DMCTC) removal rates (66.75%, 73.05%, 78.63% and 85.54%) with increasing of CTC spiked amounts (C-1, CTC-1, CTC-2 and CTC-3). These results were similar to a 57% reduction of CTC over the course of 216-day swine manure anaerobic digestion reporting by Stone et al. (2009).

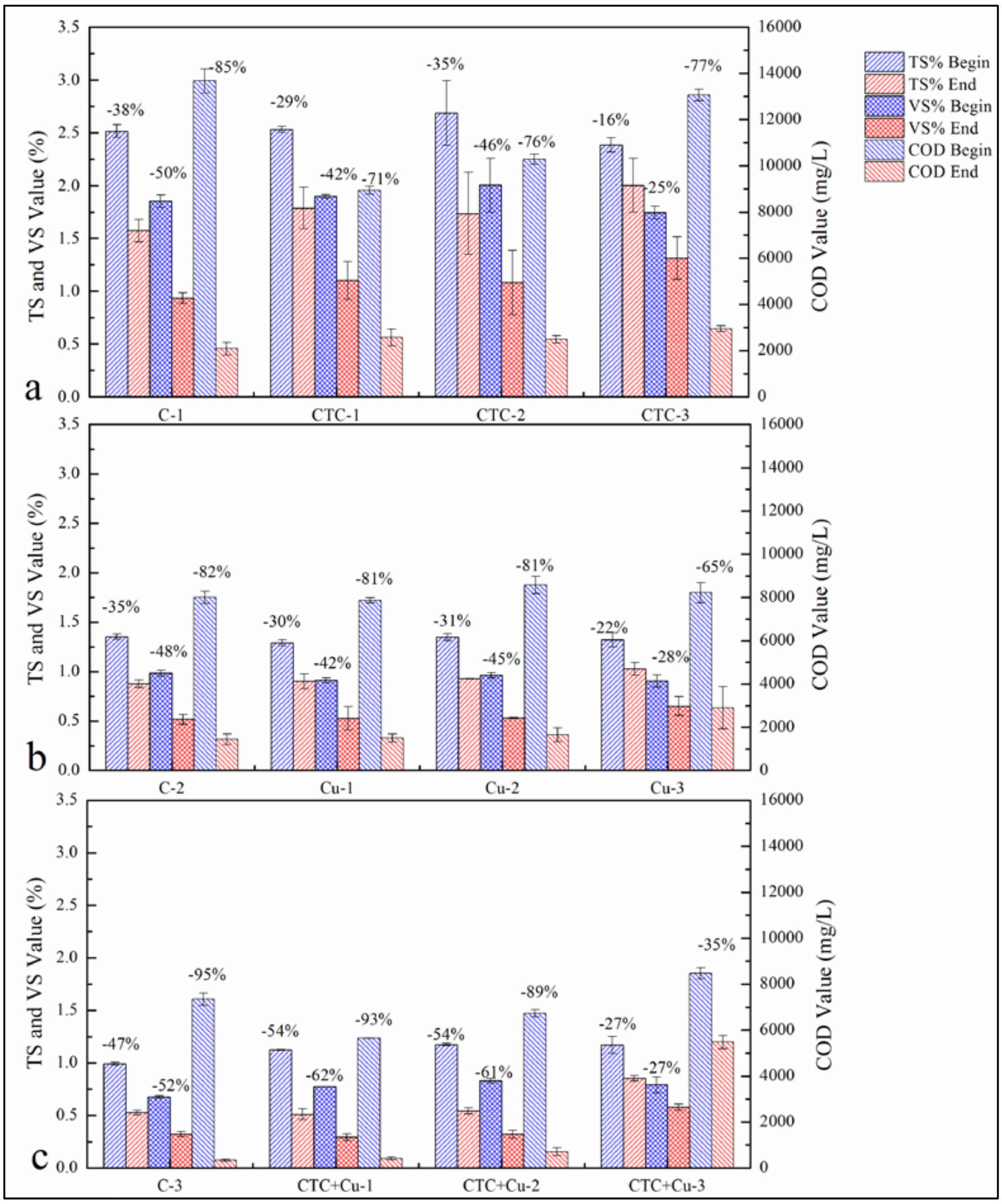

Fig. 2. TS, VS and COD values in the beginning and the end of the swine manure anaerobic digestion BMP tests in CTC (a), Cu (b) and CTC + Cu (c) treatments

The relative persistence of OTC and TC led to their concentrations increasing with degradation of organic matters during the first batch anaerobic digestion (Fig. 4a-b). However, different trends in OTC and TC concentrations were observed when Cu was spiked in the second batch (Fig. 4d-e), e.g., the removal rates of total OTC (including OTC, EOTC and β-AOTC) and TC (including TC and ETC) were 56.67%, 57.80%, 57.95%, 72.04% and 38.60%, 44.58%, 38.95%, 22.64% in the C-2, Cu-1, Cu-2 and Cu-3 experiments, respectively. These results indicated that addition of copper at different levels might improve degradation of TC and OTC during the swine manure anaerobic digestion, and further research is needed.

Furthermore, the metabolites EOTC and ETC were produced in all assays. Among copper spiked assays, OTC, EOTC and β-AOTC were accounted for 87.45%, 1.42% and 11.14%, respectively; TC and ETC were accounted for 78.94% and 21.06%, respectively during the swine manure anaerobic digestion. The order of degree of epimerisation of TCs was as follows: CTC >> TC > OTC.

3.4. Variation of copper fractions during swine manure anaerobic digestion

Concentrations of copper in the supernatant could hardly detect for all the samples of the swine manure anaerobic digestion, which indicated that copper was adsorbed on the solid. Along with the degradation of organic matter, total copper concentrations increased at the end of the BMP tests (Fig. 5). The most important changes were the decrease of both Cu-exchangeable and Cu-bound to Fe/Mn, and the increase of both Cu-organics and Cu-residual throughout anaerobic digestion. It was noticed that over 80% of total Cu was in the form of Cu combined with organics at the end of the assays. These results demonstrated that copper was transformed into more stable fractions during the swine manure anaerobic digestion.

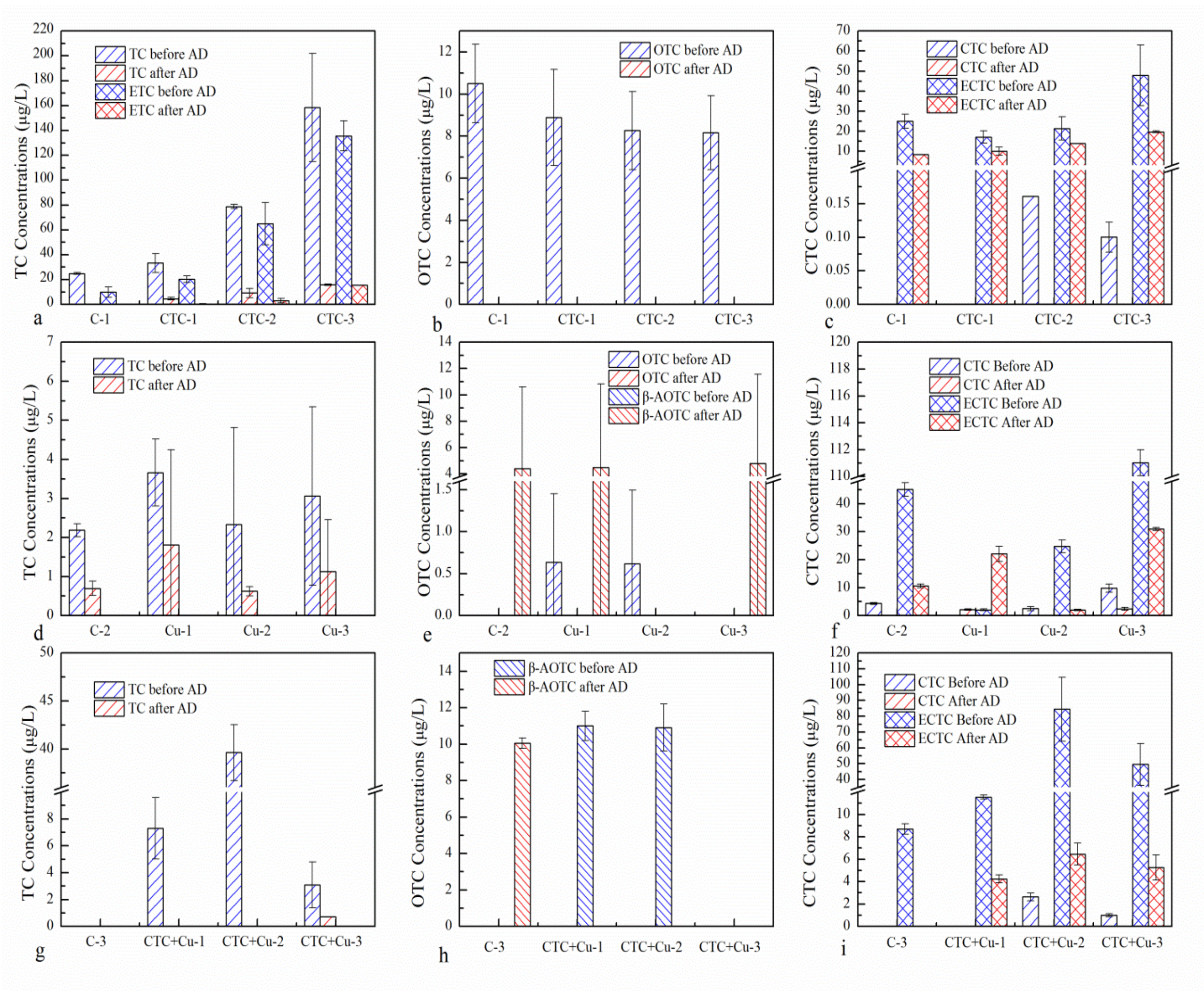

Fig. 3. TCs concentrations of CTC (a, b, c), Cu (d, e, f) and CTC + Cu (g, h, i) in the supernatant before and after the swine manure anaerobic digestion

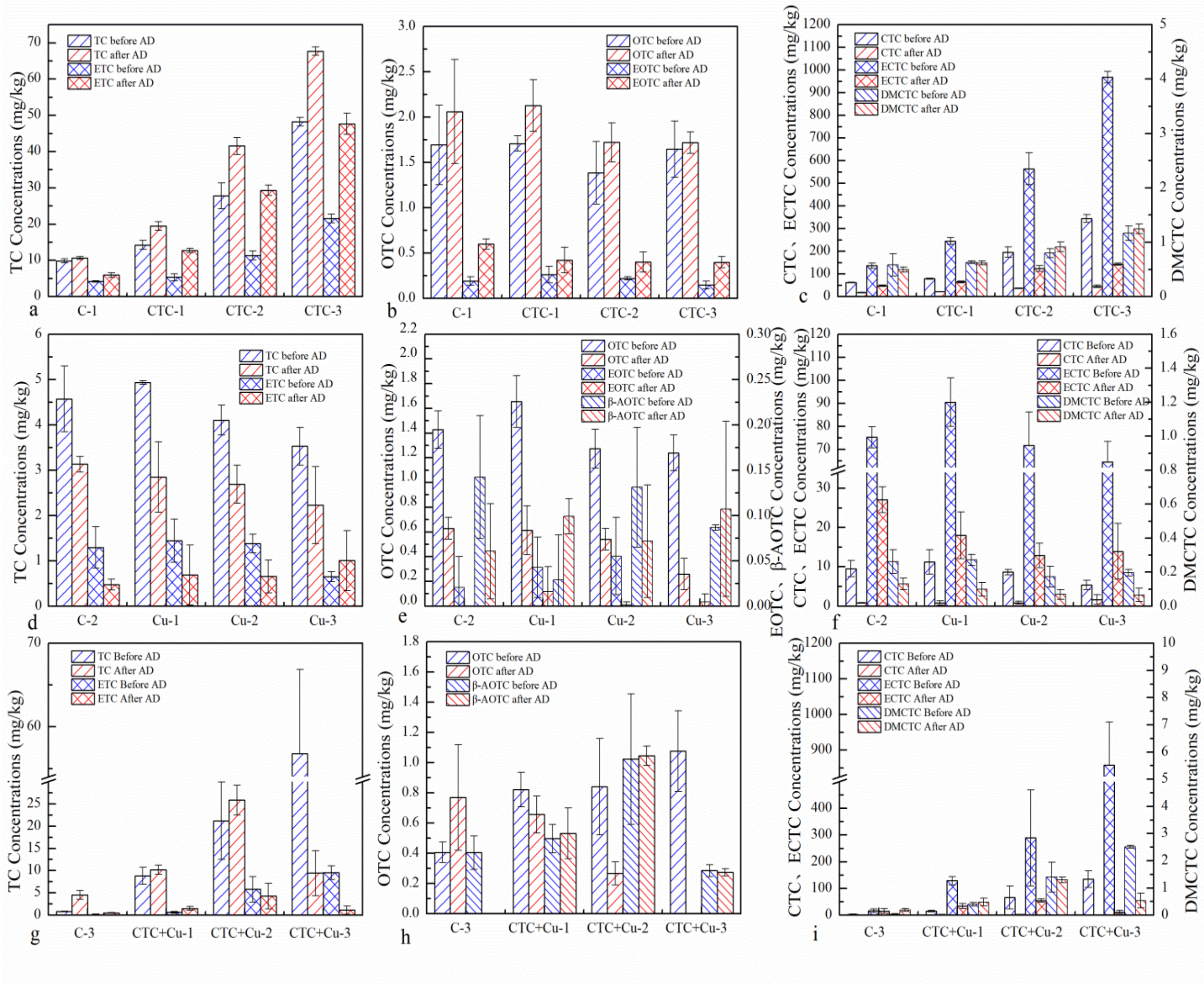

Fig. 4. TCs concentrations of CTC (a, b, c), Cu (d, e, f) and CTC + Cu (g, h, i) in the solid before and after the swine manure anaerobic digestion

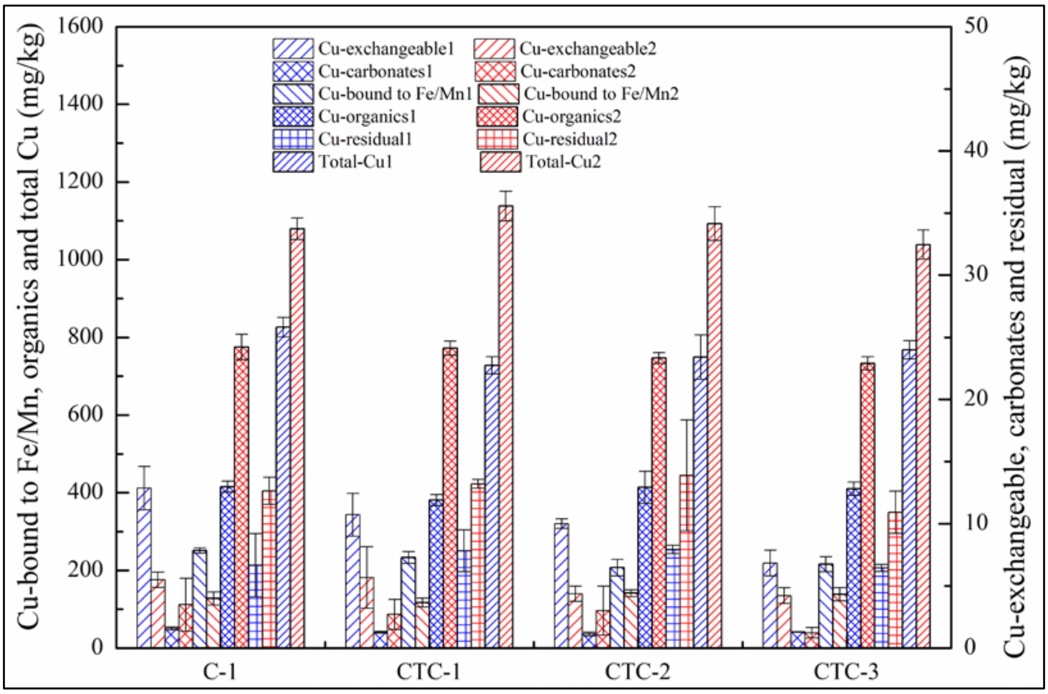

Fig. 5. Total Cu and Cu fractions in the solid with CTC spiked assays changes before and after the BMP tests (1: before anaerobic digestion; 2: after anaerobic digestion)

5. Conclusions

The inhibition of CTC and Cu spiked separately was mainly in the early stage of swine manure anaerobic digestion. High concentrations of CTC and Cu spiked had no significant influence on the total methane production (P > 0.05), but seriously delayed the daily methane production peaks. The combined effect of both CTC and Cu spiked together on the total methane production was much greater than either of them spiked separately.

There were significant differences (P < 0.05) of daily methane production in CTC+Cu-2 and CTC+Cu-3 comparing with the control assay. Furthermore, methane production was inhibited completely in CTC+Cu-3. The removal rate of CTC (including ECTC) was up to 85%. And spiked copper might improve degradation of TC and OTC during the anaerobic digestion. Both Cu-exchangeable and Cu-bound to Fe/Mn were transformed into more stable forms of Cu-organics and Cu-residual during the swine manure anaerobic digestion.

Acknowledgements
This work is financially supported by National Key Research and Development Program of China (grant number 2016YFD0501405), Special Fund for Agro-scientific Research in the Public Interest (No. 201303091), the National Natural Science Foundation of China (No. 50578156 and No.21077122).

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