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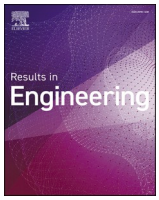

Impacts of norfloxacin on sewage sludge anaerobic digestion: Bioenergy generation and potential environmental risks

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ABSTRACT

Norfloxacin is a broad-spectrum antibiotic which has been frequently detected in the sewage sludge. However, whether norfloxacin in sewage sludge would affect the anaerobic digestion for bioenergy generation and induce environmental risks remained unclear. Therefore, this study investigated the impact of norfloxacin on sewage sludge anaerobic digestion and evaluated potential environmental risks by exploring related antibiotic resistance genes (ARGs) and norfloxacin transformation pathways. The results showed that norfloxacin at environmental relevant concentration ranges would not significantly affect methane production ($p < 0.05$) during the sludge anaerobic digestion, as the sludge solubilization and hydrolysis is unaffected. However, the individual methanogenesis process was enhanced with the increase of *Methanosaeta* abundances. Meanwhile, the increase of norfloxacin concentration will not decrease the removal efficiencies of fluoroquinolones-family ARGs by the anaerobic digestion process. However, the norfloxacin concentration is found to be increased during the anaerobic digestion process, which could probably be due to the desorption from the sludge due to cell lysis. In addition, 13 norfloxacin transformation metabolites were identified with 5 potential norfloxacin degradation pathways being constructed. The metabolites of levofloxacin/ofloxacin could be generated through Eschweiler-Clarke reaction, which may be more toxic than norfloxacin. Thus, the results of this study suggested that although norfloxacin would not affect the bioenergy generation and ARG removal during sludge anaerobic digestion, measures should be taken to deal with the potential environmental risks caused by norfloxacin release and formation of more toxic metabolites.

1. Introduction

Norfloxacin is a broad-spectrum antibiotic in type of fluoroquinolones, which can be effective against both Gram-negative and Gram-positive bacteria by imposing inhibition on the replication and transcription of bacterial DNA. It is currently of extensive use in both clinical treatment and animal husbandry and thus can be released into the environmental through various pathways. It has been reported that with an approximate ratio of 30% norfloxacin being excreted unmetabolized, norfloxacin is among the most frequently detected fluoroquinolones in the environment [1,2]. For example, the max concentrations reached 572 ng/L in Yellow River and 6620 ng/L in Pearl River of China [3], and 1150 ng/L in Australian rivers [4]. Such level of norfloxacin has raised the environmental concern due to the potential ecotoxicity. In addition, the antibiotics resistance genes (ARGs), emerging with the norfloxacin existence may pose further threats to the ecosystem. Between 2008 and 2011, a significant increase in fluoroquinolones resistance was observed by the European Centre for Disease Prevention and Control [5].

The wastewater treatment plant is one of the important pathways for

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norfloxacin transportation. However, when the wastewater was treated by the activated sludge process, nearly 70% of the norfloxacin in the wastewater can be adsorbed into the sludge [6], with the concentrations reaching at 0.1–0.01 mg/g level in sewage sludge [7,8]. The existence of norfloxacin in the sludge may pose challenge for sludge treatment, among which sludge anaerobic digestion (AD) can be a typical example. The sludge AD is one of the most recommended treatment technologies for sewage sludge especially under the net-zero emission background, as it can recover bioenergy, in the form of methane, from sewage sludge to reduce carbon emission [9–11]. However, the inhibitory effect of antibiotics on methanogenesis was often reported, but in some cases, a stimulated methane yield with the introduction of antibiotics is also observed [12,13]. However, so far, how norfloxacin affect bioenergy production during sludge anaerobic digestion is still unclear.

On the other hand, the AD process could also affect the norfloxacin abundance in the sewage sludge. In fact, antibiotics may be degraded by the microorganisms and the metabolites may also cause environmental concerns. For norfloxacin, several potential metabolites such as 6-defluoro-6-hydroxynorfloxacin, desethylene norfloxacin, 7-Amino-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic have been reported in various microbial processes [14–16]. However, to our knowledge, so far norfloxacin degradation pathways during sludge AD process is still unclear and the ecotoxicity of the potential metabolites should be identified. Moreover, the presence of norfloxacin in the sludge are likely to induce fluoroquinolones resistance. The removal efficiency of fluoroquinolones resistance genes during the sludge AD is also worthy to be investigated to fully evaluate the potential environmental risks caused by norfloxacin.

Therefore, the aim of this study is to investigate impact of norfloxacin on sludge anaerobic digestion in terms of bioenergy generation and potential environment risks due to it metabolites and antimicrobial resistance. Firstly, the effect of norfloxacin on methane production potential and each individual step of AD process will be investigated. Also, the high-throughput 16S rRNA sequencing analysis will be carried out to understand the evolution of microbial community with norfloxacin exposure. Moreover, the potential metabolic pathways and metabolites of norfloxacin during sewage sludge anaerobic digestion process will be identified based on LC/MS/MS measurement and the fluoroquinolones resistance in the WAS will be evaluated to further assess the potential impact on ecosystem. The result of this study is expected reveal the potential adverse effects and risks posed by norfloxacin in WAS that should be considered during sludge management.

2. Materials and methods

2.1. Sludge sources and norfloxacin

The waste activated sludge which was used as the substrate for the experiment was obtained from a municipal WWTP in Shanghai, China. The WAS was stored at 4 °C before use to inhibit the biotransformation. Its main characteristics are as showed below: pH 6.9 ± 0.1, total solids (TS) 30000 ± 260 mg/L, volatile solids (VS) 22000 ± 280 mg/L, total chemical oxygen demand (TCOD) 31000 ± 680 mg/L, soluble COD (SCOD) 150 ± 20 mg/L, soluble protein 49.5 ± 3.9 mg/L, soluble carbohydrates 3.6 ± 0.7 mg/L, and ammonium nitrogen (NH4+-N) 97.7 ± 10.1 mg/L.

The inoculum for the anaerobic digestion experiments was gained from a laboratory scale semi-continuous AD reactor. The reactor has a sludge retention time (SRT) of 20d and the temperature of 35.0 ± 1.0 °C. The feeding sludge of the AD reactor was collected in the same WWTP as mentioned above. The main characteristics of the inoculum are as follows: pH 7.5 ± 0.1, TS 22000 ± 300 mg/L, VS 15000 ± 160 mg/L, TCOD 27000 ± 2800 mg/L, SCOD 140 ± 20 mg/L, soluble protein 24.0 ± 2.6 mg/L, soluble carbohydrates 1.7 ± 0.7 mg/L, and ammonium nitrogen (NH4+-N) 350 ± 14 mg/L.

The norfloxacin with the purity of over 98% used in this study was purchased from Aladdin Industrial Corporation (Shanghai, China). The storage condition of the norfloxacin is 4 °C in the refrigerator.

2.2. Biochemical methane potential test

The biochemical methane potential (BMP) tests were conducted for the investigation of the effects of norfloxacin on methane production. Different dosages of norfloxacin were added into 4 batches of serum bottles to create the concentration gradient of 0, 0.03 mg/gTS, 0.3 mg/gTS and 1.7 mg/gTS. Each serum bottle has a total volume of 120 mL and was filled with 50 mL inoculum and 25 mL WAS. The bottles were sealed with butyl rubber stopper with aluminum cover after the headspace flushed by nitrogen gas to deoxygenation, and then placed in the 120 rpm shaker with the temperature of 35 ± 1 °C. The total BMP tests took 37 days and the assessment of the gas production was conducted at 1, 2, 3, 5, 8, 10, 12, 14, 18, 23, 27, 32, 37 day, respectively. The total volume recorded and the gas components detected by the gas chromatograph together showed the methane yield, reported as the volume of methane produced per kilogram of VS added (mL CH4/g VS added). After the tests, the sludge was centrifuged at 12000 rpm to separate supernatant for the measurement of the norfloxacin concentration.

2.3. Batch tests to assess each individual steps of sludge anaerobic digestion

The process of WAS anaerobic digestion can be divided into four stages, including disintegration, hydrolysis, acidogenesis and methanogenesis [17]. For each stage, a batch test was designed to investigate how norfloxacin affects the individual stage of WAS digestion by simulating the different condition of substrates.

2.3.1. Batch Test-I

Batch Test-I was aimed at assessing the produce of SCOD at the stage of disintegration when the concentration of norfloxacin differs from each other. Four groups of 250 mL serum bottles each contained 60 mL WAS and 120 mL inoculum sludge. The concentration level of each group is 0, 0.03 mg/g, 0.3 mg/g and 1.7 mg/g, respectively, which is the same as the BMP tests. Each bottle was sealed with butyl rubber stopper with aluminum cover and then placed in the 120 rpm shaker with the temperature of 35 ± 1 °C. The whole test lasts for 12 h and SCOD was assessed every 3 h. The experimental runs were performed in triplicate to ensure reliable results.

2.3.2. Batch Test- II

In order to determine the impacts of norfloxacin on hydrolysis of WAS anaerobic digestion, Batch Test- II simulate the substrate of hydrolysis stage which contains 4.0 g/L bovine serum albumin (BSA, average molecular weight 67000, a model protein compound) and 1.0 g/L dextran (average molecular weight 40000, a model polysaccharide compound). The norfloxacin levels in each group and the operational conditions were the same as Batch Test I. Sampling was conducted on 0, 0.5, 1, 1.5, 2, 3 day to observe the change of the soluble protein and soluble carbohydrates.

2.3.3. Batch Test-III

Batch Test-III was designed to investigate acidogenesis of the WAS anaerobic digestion influenced by the different concentration of norfloxacin. Batch Test-III shares the same condition as Batch Test-I and Batch Test- II, except for the substrate which was substitute by 1.0 g/L glucose (a model monosaccharide compound). The test took 9 h and the soluble carbohydrates were measured for every 3 h.

2.3.4. Batch Test-IV

So as to explore the effects norfloxacin created on the stage of methanogenesis, Batch Test- IV was carried on to assess the biogas production. Serum bottles with the total volume of 120 mL were divided

into several groups all filled with 26 mL sodium acetate with a concentration of 5.0 g/L and 52 mL inoculum. Other conditions are the same as Batch Test-I. The volume and components of gas production was assessed at 1, 2, 3, 4, 5, 6, 7 day, respectively. Then the cumulative methane production was calculated with the same method as described in the BMP tests.

2.4. Batch test for drug metabolism and microbial community analysis and ARGs assessment

This batch test is mainly used to investigate the metabolic mechanism of norfloxacin and its impacts on microorganisms and ARGs in anaerobic digestion. Two groups of 250 mL serum bottles were filled with 60 mL WAS and 120 mL inoculum sludge, and the norfloxacin concentration of each group was set at 1.7 mg/g and 3.3 mg/g, respectively. The high norfloxacin concentrations were applied here to facilitate the identification of norfloxacin degradation metabolites. The serum bottles were sealed with butyl rubber stoppers, and then were placed in a 35 ± 1 °C shaker at 120 rpm. On 0, 15, 30 and 45 day, one bottle was opened in each group, the sludge samples were centrifuged at 12000 rpm and filtered by the 0.45 μm syringe filter, and then collect the supernatant for norfloxacin and metabolites analysis.

The analysis were carried out using liquid chromatography-tandem mass spectrometry (LC-MS/MS) method with the sample preconcentration carried out by solid phase extraction (SPE). The 6 cc hydrophilic-lipophilic balance (HLB) cartridges (Waters, U.S.A.) were used for the solid phase extraction. After extraction, the samples were dissolved in the mobile phase of LC-MS/MS. The UltiMate 3000 HPLC instrument (Thermo Fisher Scientific, U.S.A.) equipped with a Hypersil Gold C18 column (100mm × 2.1 mm, 3 μm, Thermo Fisher Scientific) and the Q-Exactive Plus mass spectrometer instrument (Thermo Fisher Scientific) were used for the LC-MS/MS analysis. The flow rate for separation was 0.3 mL/min and the injection volume was 1 μL. Composition of the mobile phase was A: acetonitrile, B: 0.1% formic acid (0–1 min, 10%A; 1–8 min, 20% A; 8–18 min, 25%A; 18–20 min, 50%A; 20–23 min , 90% A; 23–25 min, 10%A). The operating conditions of the mass spectrometer in positive mode were shown as following: spray voltage, 3.20 kV; capillary temperature, 350 °C; sheath gas flow rate, 45; aux gas flow rate, 10; collision energy, 27 E/V.

2.5. High-throughput 16S rRNA sequencing

The analysis of microorganism community was conducted by high-throughput 16S rRNA sequencing method. The digested sludge sample with norfloxacin level of 0.3 mg/g and 1.7 mg/g was applied for this analysis. Firstly, the DNA samples of digested sludge were extracted by a TIANNAMP Soil DNA kit (TianGen, China) and then detected by agarose gel electrophoresis and Nano Drop 2000 (Thermo Fisher Scientific, U.S.A.). Secondly, an ABI GeneAmp® 9700 thermocycler was used for the polymerase chain reaction (PCR) amplification with the primer of 515FmodF (5′-GTGYCAGCMGCCGCGGTAA-3') and 806RmodR (5′-GGACTACNVGGGTWTCTAAT-3') . The PCR products were extracted using the AxyPrep DNA gel extraction kit (AXYGEN) and conducted by QuantiFluor™-ST blue fluorescence quantitative system (Promega). The PCR products were further processed using the TruSeq™ DNA Sample Prep Kit and subjected to Illumina Miseq PE250 sequencing.Finally, the i-sanger platform (http://www.i-sanger.com) was used for splicing the sequenced PE reads according to the overlap relationship, and then calculated the corresponding relative abundance, diversity data and the community structure at each classification level according to the results of microbial OUT cluster analysis and species taxonomy analysis. PICRUSt2 analysis was adopted for predicting metagenome information using the 16S rRNA sequencing data. Predictions were made by corresponding the marker gene data and the reference genomes in databases, Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.kegg.jp/).

2.6. Antibiotic resistance genes (ARGs) assessment

To assess the effects of norfloxacin on the abundance of ARGs in anaerobic digestion system, the class 1 integron (*intl1*) and fourteen quinolone-related resistance genes (*gyrA* , *gyrB* , *mdtH* , *mdtM* , *orpM* , *parC* , *parE* , *pmrA* , *qnrA* , *qnrB* , *qnrC* , *qnrD* , *qnrS* and *tolC*) were selected to be determined. The digested sludge sample with norfloxacin level of 0.3 mg/g and 1.7 mg/g and undigested sludge were analyzed for observing the change of ARGs relative abundance and horizontal gene transfer in anaerobic digestion system. Firstly, the total DNA of samples were extracted by TIANNAMP Soil DNA Kit (TianGen, China) and determined by the spectrophotometer (Q3000, Quawell, San Jose, CA, USA). The primer sequences for quantitative polymerase chain reaction (qPCR) in ARGs analysis were shown in Table 1. Then, the qPCR was carried out by StepOnePlus™ real time qPCR (Thermo Fisher Scientific, USA), with a qPCR system of: 1 μL DNA Sample, 0.4 μL Primer F, 0.4 μL Primer R, 5 μL TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) (2×), ROX Reference Dye (50×) and 3 μL ddH2O. Denaturation was performed at 95 °C for 30 s, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C for annealing. Finally, the relative abundances of ARGs were further calculated by the method of $2^{-\Delta C_T}$ [18].

2.7. Other analysis methods

The basic sludge parameter such as TS, VS, TCOD, SCOD, and NH_4^+-N in the test were analyzed according to standard methods [19]. For soluble organic matters in the supernatant of sludge determination, the protein was monitored according to the Lowry-Folin method with a standard of BSA [20] and the carbohydrates were detected by the Anthrone method with a standard of glucose [21]. The composition of anaerobic digestion biogas (CH_4, CO_2 and H_2) was analyzed by the gas chromatograph (GC-SP6890, Shandong Lunan Ruihong Chemical Instrument Co. Ltd., China) equipped with a thermal conductivity detector (TCD) [22]. The results shown in this study were expressed as mean ± standard deviation while all measurements of the experiments were conducted in triplicate. Furthermore, the one-way analysis of variance

Table 1
The primer sequences for qPCR in antibiotic resistance genes analysis.

Target gene	Primer sequence
gyrA	5′-CCAACAATGACCGACATCGC-3′
	5′-GCGGTTAGATGAGCGACCTT-3′
gyrB	5′-CAGACTGCCAGGAACGCGAT-3′
	5′-AGCCAAGCGCGGTGATAAGC-3′
mdtH	5′-CTGCCGTTAAATGGATGTATGC-3′
	5′-ACTCCAGCGGGCGATAGG-3′
mdtM	5′-GAGGCGTTCGGACAGACAA-3′
	5′-CCAACAGTAAGCCAACAAATGA
oprM	5′-CGCGAAGATCCAGAAGGACA-3′
	5′-GAGCTGGTAGTACTCGTCGC-3′
parC	5′-GGTGGAATATCGGTCGCCAT-3′
	5′-AAACTTCGACGGCACTTTGC-3′
parE	5′-ATGCGTGCGGCTAAAAAAGTG-3′
	5′-TCGTCGCTGTCAGGATCGATAC-3′
pmrA	5′-TTTGCAGGTTTTGTTCCTAATGC-3′
	5′-GCAGAGCCTGATTTCTCCTTTG-3′
qnrA	5′-AGGATTTCTCACGCCAGGATT-3′
	5′-CCGCTTTCAATGAAACTGCAA-3′
qnrB	5′-GGMATHGAAATTCGCCACTG-3′
	5′-TTYGCBGYYCGCCAGTCG-3′
qnrC	5′-ATTACGGGTTGTAATTTGTCTTATG-3′
	5′-ATCAGAAAATGATCCCCTACT-3′
qnrD	5′-GGAGCTGATTTTCGAGGG-3′
	5′-AGAAAAATTAGCGTAACTAAGATTTGTC-3′
qnrS	5′-GTGAGTAATCGTATGTACTTTTGC-3′
	5′-AAACACCTCGACTTAAGTCT-3′
tolC	5′-GGCCGAGAACCTGATGCA-3′
	5′-AGACTTACGCAATTCCGGGTTA-3′
intI 1	5′-CGAACGAGTGGCGGAGGGTG-3′
	5′-TACCCGAGAGCTTGGCACCCA-3′

(ANOVA) was used to analyze the data and the P value was <0.01, for assessing the significant statistical difference.

3. Results and discussion

3.1. Impact of norfloxacin on bioenergy production during sludge anaerobic digestion

The impact of norfloxacin on methane production during sludge anaerobic digestion was shown in Fig. 1A. The cumulative methane production increased gradually with the AD progress and there is no significant difference of both methane production rates and total methane productions in the tests ($P < 0.05$) with different norfloxacin dosing rates. To better understand the impacts of norfloxacin on different phases of anaerobic digestion, i.e. solubilization, hydrolysis, acidogenesis and methanogenesis, concentrations of selected indicators of each phase were measured on different time scales. As shown in Fig. 1B, the sCOD concentrations in all tests gradually increased from during the 3.0×10^3 mg/L at 0 h to 3.4×10^3 mg/L at 9 h, indicating that the solubilization process was unaffected by norfloxacin addition. Similarly, soluble carbohydrates, soluble protein and glucose degradation profiles with different norfloxacin levels were overlayed as shown in Fig. 1C–E, suggesting norfloxacin would also not affect hydrolysis and acidogenesis in sludge anaerobic digestion. However, the maximum methanogenesis rates, calculated based on the linear regression of methane productions on the first three days, increased from 5.3 mL/day to 6.7 mL/day when the norfloxacin level increased from 0 to 1.7 mg/gTS. This result suggested the norfloxacin could enhance the methanogenesis process. However, since sludge solubilization and hydrolysis was the rate limiting step of sludge anaerobic digestion, even the individual methanogenesis was promoted by norfloxacin, the overall methane production would not be increased. It has been reported that norfloxacin may pose inhibitory effect on organic waste anaerobic digestion, the different norfloxacin effect overserved in this study may because a lower norfloxacin levels was applied [23]. As the norfloxacin concentrations used in this section covered the real norfloxacin concentration in the SLUDGE, thus it can be expected the level of norfloxacin in the sludge would not pose negative effect on bioenergy generation during anaerobic digestion.

3.2. The variation in microbial communities in the sludge anaerobic digestor caused by norfloxacin

The high-throughput 16S rRNA sequencing was conducted to investigate the potential shift in microbial community structure in the sludge anaerobic digestor caused by norfloxacin exposure. The α diversities of the sludge samples with different norfloxacin exposure level after anaerobic digestion were compared in Fig. 2A. The results showed that the norfloxacin would neither significantly affect the community richness as indicated by ACE and Chao 1 indices nor change the community diversity as suggested by the Shannon and Simpson indices. The Venn diagram further compared the similarity of the microbial

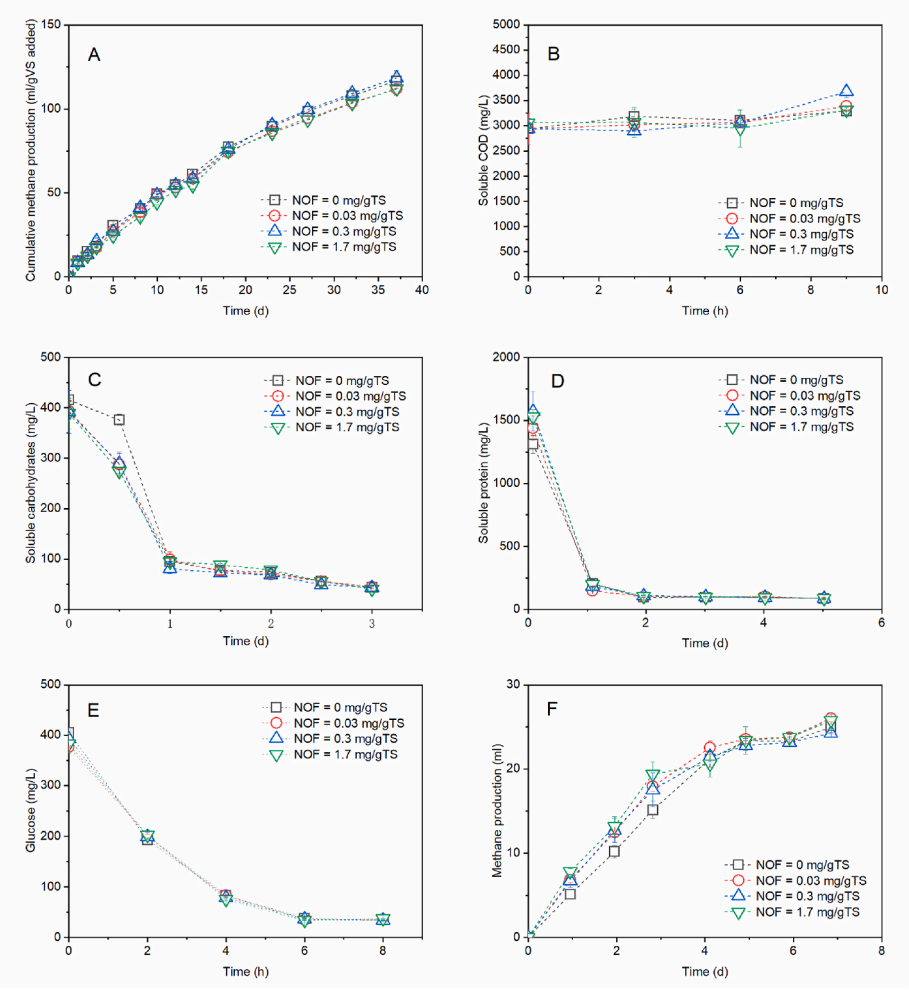


Fig. 1. The of norfloxacin on biological methane production during sludge anaerobic digestion (A) and each individual step of the anaerobic digestion processes: sludge solubilization (B), hydrolysis (C & D), acidogenesis (E) and methanogenesis (F).

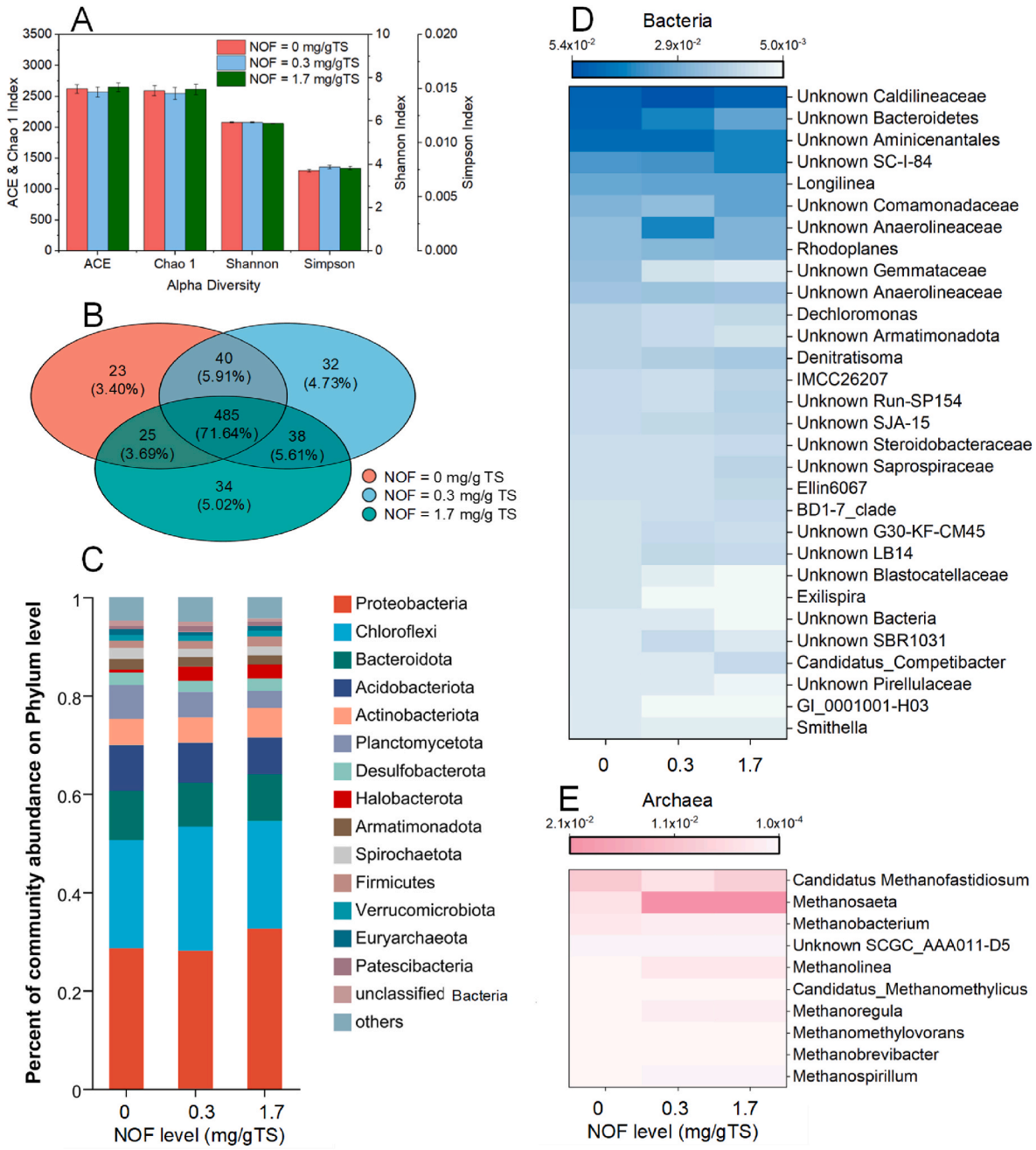


Fig. 2. The microbial community structure variation in anaerobically digested sludge sample with norfloxacin exposure levels at 0, 0.3, 1.7 mg/gTS: (A) The comparison of α diversities indices; (B) The Venn diagram comparing the microbial community structures; (C) The percent of community abundance on phylum level with the others referring to the phylum abundance less than 0.01 (D) Heatmap showing the relative abundances of top 30 genera in Bacteria domain; (E) Heatmap showing the relative abundances of top 10 genera in Archaea domain.

community structures in different samples (Fig. 2B). Overall, 71.64% of the OTU was shared by all the three sludge samples. However, with the increase of norfloxacin level, the more specified OTU can be identified. This could also be reflected by that the number of OTU shared by sludge samples of 0 mg/gTS and 1.7 mg/gTS (3.69%) was less than that shared by sludge samples of 0 mg/gTS and 0.3 mg/gTS (5.91%). This result suggested that microbial communities shifting was occurred with the norfloxacin addition.

Specifically, on phylum level (Fig. 2C), The percentages of Proteobacteria, Firmicutes Patescibacteria and Halobacterota increased from 28.6% to 32.5%, 1.5%–2.1%, 0.6%–0.9%, 0.6%–2.8% respectively, with the increase of norfloxacin levels from 0 to 1.7 mg/gTS. In contrast, the relative abundances of 6 major phyla were decreased, including Acidobacteriota (9.3%–7.4%), Planctomycetota (6.9%–3.5%), Armatimonadota (2.2%–1.9%), Spirochaetota (2.2%–1.7%), Euryarchaeota (1.2%–0.98%) and an unknown bacterium (1.1%–1.6), suggesting norfloxacin was unfavorable for their growth. To gain a deeper insight, the top 30 abundant genera in bacteria and top 10 abundant genera in archaea were selected and compared (Fig. 2 D and E). As shown in Fig. 2D, the relative abundance of 10 genera in bacteria increased for more than 10% with the norfloxacin exposure levels increasing. Among them, *Candidatus Competibacter* [24], a well known glycogen-accumulating organism, showing the highest increase of 34% and *Denitratisoma*, *Ellin6067* and an unknown *Saprospiraceae* increasing over 20%. On the other hand, 8 genera decreased for more than 10% with norfloxacin addition and *Exilispira* decreasing the most about 60%.

As for archaea, a significant increase was shown in the genus of *Methanosaeta*, as its relative abundance increased from 0.5% to 2.1% with the increasing norfloxacin level. *Methanosaeta* belongs to the phylum of Halobacterota and it is a well-known acetoclastic methanogen using acetate as the sole substrate for methanogenesis [25,26]. Similarly, some other methanogens in Halobacterota, such as *Methanolinea Methanoregula, Methanomethylovorans* and *Methanospirillum* were also increased, despite that the abundance of each genus was less than 0.5%. Among these genera, *Methanolinea Methanoregula, and Methanospirillum* were well known hydrogenotrophic methanogens and *Methanomethylovorans* is typical methylotrophic methanogen [27–30]. The increase of these methanogens was in accordance with the increase of the Halobacterota as shown in Fig. 2C and it may also explain the enhance of methanogensis rate as shown in Fig. 1F. On the other hand, the relative abundance of *Candidatus Methanofastidiosum* (methylotrophic) and *Methanobacterium* (hydrogenotrophic) belonged to the phylum of Euryarchaeota decreased with the increase of norfloxacin level, and the relative abundance of *Candidatus Methanomethylicus* (methylotrophic) in the phylum of Crenarchaeota also dropped [31,32].

3.3. Changes in microbial transformation pathways in the anaerobic digestor after norfloxacin exposure

The abundances of functional genes involved in metabolisms, genetic information processing, environmental information processing and cellular processes as indicated in KEGG level 2 pathway in different reactors were predicted by PICRUSt2 and compared in Fig. 3. The results showed that norfloxacin exposure would not significantly affect the most of these processes as the variations relative abundances were within −2%–4% when the norfloxacin dosage increase from 0 to 1.7 mg/gTS expect for the pathway of cellular community-eukaryotes. In comparison, the relative abundance of cellular community-eukaryotes pathway enriched 1.7 times with norfloxacin addition. The cellular community-eukaryotes pathway mainly include adhesion, junction and signaling of the eukaryotes. The enhancement indicated that eukaryotes were likely to gather after norfloxacin exposure, which may explain the resistance of eukaryotes to norfloxacin treatment.

Although the overall microbial transformation was insignificantly affected, the methanogensis was improved by norfloxacin dosing as showing Fig. 1F. Therefore, the functional genes involved in methanogenesis were explored in detail. Fig. 4 summarized four methanogensis pathways as presented in KEGG module and the relative abundances of genes involved each process (summarized in Table 2) were compared.

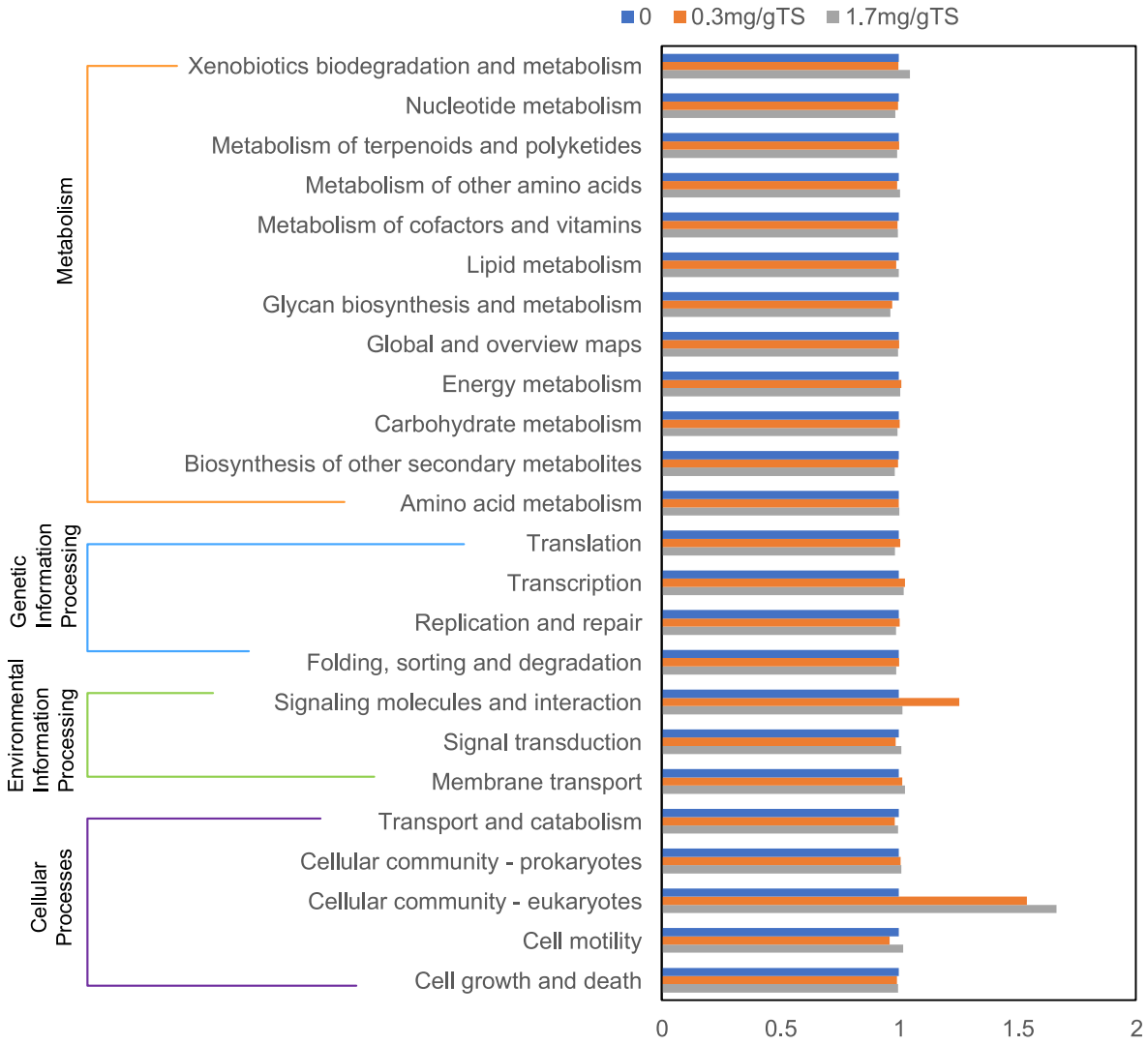


Fig. 3. Relative abundance of KEGG level 2 pathways in different reactors were predicted by PICRUSt2. The abundances of enzymes at norfloxacin dosing rates 0.3 and 1.7 mg/gTS were presented as the ratios to those without norfloxacin dosing, which were assumed as 1.

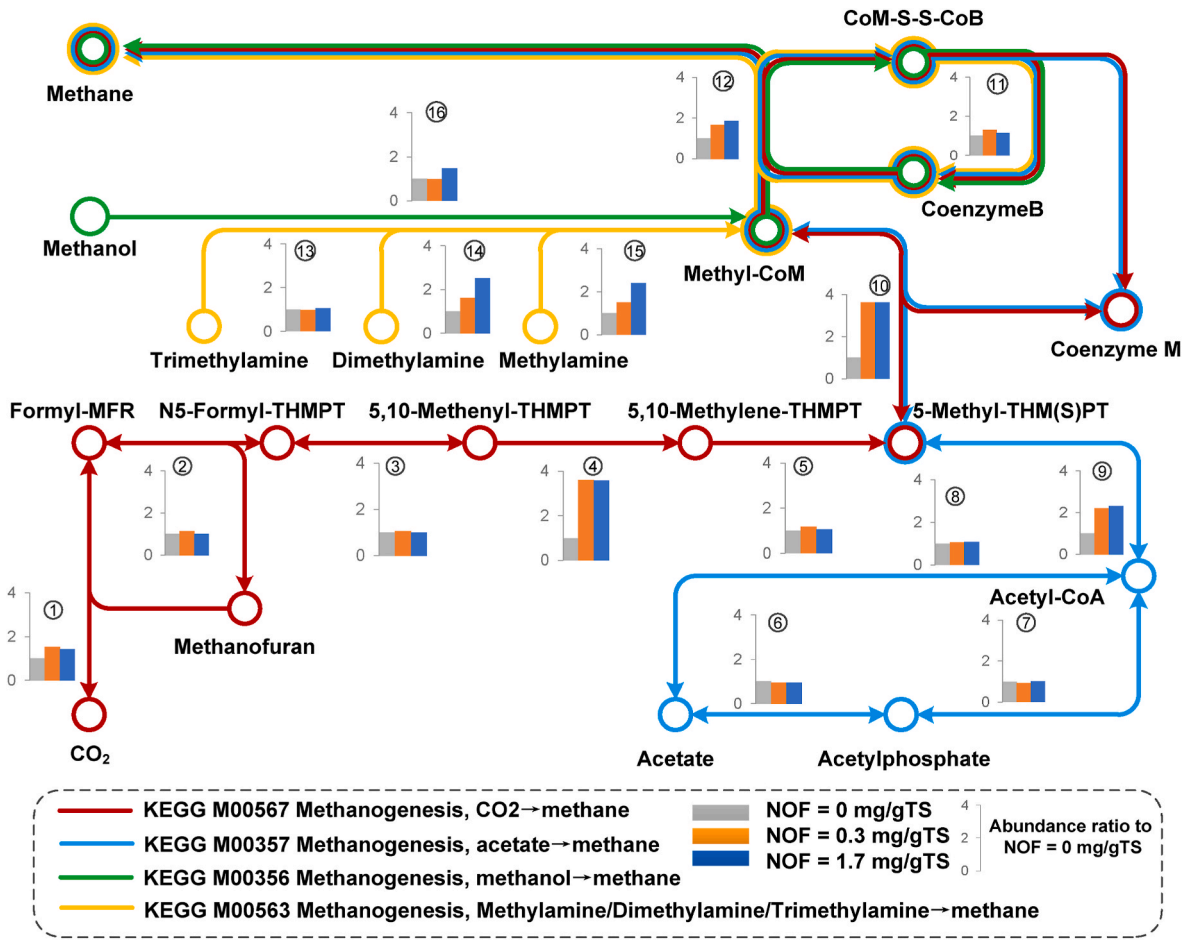


Fig. 4. The microbial pathways and functional profiles of methanogensis processes in the anaerobic digestors with different norfloxacin dosing rates predicted by PICRUSt2. The dots refer to metabolic products and the arrows refer to transformation paths. The inserts - compared the total abundances of enzymes detected in each reactor which involved in the corresponding pathway, with the enzyme commission number listed in Table 2. The abundances of enzymes at norfloxacin dosing rates 0.3 and 1.7 mg/gTS were presented as the ratios to those without norfloxacin dosing, which were assumed as 1.

Table 2
Enzymes involved in methanogenesis pathways eq \o\ac(○,1)- eq \o\ac(○,16)as presented in Fig. 4 according to KEGG database.

No.	Pathway description	Enzymes involved
1	CO2 → Formyl-MFR	1.2.7.12
2	Formyl-MFR → N5-Formyl-THMPT	2.3.1.101
3	N5-Formyl-THMPT ↔ 5,10-Methenyl-THMPT	3.5.4.27
4	5,10-Methenyl-THMPT ↔ 5,10-Methylene-THMPT	1.12.98.2; 1.5.98.1
5	5,10-Methylene-THMPT → 5-Methyl-THM(S)PT	1.5.98.2
6	Acetate ↔ Acetylphosphate	2.7.2.1
7	Acetylphosphate ↔ Acetyl-CoA	2.3.1.8
8	Acetate ↔ Acetyl-CoA	6.2.1.1
9	Acetyl-CoA ↔ 5-Methyl-THM(S)PT	ACDS
10	Methyl-CoM ↔5,10-Methyl-THM(S)PT + Coenzyme M	2.1.1.86
11	CoM-S-S-CoB → Coenzyme B	1.8.7.3; 1.8.98.1; 1.8.98.4; 1.8.98.5; 1.8.98.6
12	Coenzyme B + Methyl-CoM → CoM-S-S-CoB + Methane	2.8.4.1
13	Trimethylamine → Methyl-CoM	MfbA; MttB; MttC
14	Dimethylamine → Methyl-CoM	MfbA; MfbB; MfbC
15	Methylamine → Methyl-CoM	MfbA; MtmB; MtmC
16	Methanol → Methyl-CoM	MtaA; MtaB; MtaC

Specifically, the acetoclastic pathway involved four major steps, i.e. acetate→acetyl-CoA→5-Methyl-THM(S)PT→Methyl-CoM→Methane. Three of these steps were significantly upregulated by 1.9–3.6 times with the norfloxacin dosage increased to 1.7 mg/gTS. This is in accordance with the highest increase of *Methanosaeta* abundance among all the methanogens as shown in Fig. 2E. The hydrogenotrophic with CO_2 reduction pathway as well as the methylotrophic pathway were also upregulated in some genes. For example, the genes abundances involved in 5,10-Methenyl-THMPT → 5,10-Methylene-THMPT, Dimethylamine → Methyl-CoM and Methylamine → Methyl-CoM processes increased by 3.6, 2.5 and 2.4 times after norfloxacin dosage increased to 1.7 mg/gTS. However, the hydrogenotrophic and methylotrophic were not always increased with the increase of norfloxacin dosing. Therefore, enrich of the methanogen by norfloxacin may not directly related to their substrate. However, the enrichment may depend on the phylogenetic characteristics [33], as the abundances of most methanogens in the phylum Halobacterota were enhanced with the norfloxacin addition.

3.4. The impact of norfloxacin on ARGs removal during sludge anaerobic digestion

The anaerobic digestion is identified as an effective way to remove ARGs in sewage sludge to prevent antimicrobial resistance issues. The potential impact of norfloxacin on ARG removal was evaluated by comparing the abundances of quinolone-related resistance genes in the sludge before anaerobic digestion and after digestion with different

dosing rates. Overall, eight quinolone-related resistance genes, i.e. *qnrB, qnrS, gyrB, gryA, mdtM, parC, parE,* and *pmrA* as well as the class 1 integron (*intI1*) were identified in the sludge samples. Among the identified genes, *gyrA*, *gyrB* can ecode DNA gyrase and *parC* and *parE* can ecode topoisomerase IV proteins, which resulted in one of the main mechanisms of fluoroquinolone resistance, i.e., amino acid substitutions in the DNA gyrase and topoisomerase IV proteins [34]. In addition, *qnrB* and *qnrS* would cause plasmid-mediated fluoroquinolone resistance, by coding for proteins of the pentapeptide repeat family that protects DNA gyrase and topoisomerase IV from quinolone inhibition [35]. On the other hand, *mdtM* and *pmrA* are associated with the efflux pumps of antibiotics and *intI1* was related to horizontal gene transfer [36,37].

As shown in Fig. 5, the relative abundances of eight genes reduced significantly after anaerobic digestion. The reduction of *intI1*, *parC* and *gyrB* were 31%, 36% and 50%, respectively in the case of norfloxacin level at 1.7 mg/gTS. As for *qnrB, qnrS, mdtM, parE* and *pmrA,* the relative abundances could become negligible after anaerobic digestion. As for *gyrA*, the abundance decreases by 15% in the 1.7 mg/gTS norfloxacin case but slightly increased by 10% in 0.3 mg/gTS norfloxacin case, suggested that *gyrA* is more persistent than other genes during anaerobic digestion. The comparison of ARG abundances in the digested sludge with 0.3 and 1.7 mg/gTS norfloxacin suggested that the increase of norfloxacin level did not significantly affect the ARGs removal rate. As 1.7 mg/gTS is over ten to a hundred times above the concentration identified in real sewage sludge [7,8], our result suggested the norfloxacin in the sludge would not pose additional risks of antibiotic resistance as long as anaerobic digestion was applied.

3.5. Metabolism of norfloxacin during sludge anaerobic digestion

The metabolism of norfloxacin during anaerobic digestion was conducted on the basis of LC-MS/MS analysis with supporting fragmentation patterns from preceding experiments. Surprisingly, the norfloxacin concentration detected in the sludge liquor was increased with the anaerobic digestion process (Fig. 6). It has been reported that norfloxacin can be easily adsorbed on sludge due to its high partition coefficients ($k_{d,\ sludge}$ = 367–5123 L/kg) [38]. The increase of norfloxacin concentration probably because that the adsorbed norfloxacin were released to the liquid phase due to the change of sludge characteristics with the anaerobic digestion progress. It is also likely that norfloxacin uptaken by the microorganisms were released as the cell lysis during the anaerobic digestion.

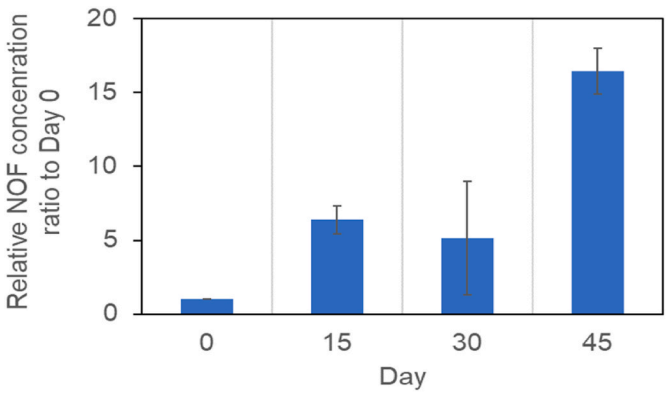


Fig. 6. Relative NOF concentration in the anerobic digestion reactor to Day 0 as detected by LC-MS/MS.

The main 13 metabolic and transformation products are shown in Table 3. Based on these products, 5 potential metabolic pathways are proposed and illustrated in Fig. 7. In the Path A: norfloxacin was deflorinated and the hexatomic ring of core quinolone group was decomposed, forming NOR-8. Alternatively, in Path B, piperazine ring cleavage took place and potential intermediate I was formed [39,40] followed by further deflorination and ring opening which generated NOR-9 and NOR-6 with a subsequent loss of ethyl. In Path C, NOR-11 was derived from an ensuing elimination of ethyl from a previously reported intermediate II formed by the rupture of C–F bond and piperazine ring breakage [41]. At this point, the loss of N atom in the hexatomic ring could generate NOR-10 as shown in path C1, and that of the substituent group from former piperazine ring on benzene ring could form NOR-5 in path C2. NOR-2 would be then formed after the

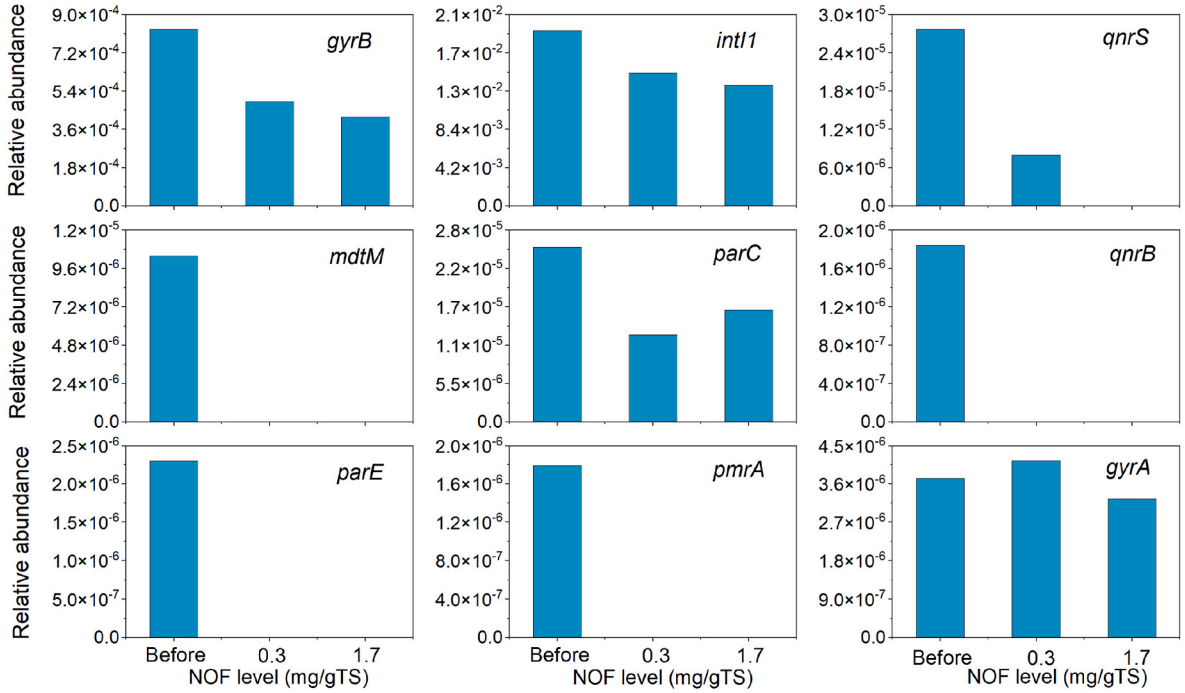
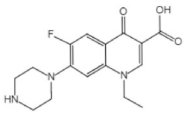
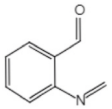
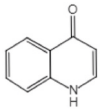
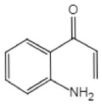
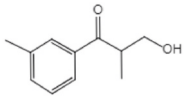
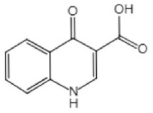
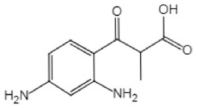
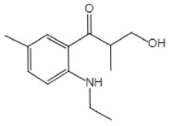
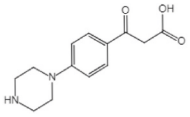
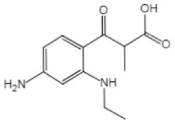
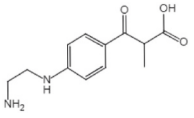
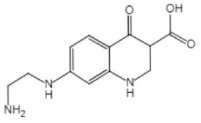
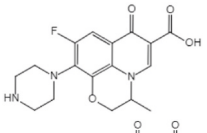
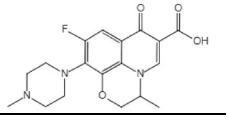


Fig. 5. The relative abundance of quinolone-related resistance genes detected in the sludge before and after anaerobic digestion with different norfloxacin exposure level.

Table 3
The main metabolic and transformation products of norfloxacin during sludge anaerobic digestion.

	Proposed Formula	*m/z*	Retention Time(min)	Chemical Structure
NOR	$C_{16}H_{18}FN_3O_3$	319.13	4.331	
NOR-1	C_8H_7NO	133.05	5.217	
NOR-2	C_9H_7NO	145.05	5.336	
NOR-3	C_9H_9NO	147.07	6.328	
NOR-4	$C_{11}H_{14}O_2$	178.10	21.112	
NOR-5	$C_{10}H_7NO_3$	189.04	23.259	
NOR-6	$C_{10}H_{14}N_2O_3$	210.10	2.396	
NOR-7	$C_{13}H_{19}NO_2$	221.14	2.415	
NOR-8	$C_{13}H_{16}N_2O_2$	232.12	4.672	
NOR-9	$C_{12}H_{18}N_2O_3$	238.13	2.403	
NOR-10	$C_{12}H_{18}N_2O_3$	238.13	2.403	
NOR-11	$C_{12}H_{15}N_3O_3$	249.11	19.552	
NOR-12	$C_{17}H_{18}FN_3O_4$	347.13	8.941	
NOR-13 (Levofloxacin/Ofloxacin)	$C_{18}H_{20}FN_3O_4$	361.14	9.897	

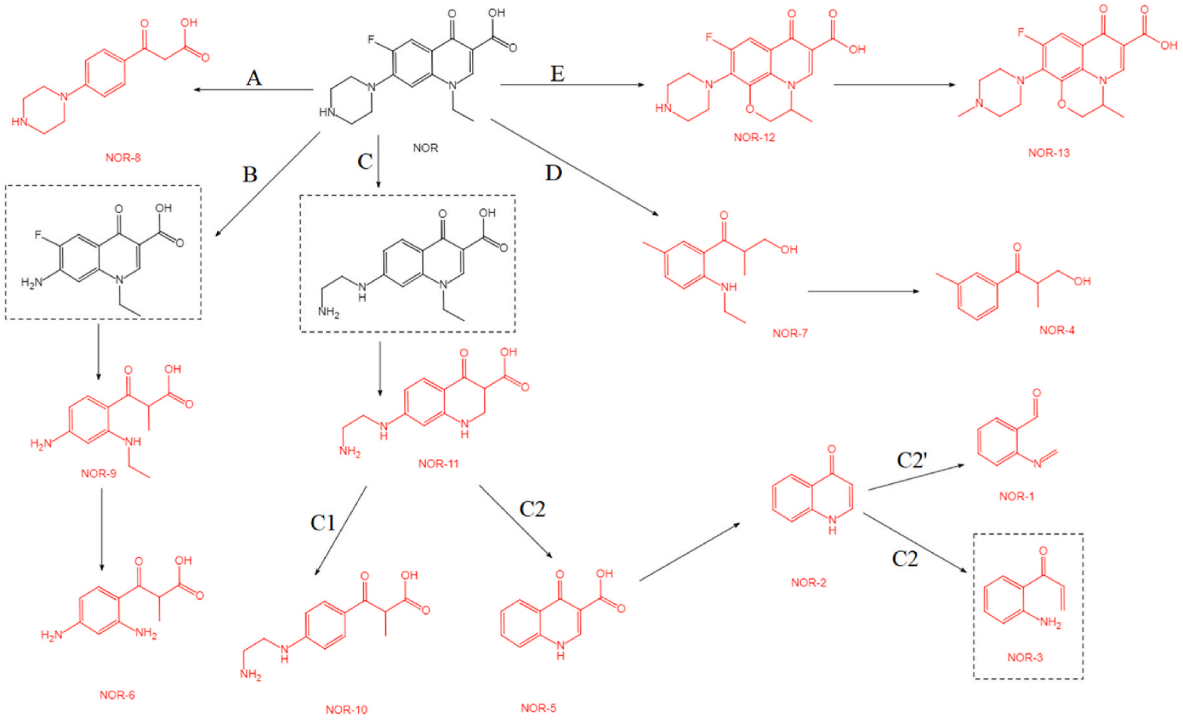


Fig. 7. Proposed metabolic pathways of norfloxacin during sludge anaerobic digestion.

decarboxylation of NOR-5, and the ensuing ring opening resulted in 2 metabolites, NOR-1 and NOR-3, of which the latter was reported by Ref. [42] In Path D, in NOR-7, the F atom was substituted by methyl, the piperazine ring was lost, and the core quinolone group was modified with ring opening and decarboxylation. However, intermediates not detected which should exist before the formation of NOR-7. Then, with the loss of substituent group containing N atom on benzene ring, NOR-4 was formed. For Path E, the formation of morpholine moiety resulted in NOR-12, and with the introduction of methyl on piperazine ring, NOR-13 was generated which was identified as Levofloxacin/Ofloxacin.

According to the proposed pathways, the piperazine ring, core quinolone group and F atom are main targets during the anerobic digestion process. Notably, most intermediates are deflorinated which was rarely occurred according to previous studies [39,40], indicating the distinctiveness degradation property of norfloxacin in the anaerobic digestion system. It should also be noted that one main metabolic product detected in this study, i.e. NOR 13, is highly likely to be Levofloxacin/Ofloxacin, another kind of fluoroquinolone antibiotic, along with the intermediate NOR-12 (Ofloxacin impurity E). This result provides collective evidence for a potential transformation within fluoroquinolone class. With regard to the transformation from NOR-12 to NOR-13, the Eschweiler-Clarke reaction may occur. During the norfloxacin degradation process, the decarboxylation would generate formic acid and some intermediates might be produced through the loss of formaldehyde molecule. The formic acids and formaldehyde were served as important reactant for Eschweiler-Clarke reaction. It has been reported that some norfloxacin degradation intermediates, such as NOR 11 and NOR 3 detected in this study exert a significantly lower toxicity on aqueous organisms [42]. However, Levofloxacin/Ofloxacin was reported to be more toxic than norfloxacin for some aquatic species as indicated by the lower lowest-observable-effect concentration and 50% effective concentration [43,44]. In addition, the toxicities of other intermediates are worthy to be investigated in future to fully understand the environmental impact of norfloxacin after anaerobic digestion.

4. Conclusions

This study investigated the impact of norfloxacin on sewage sludge anaerobic digestion in term of bioenergy production and potential environmental risks. The following conclusion can be drawn based on the results of this study.

- Norfloxacin concentration at the environmental relevant level will not significantly affect the total bioenergy production during the sludge anaerobic digestion.
- The methanogenesis process will be enhanced by norfloxacin with the increase of *Methanosaeta* abundance, a well-known acetoclastic methanogenic archaea.
- The presence of norfloxacin at the environmental relevant level will not decrease the removal efficiencies of fluoroquinolones-family ARGs by the anaerobic digestion process.
- Thirteen metabolites of norfloxacin degradation during sludge anaerobic digestion have been identified, with most being defluorinated, and five different degradation pathways, including Eschweiler-Clarke reaction, were proposed.
- The potential environmental risks caused by the increase of the norfloxacin concentration after anaerobic digestion due to cell lysis and the toxicity of its degradation metabolites should be considered in practices.

CRediT authorship contribution statement

Shuting Zeng: Investigation, Methodology, Validation, Formal analysis, Writing – original draft. **Jing Sun:** Conceptualization, Methodology, Resources, Writing – review & editing, Project administration, Funding acquisition. **Xuyang Lü:** Investigation, Visualization. **Zitong Peng:** Investigation. **Bin Dong:** Writing – review & editing. **Xiaohu Dai:** Writing – review & editing, Resources. **Bing-Jie Ni:** Resources, Funding acquisition, Supervision.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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