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Biodegradation mechanism of typical fluoroquinolones in sewage-sludge composts

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ABSTRACT

The biodegradation mechanism of fluoroquinolones (FQs) during composting is the key information to accurately assess and effectively control the environmental risk caused by FQs in sewage sludge. This study investigated the biodegradation of two FQs (norfloxacin [NOR] and ofloxacin [OFL]) using a series of sludge composts over a 56-d incubation period. Sludge composts were collected at the mesophilic, thermophilic, and curing phases from the matrix of a pilot-scale sewage sludge composter; spiked with NOR and OFL (each 5 mg/kg); and incubated at 35 (mesophilic), 55 (thermophilic), and 40°C (curing). Compost samples were evaluated by determining FQ concentration, intermediate metabolites, enzymatic activity (laccase, manganese peroxidase, and lignin peroxidase), and bacterial and fungal communities. Results showed that NOR was likely to be predominantly biodegraded via the acetylation route and the routes for OFL in each phase varied, while the main metabolites from both NOR and OFL retained the FQ core structure related to the antibacterial activity. Only manganese peroxidase had a significant correlation with FQ residue during the mesophilic phase. Proteobacteria and Ascomycota were the dominant bacterial and fungal phyla in the incubated sludge compost, respectively, and *Pseudomonas* was the most involved bacterial genus for FQ biodegradation in the sludge composts with *Exophiala*, *Dissoconium*, *Talaromyces* as the fungal genera.

Keywords: Sewage sludge; Antibiotics; Composting; Biodegradation; Microbial community

1. Introduction

Antibiotics resistance genes (ARGs) have attracted significant attention in recent decades due to their ubiquitous presence in the environment, including surface waters [1], soil [2], and the atmosphere [3]. Antibiotic residue is a primary inducer of ARGs in environment [4]. For example, Zhu et al. [2] found that the application of manure compost with a high antibiotic residue enhanced the abundance and activity of ARGs in the soil. Furthermore, antibiotics used by human beings are absorbed into sewage sludge at wastewater treatment plants [5,6] and discarded into the soil via the application of sewage sludge following composting or anaerobic digestion [7,8]. Consequently, sewage

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sludge is considered to be the main pollution source of anthropogenic antibiotics in soil.

Fluroquinolones (FQs) are high-risk antibiotics in sewage sludge from China [9–11]. They are also widely used as human antibiotics in China, accounting for approximately one third of antibiotic sales, and have a high residue in the environment [12]. A national investigation by Chen et al. [9] showed that FQs were the key antibiotics in Chinese sewage, with a detection frequency of 92%–100% and a residue of approximately 2.0–6.0 mg/kg. Considering that composting is the main treatment for sewage sludge before its land application in China [13], it is important to investigate the occurrence and fate of FQs in sewage sludge during the composting process for minimizing its potential ecological risk.

A researcher found that FQs of low concentration (~mg/kg) in sewage sludge are not eliminated by composting [14], whereas FQs of high concentration (~mg/kg) can be effectively removed from sewage sludge by composting, and biodegradation is considered to be the most important pathway for FQ removal [15,16]. Zhang et al. [16] investigated the dissipation of the FQs norfloxacin (NOR) and ofloxacin (OFL) during the composting of sewage sludge for 30 d when their concentrations were high (5 mg/kg), and also observed high removal efficiencies (87%–95%), mainly through biodegradation. To illustrate FQ biodegradation mechanisms, studies have investigated different types of fungi and bacteria. Fungi, such as white-rot fungi [17,18], brown-rot fungi [19], and ascomycete [20], can effectively degrade or even mineralize FQs using non-specificity enzymes such as laccase (Lac), manganese peroxidase (MnP), and lignin peroxidase (LiP). Other studies found that bacteria including *Labrys portucalensis* [21] and *Rhodococcus* sp. [22] can also biodegrade FQs. However, the removal of FQs by biodegradation does not mean the elimination of environmental risk from FQs, since recent researches found that the FQ metabolites after biodegradation can still retain a high-level antibacterial activity [17,23]. So, the biodegradation mechanism of FQs during composting is the key information for the accurate assessment and effective control of the environment risk caused by FQs in sewage sludge.

Notwithstanding, uncertainty still exists regarding the biodegradation mechanism of FQs in sewage sludge during composting, specifically with respect to the biodegradation pathways, intermediate chemicals, corresponding enzymes, and microbial communities involved. In this study, we investigate FQ biodegradation mechanisms in sewage sludge composting by conducting a bench-scale series of 0.5 L incubation experiments using compost with the addition of 5 mg/kg each of NOR and OFL. Samples from the incubation experiments were then used to detect the FQ concentration, intermediate products of FQ biodegradation, enzymatic activity of Lac/MnP/LiP, and the bacterial and fungal communities. Using this information, we explore the biodegradation mechanisms of FQ during the aerobic composting of sewage sludge. This information can be used to control and manage the environmental risk from FQs in sewage sludge.

2. Materials and methods

2.1. Raw materials and chemical reagents

Mechanically dewatered sewage sludge was collected from a municipal wastewater treatment plant in Guilin, China. Sawdust purchased from a suburban wood-processing manufactory was screened to remove particles >10 mesh (2.00 mm) and then dried in the oven at 50°C for 24 h. The dried sawdust was used as an amendment to improve the physicochemical properties of the sludge. The properties of the raw materials are listed in Table 1.

Ultrapure water was obtained using an ultrapure water system (Milli-Q A10, Millipore, the United States). Methanol and acetonitrile of pesticide-residue-grade-analysis quality were purchased from Tedia (Fairfield, the United States), while standard FQs (NOR and OFL; 99%) from Aladdin (Shanghai, China) were used to prepare stock solutions (100 mg/L NOR and 100 mg/L OFL) using methanol as a solvent. An Oasis HLB cartridge (500 mg, 6 mL) and a SAX cartridge (500 mg, 6 mL) were purchased from Waters (Milford, MA, the United States) and Agilent (Santa Clara, CA, the United States), respectively, for cleaning the solid sample extracts. Prior to use, the SAX and HLB cartridges were pre-conditioned in sequence with 6 mL MeOH and 6 mL ultrapure water.

2.2. Experimental design

2.2.1. Design of the experiments

Pilot sewage sludge composting experiments without the addition of FQs were carried out in a polyvinyl-chloride (PVC) frustum-shaped bioreactor of 60 L (height 520 mm, top diameter 450 mm, bottom diameter 320 mm). The bioreactor was coated in a layer of mineral

Table 1
Physicochemical properties of raw materials for aerobic composting

Materials	Sewage sludge	Sawdust
Moisture content (%)	83.0 ± 0.2	11.5 ± 0.1
Volatile solid (db[a], %)	70.3 ± 0.9	96.2 ± 0.2
Total C (db[a], %)	29.6 ± 0.3	45.0 ± 0.3
Total N (db[a], %)	5.19 ± 0.04	0.88 ± 0.05
C/N	5.7	52.2
Fluoroquinolones (db[a], mg/kg)	1.01 ± 0.62(NOR), 1.35 ± 0.33(OFL)	0.09 ± 0.03(NOR), <0.1(OFL)

[a]db indicates dry basis.

wool 20 mm thick to prevent heat loss. At the bottom of the bioreactor, plastic hollow balls with a diameter of 5 mm were placed to set the ventilation tube, which was connected to a pump for aeration with ambient air. The mixing ratio of sewage sludge to sawdust was 3.5:1 (*w*:*w*), resulting in a total weight of 18.0 kg and moisture content of 64.5%. The aeration rate was 0.5 L/min, adjusted by an air flow meter (0.3–2.7 L/min). The pilot matrix was mixed once every 2 or 3 d, and the experiment lasted for 30 d. After thorough mixing, three 10-g composts were collected from the center of matrix (height, 160–350 mm) on days 1, 3, 5, 7, 13, 15, 18, 21, 24, and 27. Before lyophilization, the moisture of the compost samples was determined. The treatment was run in duplicate.

Bench incubation experiments were carried out with the compost collected from the mixed pilot-scale matrix in three phases: the mesophilic (day 1, 35°C), thermophilic (day 5, 55°C), and curing phases (day 14, 40°C). Each 180 g compost was put into a 0.5 L bioreactor, and mixed after being spiked with the FQ stock solutions to increase a concentration of 5.0 mg/kg (based on dry solid weight of the compost). Three incubation treatments of the mixed composts were run with an aeration rate of 15 mL/min at different temperatures according to the corresponding composting phases: 35°C (mesophilic phase), 55°C (thermophilic phase), and 40°C (curing phase). For each treatment, a sterilized treatment was run as the control, in which the sludge compost was sterilized twice for 30 min at 121°C before it was placed in the 0.5 L bioreactor. Sterilized ultrapure water was added weekly to the bioreactors to maintain the moisture of composts at approximately 60%. Each incubation lasted for 56 d in the dark to keep FQs from the potential photodegradation [24], and all experiments were conducted in duplicate.

2.3. Analysis methods

2.3.1. Physicochemical properties

The moisture content and organic matter levels were analyzed using the standard methods from Miner [25]. The total C and N levels were determined using an element analyzer (EA2400II, PerkinElmer, MA, the United States), and the C/N ratio was calculated from those values. The temperature of the pilot-scale matrix was obtained by averaging the temperatures measured at three depths (10, 25, and 40 cm) twice a day, at 09:00 and 21:00.

2.3.2. FQs and intermediate products

The raw material and incubated compost samples were collected and lyophilized from the 0.5 L bioreactors on days 0, 7, 14, 21, 28, and 56 to determine the FQ concentration, and samples on days 7 and 21 were used to determine the intermediate products. Lyophilized samples were mortared to pass through a 60 mesh (0.25 mm) nylon sieve, and stored at 4°C before being extracted. A 0.5 g sample was weighed for the extraction and determination of FQs and intermediate products. The sample was extracted, cleaned, concentrated, and resolved using sonic-wave assisted extraction combined with the solid-phase microextraction method described by Zhang et al. [16]. The resolved extract was passed through a 0.22 μm organic membrane filter and sonicated for approximately 1 min before the determination of target chemicals. The FQ concentration was determined using high-performance liquid chromatography equipped with a fluorescence detector (1260, Agilent, the United States), and intermediate products were analyzed using a high-performance liquid chromatography equipped tandem mass spectrometer (6495, Agilent, the United States). Each filtered sample (20 μL) was injected using splitless mode into a column filled with C18 (XBridge, 250 mm × 4.6 mm, 5 μm film thickness; Waters, MA) at 30°C. The mobile phase was a mixture of methanol and phosphate buffer (*v*:*v*, 20:80), and passed through the column at a rate of 1.0 mL/min. The excitation and emission wavelengths of the fluorescence detector were 280 and 450 nm, respectively. For the intermediate product of FQs, each filtered sample (15 μL) was injected using splitless mode into a column filled with C18 column (ZORBAX Eclipse XDB, 150 mm × 4.6 mm, 3.5 μm film thickness, Agilent, the United States) at 25°C. The mobile phases A and B were prepared by mixing acetonitrile and ultrapure water at ratios of 5:95 and 95:5 (acetonitrile: ultrapure water, *v*:*v*), respectively, and then 0.5% formic acid (volume) was added into both A and B. A gradient elution procedure was applied with the ratios of A:B as follows: 90:10 during 0–1 min and 90:10 to 10:100 during 1–30 min. The electrospray ion source was used with positive ion scanning mode at 275°C, a gas volume rate of 9 L/min, capillary voltage of 4.0 kV, atomizer pressure of 310 kPa, and collision energy of 30 eV. Ion detection was performed in multiple reaction monitoring mode. The key parameters for each compound are listed in Table 2, including structure formula, retention time, parent ion, and most intensive fragment ion.

2.3.3. Enzymatic activity and microbial community

The enzymatic activity of Lac, MnP, and LiP was measured for the initial and incubated compost samples collected from the 0.5 L bioreactors on days 0, 3, 7, 14, 21, 28, 35, 49, and 56. The collected samples were pretreated to obtain the enzyme solution using a modified method from Zhao et al. [26], which included water extraction, centrifugation and filtration. A 1.0-g compost sample was extracted via shaking for 30 min using ultrapure water with a ratio of 1:9 (w:v) at 180 rpm, 28°C. The extracted sample was centrifuged for 10 min at 4,000 rpm and 4°C, and the supernatant was filtered with a 0.45 μm filter membrane. Filtrate was diluted with ultrapure water to a volume of 10 mL. The enzymatic activities of Lac, MnP, and LiP in the pretreated samples were measured using the ABTS, $MnSO_4$-H_2O_2, and veratrole alcohol methods, respectively [26]. ABTS method: 1.6 mL of 0.5 mmol/L ABTS solution with an acetate acid–sodium acetate buffer (0.15 mol/L, pH 4) was placed into the cuvette and 1.6 mL of the pretreated sample was added into the cuvette to activate enzymatic action for 3 min at 30°C. The ABTS concentration was measured by observing the absorbance of the mixed solution at a wave length of 420 nm using an ultraviolet spectrophotometer (V-5800PC, Metash Instruments, China). The difference in ABTS concentration was used to calculate the enzymatic activity of Lac; one unit (U) of Lac activity referred to the amount of

Table 2
Metabolite abbreviations, structures, retention times, mass of the protonated molecules, and product ions determined using HPLC-MS/MS (ESI+) of the respective protonated molecule for norfloxacin (NOR) and ofloxacin (OFL)

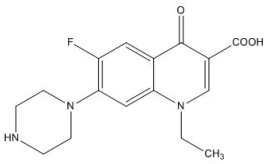
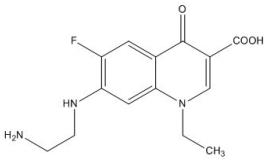
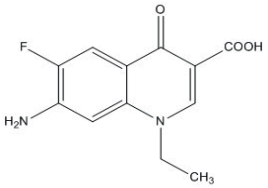
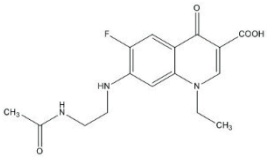
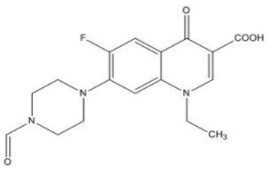
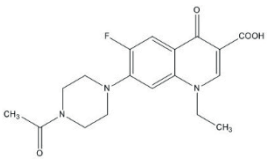
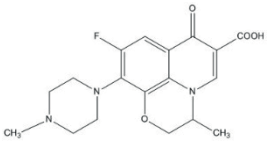
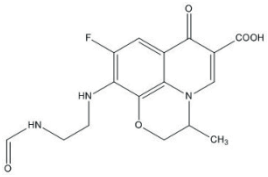
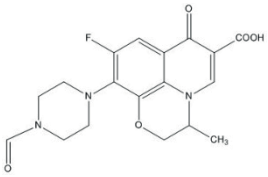
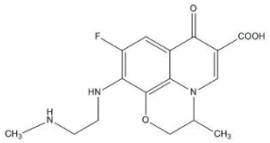
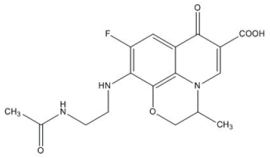
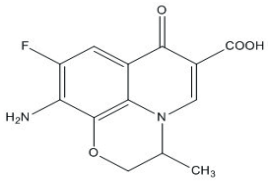
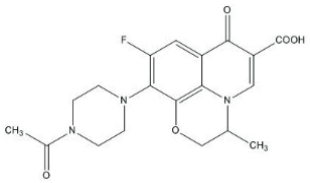
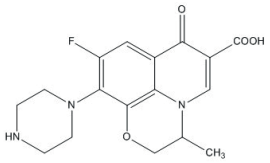
Products	Structure	Chemical names	t_R (min)	$[M+H]^+$ (m/z)	Product ions (m/z)
NOR		Norfloxacin	4.4	320	302, 276, 231
N1		Desethylene-N-norfloxacin	7.8	294	276
N2		7-Amino-1-ethyl-6-fluoro-4-oxo-1, 4-dihydroquinoline-3-carboxylic acid	7.9	251	233, 205, 177, 149
N3		Desethylene-N-acetylnorfloxacin	9.3	336	318
N4		N-Formylnorfloxacin	9.4	348	330, 274
N5		N-Acetylnorfloxacin	7.5	362	344, 274
OFL		Ofloxacin	4.5	362	344, 318, 261
O1		9-Fluoro-10-[(2-formamidoethyl) amino]-3-methyl-7-oxo-2,3-dihydro-7H-[1,4]oxazino[2,3,4-ij] quinoline-6-carboxylic acid	5.6	350	318, 298
O2		9-Fluoro-10-(4-formylpiperazin-1-yl)-3-methyl-7-oxo-2,3-dihydro-7H-[1,4] Oxazino[2,3,4-ij]quinoline-6-carboxylic acid	5.7	376	344, 319, 287

Table 2 *Continued*

Products	Structure	Chemical names	t_R (min)	$[M+H]^+$ (m/z)	Product ions (m/z)
O3		Desethylene-Nofloxacin	4.8	336	318, 298, 261
O4		10-[(2-Acetamidoethyl)amino]-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7H-[1,4]oxazino[2,3,4-ij]quinoline-6-carboxylic acid	3.3	364	-
O5		10-Amino-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7H-[1,4]oxazino[2,3,4-ij]quinoline-6-carboxylic acid	9.3	279	261
O6		10-(4-Acetylpiperazin-1-yl)-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7H-[1,4]oxazino[2,3,4-ij]quinoline-6-carboxylic acid	21.1	390	372
O7		Desmethyl-N-ofloxacin	4.3	348	330, 304, 261

enzyme that catalyzed the conversion of 1 μmol of ABTS per minute. 1.6 mL ultrapure water was analyzed as a blank sample using the same procedure as the pretreated sample. $MnSO_4$-H_2O_2 method: 2.4 mL of 1.25 mmol/L $MnSO_4$ solution with a tartrate buffer (0.50 mol/L, pH 4.5) and 0.6 mL of the pretreated sample was mixed in a colorimetric tube, added into the cuvette, and then 60 μL of 20 mmol/L hydrogen peroxide was injected into the mixed liquor to activate enzymatic action for 3 min at 30°C. The concentration of Mn^{2+} was determined by measuring the absorbance of the mixed solution at a wave length of 290 nm with the V-5800PC ultraviolet spectrophotometer. The enzyme activity of MnP was obtained by calculating the difference in Mn^{2+} concentration; one unit (U) of MnP activity is referred to the amount of enzyme that catalyzed the conversion of 1 μmol of Mn^{2+} per minute. The mixed liquor without the addition of hydrogen peroxide was used as the blank sample. Veratrole-alcohol method: 0.6 mL of 10 mmol/L veratrole alcohol, 1.2 mL of 250 mmol/L tartrate buffer (pH = 3), and 1.2 mL of the pretreated sample was mixed and added into the cuvette, and then 60 μL of 20 mmol/L hydrogen peroxide was injected into the mixed liquor to activate enzyme action for 3 min at 30°C. The concentration of veratrole alcohol was determined by measuring the absorbance of the mixed solution at a wave length of 310 nm using the V-5800PC ultraviolet spectrophotometer. The enzymatic activity of LiP was obtained by calculating the difference in the veratrole alcohol concentrations; one unit (U) of LiP activity is referred to the amount of enzyme that catalyzed the conversion of 1 μmol of veratrole alcohol per minute. The mixed liquor without hydrogen peroxide was used as the blank sample.

The enzymatic activities of Lac, MnP, LiP were calculated with Eq. (1) as follows:

$$U = \frac{V_r \times \Delta A \times 10^6}{V_{en} \times \varepsilon \times t \times L} \times \frac{V_{ex}}{V_{en} \times m} \quad (1)$$

where U is the enzymatic activity, U/g; V_r is the total volume of reaction assay, which is 3.2, 3.06, 3.06 mL for Lac, MnP, and LiP, respectively; V_{en} is the volume of enzyme used, which is 1.6, 0.6, 1.2 mL for Lac, MnP, and LiP, respectively; V_{ex} is the volume of extracted enzyme solution, which is 9 mL for each enzyme; ΔA is the change of absorbance of each reaction assay during the reaction, dimensionless; ε is the molar extinction coefficient of the oxidized substrate in each reaction assay, which is 36,000, 11,590, and 9,300 L/(mol cm) for Lac, MnP, and LiP, respectively; t is the reaction duration time, 3 min; L is the length of cuvette, 1 cm; m is the mass of extracted compost, 1 g.

The microbial community was measured for the initial and incubated compost samples collected from the 0.5 L bioreactors on days 0, 21, and 56. A 5.0 g compost was collected and kept in a sterilized Eppendorf tube at 4°C for no more than 2 d before the follow-up treatment. The sample was treated according to the following steps: extraction, DNA purification, DNA PCR amplification (GeneAmp PCR System 9700, Thermo Fisher, the United States), quantification of the recycled PCR product (Qubit 2.0, Thermo Fisher, the United States), and sequencing of the 16S RNA/ITS amplicon (Miseq PE250, Illumina, the United States). The abundance and diversity of microorganisms were analyzed based on the sequence information and gene-banks of RDP, SILVA, and Greengenes.

2.4. Quality assurance and quality control

Quality control was used to guarantee reliable results for the quantification of FQs in the solid samples. Recovery tests were performed by spiking the diluted stock solution of FQs into the sample at 2.5 and 5 mg/kg (based on the dry weight). The average recoveries were 110.6% ± 4.4% (2.5 mg/kg) and 98.5% ± 5.0% (5.0 mg/kg), and 120.9% ± 1.8% (2.5 mg/kg) and 107.9% ± 4.0% (5.0 mg/kg) for NOR and OFL, respectively.

2.5. Statistical analysis

FQ residue and enzymatic activity were analyzed for a statistical relationship using correlation analysis at a significance level of $\alpha = 0{:}05$ using R software [27].

3. Results and discussion

3.1. Temperature and moisture during pilot-scale composting

Temporal changes of temperature and moisture showed a typical trend during the pilot-scale composting of sewage sludge without the addition of FQs (Fig. 1). There were three phases in the temperature profile of the matrix; the mesophilic (20°C–40°C), thermophilic (40°C–60°C), and curing phases (≤40°C), and they lasted 2, 10, and 16 d, respectively. The accumulated time when temperature of matrix exceeded 50°C (t_{50}) was 6.5 d, which satisfied the sanitary standard for the composting of night soil in China. The peak temperature 59.6°C was reached on day 4, consistent with the temperature profiles of the 60-L aerobic composting of sewage sludge with the addition of FQs of 5 mg/kg^{-1} by Zhang et al. [16]. The matrix moisture increased initially from 60.9% to 64.9%, and then decreased slowly to 50.5% at the end of the experiment. Moisture increases during the mesophilic phase were also observed by Liu et al. [28], and the was attributed to more water production from the aerobic biodegradation of low-molecular organic matter rather than the loss of water vapor through aeration.

3.2. FQ biodegradation

3.2.1. FQ removal

The removal efficiencies of FQs during the three composting phases were thermophilic (48.4%) > mesophilic (43.5%) > curing (31.3%) for NOR (Figs. 2A–C), and mesophilic (38.2%) > thermophilic (31.8%) > curing (16.3%) for OFL (Figs. 2D–F). After subtracting the results from the sterilized control treatment, the removal efficiencies of FQs through biodegradation were thermophilic (32.1%) > mesophilic (29.8%) > curing (14.5%) for NOR and mesophilic (27.5%) > thermophilic (18.9%) > curing (7.0%) for OFL. FQs demonstrated high biodegradation removal efficiency during both the mesophilic and thermophilic phases, showing that both mesophilic and thermophilic microorganisms could excrete enzymes to effectively biodegrade FQs. Conversely, biodegradation in curing phases composts was very low, which may be attributed to exhaustion of the organic substrate and nutrition depletion. Ho et al. [29] and Wang et al. [30] also found that most FQ removal occurred during the mesophilic and thermophilic phases. Compared with NOR, OFL had a lower removal efficiency in the same compost in all three phases, attributed to the higher molecular mass and more complex structure of OFL [18].

3.2.2. Biodegradation pathways

FQ biodegradation initially occurred at piperazine, and relatively stable intermediate products were achieved after approximately two to four steps, the routes of which are shown in Fig. 3. With bacteria or fungi, NOR was decomposed along two pathways N-acylation or N-dealkylation as three routes to five main intermediate chemicals [17,18,31,32]. These routes included the following steps: (1) the

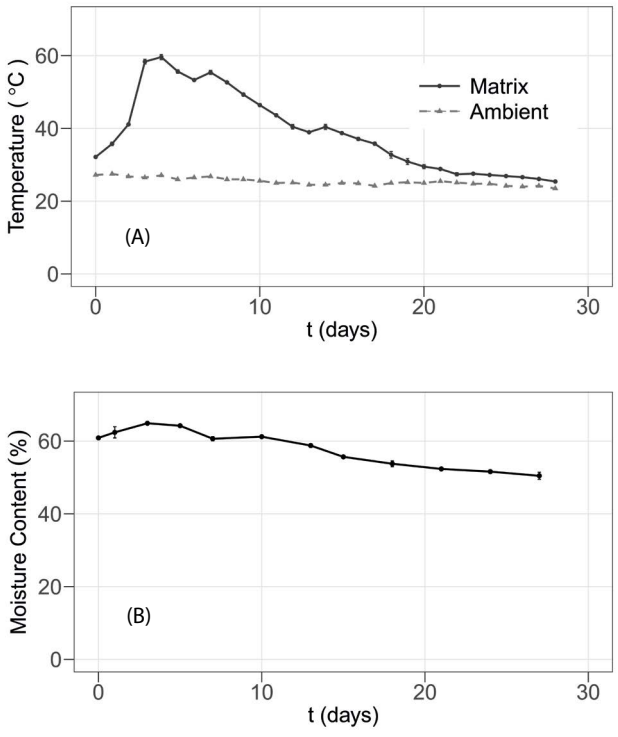


Fig. 1. Temporal changes in the temperature (A) and moisture content (B) during the composting of sewage sludge without the addition of fluoroquinolones (FQs).

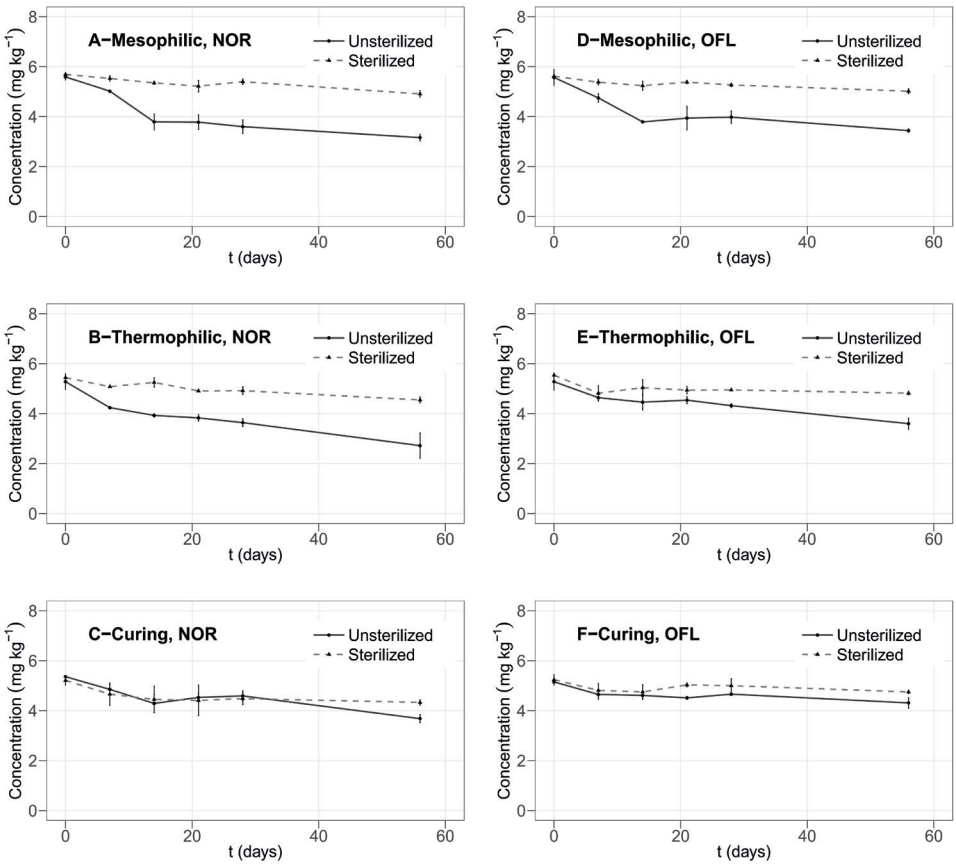


Fig. 2. Concentrations of the FQs norfloxacin (NOR) and ofloxacin (OFL) during different phases of sludge composting: (A, B, and C) NOR concentrations in mesophilic, thermophilic, and curing phase composts, respectively, and (D, E, and F) OFL concentrations in mesophilic, thermophilic, and curing-phase composts, respectively.

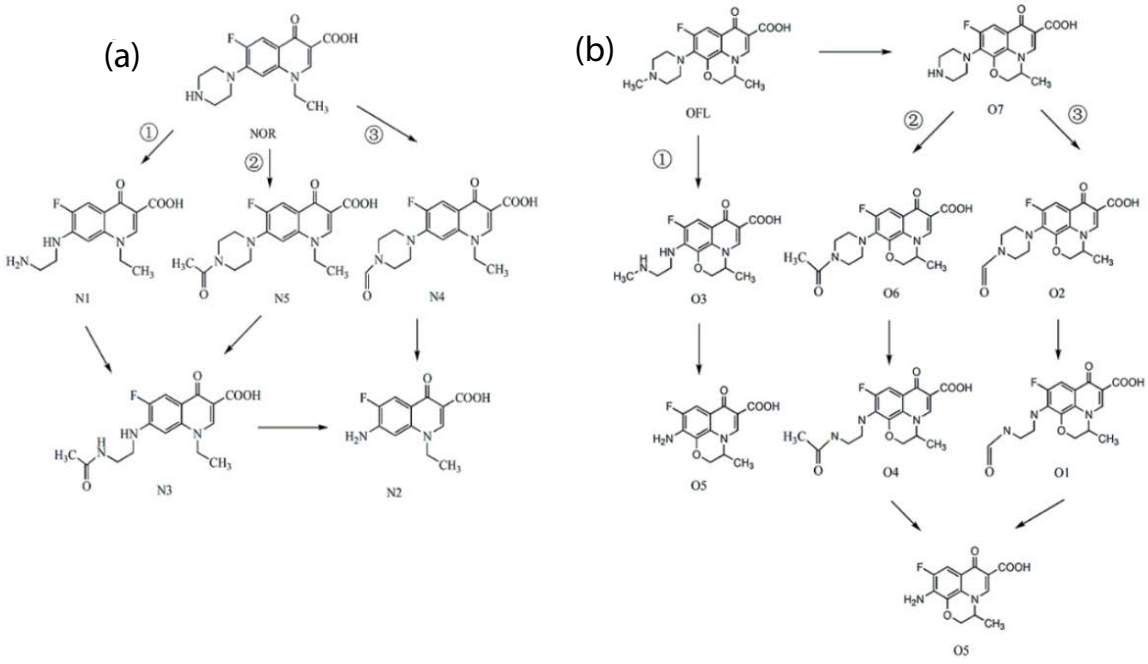


Fig. 3. Main biodegradation pathways for NOR (a) and OFL (b) by lignin-biodegrading microbes [17,18,31,32].

desethylation route, NOR → desethylene-N-norfloxacin (N1) → desethylene-N-acetylnorfloxacin (N3) → 7-amino-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (N2); (2) the acetylation route, NOR → N-acetylnorfloxacin (N5) → N3 → N2; and (3) the formylation route, NOR → N-formylnorfloxacin (N4) → N2. Similar to NOR, OFL could be biodegraded by lignin-degrading microbes to seven intermediate products via three routes [17]: (1) the desethylation route, OFL → desethylene-o (O3) → 10-amino-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7H-[1,4] oxazino[2,3,4-ij] quinoline-6-carboxylic acid (O5); (2) the demethylation–acetylation route, OFL → desmethyl-N-ofloxacin (O7) → 10-(4-acetylpiperazin-1-yl)-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7H-[1,4]oxazino [2,3,4-ij]quinoline-6-carboxylic acid (O6) → 10-[(2-acetamidoethyl)amino]-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7H-[1,4]oxazino[2,3,4-ij]quinoline-6-carboxylic acid (O4) → O5; and (3) the demethylation–formylation route, OFL → O7 → 9-fluoro-10-(4-formylpiperazin-1-yl)-3-methyl-7-oxo-2,3-dihydro-7H-[1,4]oxazino[2,3,4-ij] quinoline-6-carboxylic acid (O2) → 9-fluoro-10-[(2-formamidoethyl)amino]-3-methyl-7-oxo-2,3-dihydro-7H-[1,4]oxazino[2,3,4-ij]quinoline-6-carboxylic acid (O1)→ O5. The FQs biodegradation pathways are different from those observed in the advanced oxidation treatments such as photocatalytic degradation [33], ferrous-activated persulfate oxidation [34], and electrochemical oxidation [35]. During the advanced oxidation process, N-dealkylation and hydroxylation of piperazinyl ring are the two main pathways.

The temporal changes of FQ in most incubation had a similar temporal change (Fig. 2): FQs degraded fast during the first 7 d and the degradation had almost finished at day 21. So, days 7, 21 were chosen as the key timepoints to investigate the intermediates formation. Based on the peak area of intermediate products from high-performance liquid chromatography/mass spectrometry (HPLC/MS/MS; Fig. 4), the main metabolites and routes of FQ biodegradation were identified in the different phases of sludge compost during 0–21 d of incubation (Table 3). The acetylation route was the major biodegradation route in all three sludge composts (Figs. 4A–E). On day 7, N3 was the major metabolite followed by N2, N4, and N5, showing that the acetylation route was the most important route followed by the formylation route during the first 7 d. By day 21, N2 became the main metabolite in both mesophilic and

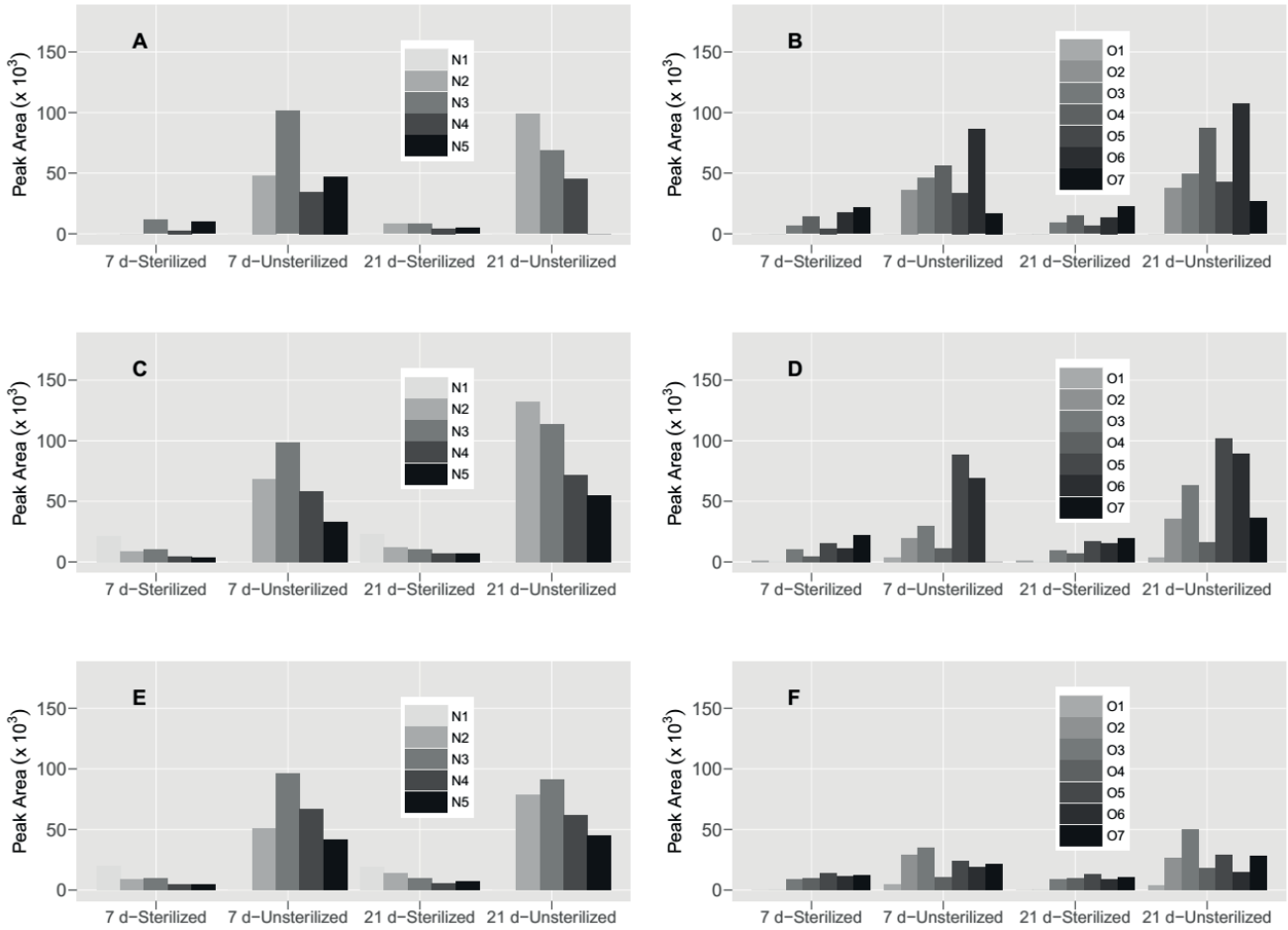


Fig. 4. Peak area of intermediate metabolites for NOR and OFL during incubation of the three-phase sludge composts: A, NOR in mesophilic-phase compost; B, OFL in mesophilic-phase compost; C, NOR in thermophilic-phase compost; D, OFL in thermophilic-phase compost; E, NOR in curing-phase compost; F, OFL in curing-phase compost; the samples was coded as "incubation time + compost treatment" when the sterilized compost was used as the blank treatment.

Table 3
Main metabolites and biodegradation routes of fluoroquinolones (FQs) in the bench incubations of different-phase sludge composts

FQs	Sludge compost	Main metabolites		Main biodegradation routs
		0–7 d	7–21 d	
NOR	Mesophilic-phase compost	N3	N2	Acetylation route
	Thermophilic-phase compost	N3	N2	Acetylation route
	Curing-phase compost	N3	N3	Acetylation route
OFL	Mesophilic-phase compost	O6	O6	Demethylation–acetylation route
	Thermophilic-phase compost	O5/O6	O5/O6	Demethylation–acetylation route, Desethylation
	Curing-phase compost	O3	O3	Desethylation

thermophilic phase compost, and N3 was the main metabolite in curing phase compost. The variation in peak areas for NOR metabolites showed that the acetylation route was still the main biodegradation route in all three composts; however, the levels differed. The rate-limiting steps were the transformation from N3/N4 to N2 and from N5/N4 to N3/N2 for the mesophilic and thermophilic phase composts, respectively. All steps in the curing phase compost were slower compared with the other two composts. The main metabolite and biodegradation routes differed between three types of compost for OFL (Figs. 4B, D, and F). In mesophilic phase compost, O6 was the dominant metabolite, the demethylation–acetylation route was the main biodegradation pathway, and the transformation from O4 to O5 was the rate-limiting step because more O4 and O6 accumulated compared with O5 between days 0 and 21. In thermophilic phase compost, O5 and O6 were the dominant metabolites, the desethylation and demethylation–acetylation routes were the main biodegradation pathways, and the rate-limiting step was the transformation from O6 to O4. In curing phase compost, O3 was the dominant metabolite, the while desethylation route was the main biodegradation pathway, and the rate-limiting step as the transformation from O3 to O5. It has been widely reported that the metabolites from FQ degradation still result in a considerable antibacterial activity [17,24,35]. Since the FQ core structure related to antibacterial agents has not been largely damaged in the biodegradation, the major metabolites of FQs in sewage-sludge compost could retain the antibacterial activity and potentially enhance proliferation of resistance genes. Therefore, more attention should be paid on the probable environmental risk caused by FQ metabolites in sewage-sludge compost.

3.3. Enzyme activity

The enzymatic activities of Lac, MnP, and LiP had different temporal trends in the different composts (Fig. 5). Lac had similar temporal profiles during the incubation of the mesophilic phase compost with that in the curing phase compost; increasing rapidly at first and then decreasing rapidly. There was limited enzyme activity with respect to Lac in the thermophilic phase compost (Fig. 5A). Because Lac can utilize both phenol and non-phenol structures, the difference in Lac activity between the three phases can be attributed to Lac being excreted by mesophilic fungi [36]. MnP activity decreased gradually with incubation time, and tended to decrease initially and then increase during the other two phases (Fig. 5B). Considering that most of the substrates inducing MnP excretion from microorganisms are phenol structures that tend to be released from degraded lignin during the thermophilic and curing phases [37], the decomposition of lignin was beneficial to the synthesis and activation of MnP during these two phases. Compared with MnP activity, LiP displayed similar trends during incubation (Fig. 5C). Because the induced substrate was a non-phenol structure for the secretion of LiP [38], the similarity of the MnP and LiP profiles can be attributed to the likelihood that they shared the same substrate–lignin. The humped shape in both the MnP and LiP activity profiles can be attributed to inhibition caused by the high concentration of toxic FQs following the initial incubation, which reduced with the gradual reduction of residual FQs.

3.3.1. Relationship between FQ residue and enzyme activity

On the basis of Pearson's correlation analysis, MnP activity in the mesophilic phase sludge compost had a significant positive relationship with FQ residue (Fig. 6A). The correlation between FQ residue and MnP activity was 0.90 and 0.94 for NOR and OFL, respectively, indicating that FQ residue had strong positive relationship with MnP. No significant relationship was observed between Lac and LiP activity and FQ residue or between MnP activity and FQ residue in thermophilic and curing phase composts (Figs. 6B and C). Consequently, the removal of FQ from mesophilic phase compost can be attributed to MnP. This attribution was in accordance with the findings of Čvančarová et al. [17], who found that MnP was the main peroxidase in the biodegradation of FQs when investigating changes in Lac, LiP, and MnP activities during the FQ biodegradation process using five different fungi.

3.4. Microbial community

Bioinformatics analysis was applied to analyze operational taxonomic units (OTUs) in all samples with a similarity of 97%.

3.4.1. Bacteria

In all compost phases, five main bacterial phyla were identified totaling a relative abundance of 90% and included Proteobacteria, Firmicutes, Acidobacteria,

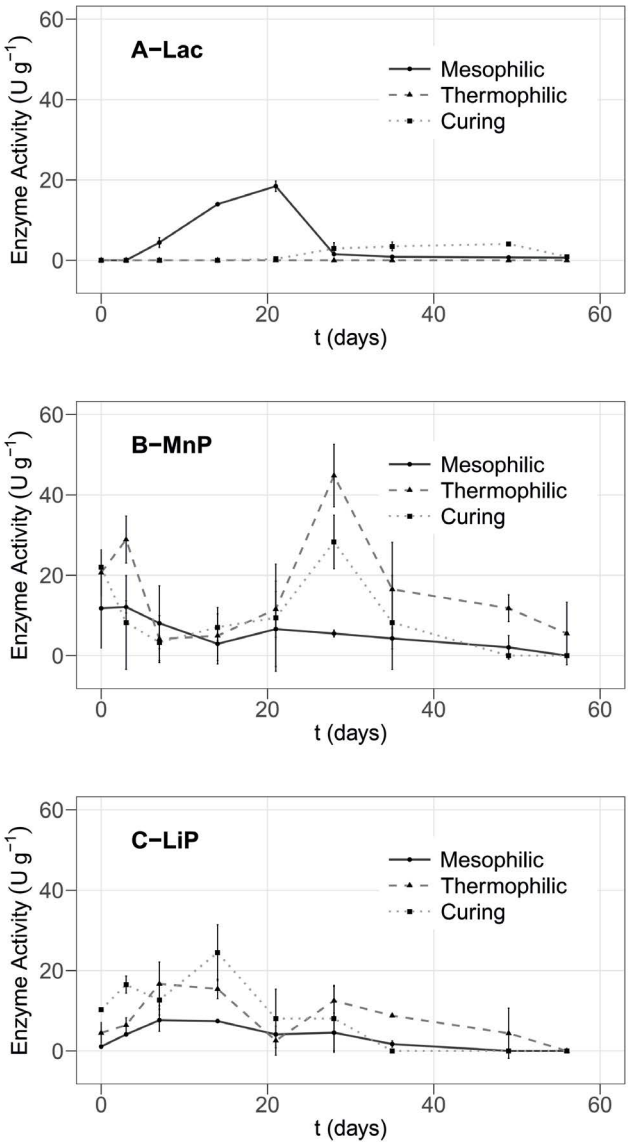


Fig. 5. Temporal changes in the enzyme activities of laccase (Lac), manganese peroxide (MnP), and lignin peroxidase (LiP) during the different phases of sludge composting with FQs.

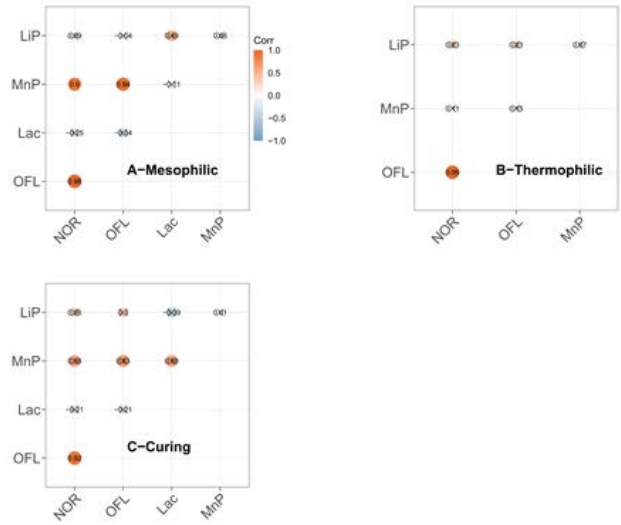


Fig. 6. Pearson coefficient matrix based on enzyme activities and FQ residue at the level p = 0.05 for (A) mesophilic, (B) thermophilic, and (C) curing-phase composts.

Chloroflexi, and Actinobacteria (Fig. 7A). Among these phyla, Proteobacteria had the highest abundance, with mean values of 82.4%, 77.7%, and 81.5% for mesophilic, thermophilic, and curing phase composts, respectively. During incubation, the abundance of Proteobacteria increased steadily in mesophilic phase compost, increased and then decreased in thermophilic phase compost, and decreased gradually in curing phase compost. Similarly, Meng et al. [39] also observed that the rapid biodegradation of FQs was related to the domination of Proteobacteria and Bacteroidetes in a membrane bioreactor. Consequently, Proteobacteria could be the dominant bacteria in the degradation of FQs. Actinobacteria could also be involved the biodegradation of FQs during the thermophilic phase considering its high relative abundance during this phase. Seven bacterial genera were identified as the main phyla (relative abundance > 1%) in the composts, including *Pseudomonas*, *Escherichia-Shigella*, *Bordetella*, *Sphingomonas*, *Stenortrophomonas*, *Blastococcus*, and *Sphingobium* (Fig. 7B). *Pseudomonas* had a higher relative abundance compared with other genera in the majority of the sludge composts during the incubation. The results from correlation analysis showed that *Pseudomonas* and *Escherichia-Shigella* had the highest correlation coefficients with the FQ residue (Fig. 8A). Besides, *Pseudomonas* had the highest relationship with the enzymatic activity of MnP, which has been identified as the main peroxidase involved in the biodegradation of FQs. Though MnP was traditionally observed from the fungi, the recent studies showed that it also can be produced by some bacteria especially *Pseudomonas* spp. [40]. So, *Pseudomonas* can be considered as the major genus related to the biodegradation of FQs in the sludge composts, and it was speculated to accelerate the biodegradation of NOR and OFL by oxidizing the polycyclic structure with the extracellular MnP.

3.4.2. Fungi

Five fungi phyla were identified as the main phyla in all compost phases, including Ascomycota, Basidiomycota, Rozellomycota, Zygomycota, and Chytridiomycota (Fig. 7C). Ascomycota was the dominant fungi phylum and there were marked differences between the five phyla. During incubation, the abundance of Ascomycota increased steadily in mesophilic and curing phase composts, and increased initially and then decreased in thermophilic phase compost. Because Ascomycota had the highest relative abundance in all samples, this phylum is likely to be the most important fungi in the biodegradation of FQs during sludge composting. A similar result was observed by Rusch et al. [20,41] and Čvančarová et al. [17], who found that *Xylaria longipes* (a species of Ascomycota) effectively decomposed FQs including NOR, OFL, and

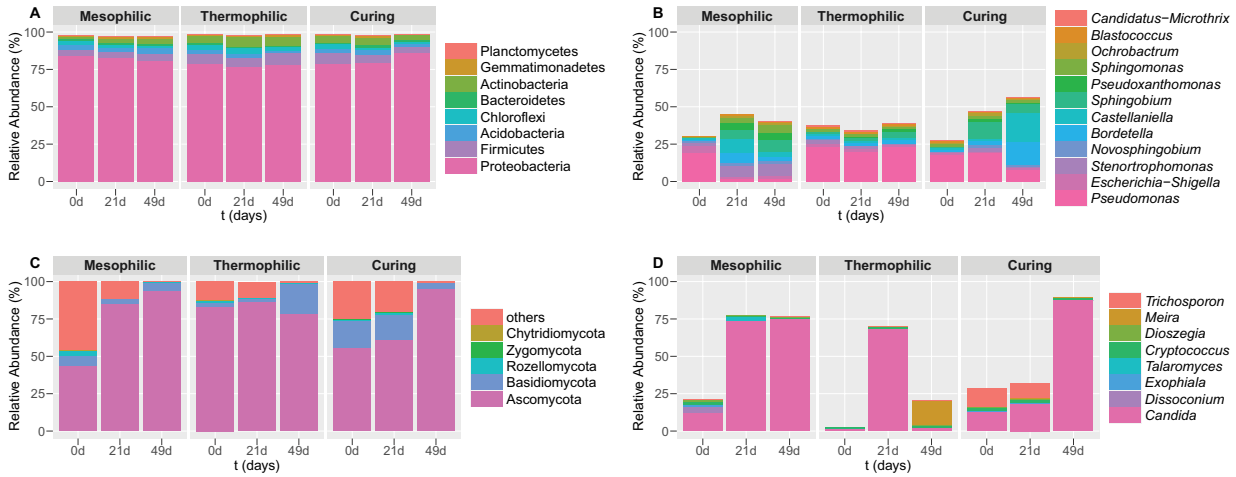


Fig. 7. Relative abundance (>1%) of microbial communities in samples during the composting phases of sludge with FQs: (A) bacteria at the phylum level, (B) bacteria at the genus level, (C) fungi at the phylum level, and (D) fungi at the genus level.

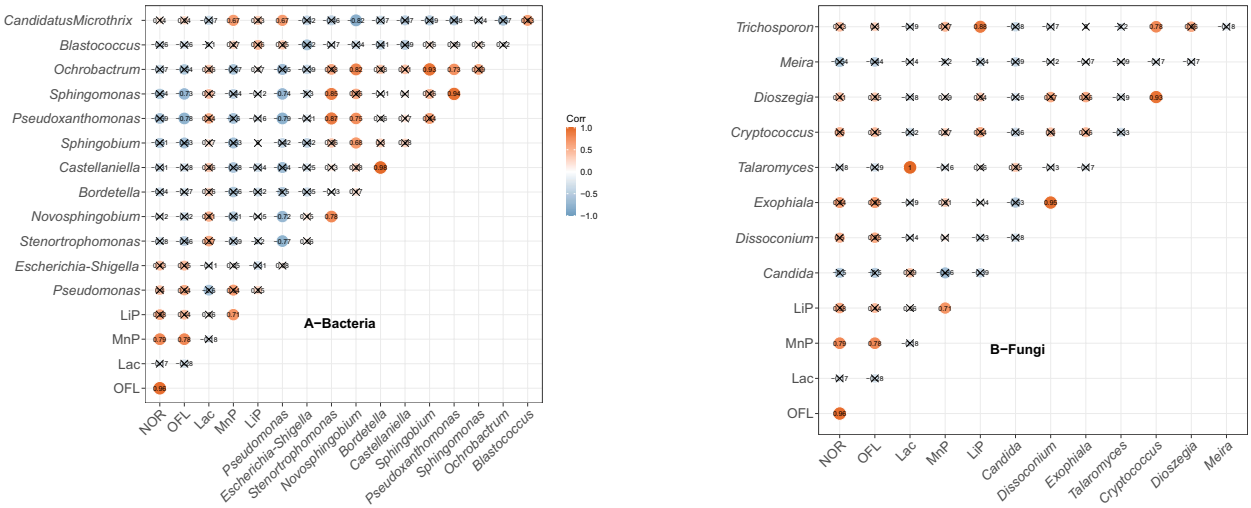


Fig. 8. Pearson coefficient matrix based on enzyme activities, FQ residue and relative abundancy of microbial genera during incubation of sludge composts at the level $p = 0.05$ for (A) bacteria, and (B) fungus.

CIP. Eight fungi genera were identified in the composts, including *Candida*, *Trichosporon*, *Dissoconium*, *Exophiala*, *Talaromyces*, *Cryptococcus*, and *Meira* (Fig. 7D). Even though *Candida* was the dominant fungus genera in most of the sludge composts during the incubation, it had a negative correlation with FQ residue, which meant that *Candida* could be not attributed to the main fungus for the biodegradation of FQs in the sludge composts. By contrast, *Exophiala*, *Dissoconium*, *Talaromyces* showed a relative higher positive correlation coefficient than the others (Fig. 8B), which implied that these fungal genera could have the more attribution for the biodegradation of FQs in the sludge composts.

4. Conclusion

- For all sludge compost phases, the main metabolites of NOR were N3 at days 0–7 and N2 at days 7–21, and the transformation from N3/N4 to N2 and N5/N4 to N3/N2 were the limiting reactions in the mesophilic and thermophilic phases, respectively, when the acetylation route was speculated as the potential route for NOR biodegradation. The dominant metabolites during OFL degradation were O6, O5/O6, and O3 in the mesophilic, thermophilic, and curing phases, respectively, corresponding to the demethylation–acetylation, desethylation and demethylation–acetylation, and desethylation decomposition routes as the possible routes for OFL biodegradation in the mesophilic, thermophilic, and curing phases, respectively.
- Only MnP activity displayed a significant positive relationship ($p < 0.05$) with FQ residue during incubation in mesophilic phase sludge compost, but not for Lac and LiP. Throughout the composting process, Proteobacteria and Ascomycota always were the dominant bacteria and fungus phyla, respectively, and *Pseudomonas* was the

majorly involved bacterial genus for FQ biodegradation in the sludge composts while *Exophiala*, *Dissoconium*, *Talaromyces* as the involved fungal genera.
- The future research is suggested to focus on investigating the antibacterial activity of FQs' metabolites in sewage-sludge composts.

Acknowledgments

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References

[1] G. Wang, S. Zhou, X. Han, L. Zhang, S. Ding, Y. Li, D. Zhang, K. Zarin, Occurrence, distribution, and source track of antibiotics and antibiotic resistance genes in the main rivers of Chongqing city, southwest China, J. Hazard. Mater., 389 (2020) 122110, doi: 10.1016/j.jhazmat.2020.122110.
[2] Y.G. Zhu, T.A. Johnson, J.Q. Su, M. Qiao, G.X. Guo, R.D. Stedtfeld, S.A. Hashsham, J.M. Tiedje, Diverse and abundant antibiotic resistance genes in Chinese swine farms, Proc. Natl. Acad. Sci. USA, 110 (2013) 3435–3440.
[3] W. Ouyang, B. Gao, H. Cheng, L. Zhang, Y. Wang, C. Lin, J. Chen, Airborne bacterial communities and antibiotic resistance gene dynamics in PM2.5 during rainfall, Environ. Int., 134 (2020) 105318. https://doi.org/10.1016/j.envint.2019.105318.
[4] M.-H. Huang, W. Zhang, C. Liu, H.-Y. Hu, Fate of trace tetracycline with resistant bacteria and resistance genes in an improved AAO wastewater treatment plant, Process Saf. Environ. Prot., 93 (2015) 68–74.
[5] S. Hu, J. Hu, B. Liu, D. Wang, L. Wu, K. Xiao, S. Liang, H. Hou, J. Yang, In situ generation of zero valent iron for enhanced hydroxyl radical oxidation in an electrooxidation system for sewage sludge dewatering, Water Res., 145 (2018) 162–171.
[6] R. Mailler, J. Gasperi, D. Patureau, E. Vulliet, N. Delgenes, A. Danel, S. Deshayes, V. Eudes, S. Guerin, R. Moilleron, G. Chebbo, V. Rocher, Fate of emerging and priority micropollutants during the sewage sludge treatment: case study of Paris conurbation. Part 1: Contamination of the different types of sewage sludge, Waste Manage., 59 (2017) 379–393.
[7] J.Q. Su, B. Wei, W.Y. Ou-Yang, F.Y. Huang, Y. Zhao, H.J. Xu, Y.G. Zhu, Antibiotic resistome and its association with bacterial communities during sewage sludge composting, Environ. Sci. Technol., 49 (2015) 7356–7363.
[8] L. Wang, Z. Qiang, Y. Li, W. Ben, An insight into the removal of fluoroquinolones in activated sludge process: sorption and biodegradation characteristics, J. Environ. Sci. (China), 56 (2017) 263–271.
[9] Y. Chen, G. Yu, Q. Cao, H. Zhang, Q. Lin, Y. Hong, Occurrence and environmental implications of pharmaceuticals in Chinese municipal sewage sludge, Chemosphere, 93 (2013) 1765–1772.
[10] L. Gao, Y. Shi, W. Li, H. Niu, J. Liu, Y. Cai, Occurrence of antibiotics in eight sewage treatment plants in Beijing, China, Chemosphere, 86 (2012) 665–671.
[11] W. Li, Y. Shi, L. Gao, J. Liu, Y. Cai, Occurrence, distribution and potential affecting factors of antibiotics in sewage sludge of wastewater treatment plants in China, Sci. Total Environ., 445–446 (2013) 306–313.
[12] Q.Q. Zhang, G.G. Ying, C.G. Pan, Y.S. Liu, J.L. Zhao, Comprehensive evaluation of antibiotics emission and fate in the river basins of China: source analysis, multimedia modeling, and linkage to bacterial resistance, Environ. Sci. Technol., 49 (2015) 6772–6782.
[13] J. Zhang, Q. Sui, J. Tong, H. Zhong, Y. Wang, M. Chen, Y. Wei, Soil types influence the fate of antibiotic-resistant bacteria and antibiotic resistance genes following the land application of sludge composts, Environ. Int., 118 (2018) 34–43.
[14] A. Khadra, A. Ezzariai, G. Merlina, M.-J. Capdeville, H. Budzinski, H. Hamdi, E. Pinelli, M. Hafidi, Fate of antibiotics present in a primary sludge of WWTP during their co-composting with palm wastes, Waste Manage., 84 (2019) 13–19.
[15] A. Ezzariai, M. Hafidi, A. Khadra, Q. Aemig, L. El Fels, M. Barret, G. Merlina, D. Patureau, E. Pinelli, Human and veterinary antibiotics during composting of sludge or manure: global perspectives on persistence, degradation, and resistance genes, J. Hazard. Mater., 359 (2018) 465–481.
[16] J. Zhang, Y. Bao, Y. Jiang, H.T. Liu, B.D. Xi, D.Q. Wang, Removal and dissipation pathway of typical fluoroquinolones in sewage sludge during aerobic composting, Waste Manage., 95 (2019) 450–457.
[17] M. Čvančarová, M. Moeder, A. Filipová, T. Cajthaml, Biotransformation of fluoroquinolone antibiotics by ligninolytic fungi - metabolites, enzymes and residual antibacterial activity, Chemosphere, 136 (2014) 311–320.
[18] A. Prieto, M. Möder, R. Rodil, L. Adrian, E. Marco-Urrea, Degradation of the antibiotics norfloxacin and ciprofloxacin by a white-rot fungus and identification of degradation products, Bioresour. Technol., 102 (2011) 10987–10995.
[19] H.G. Wetzstein, J. Schneider, W. Karl, Metabolite proving fungal cleavage of the aromatic core part of a fluoroquinolone antibiotic, AMB Express, 2 (2012) 1–7.
[20] M. Rusch, A. Spielmeyer, J. Meißner, M. Kietzmann, H. Zorn, G. Hamscher, Efficient reduction of antibacterial activity and cytotoxicity of fluoroquinolones by fungal-mediated N-oxidation, J. Agric. Food Chem., 65 (2017) 3118–3126.
[21] C.L. Amorim, I.S. Moreira, A.S. Maia, M.E. Tiritan, P.M.L. Castro, Biodegradation of ofloxacin, norfloxacin, and ciprofloxacin as single and mixed substrates by *Labrys portucalensis* F11, Appl. Microbiol. Biotechnol., 98 (2014) 3181–3190.
[22] A.S. Maia, A.R. Ribeiro, C.L. Amorim, J.C. Barreiro, Q.B. Cass, P.M.L. Castro, M.E. Tiritan, Degradation of fluoroquinolone antibiotics and identification of metabolites/transformation products by liquid chromatography-tandem mass spectrometry, J. Chromatogr. A, 1333 (2014) 87–98.
[23] D. Becker, S. Varela Della Giustina, S. Rodriguez-Mozaz, R. Schoevaart, D. Barceló, M. de Cazes, M.P. Belleville, J. Sanchez-Marcano, J. de Gunzburg, O. Couillerot, J. Völker, J. Oehlmann, M. Wagner, Removal of antibiotics in wastewater by enzymatic treatment with fungal laccase – degradation of compounds does not always eliminate toxicity, Bioresour. Technol., 219 (2016) 500–509.
[24] M. Sturini, A. Speltini, F. Maraschi, L. Pretali, A. Profumo, E. Fasani, A. Albini, R. Migliavacca, E. Nucleo, Photodegradation of fluoroquinolones in surface water and antimicrobial activity of the photoproducts, Water Res., 46 (2012) 5575–5582.
[25] G. Miner, Standard Methods for the Examination of Water and Wastewater, 21st Edition, J. Am. Water Work. Assoc., (2006) 130.
[26] M.-H. Zhao, G.-M. Zeng, D.-L. Huan, C.-L. Feng, C. Huang, S. Hu, F.-F. Su, C. Lai, Z. Wei, Research on sorption and transport characteristics of ligninolytic enzymes in different compost substances, Environ. Sci., 31 (2010) 1647–1654. (In Chinese)
[27] R.C. Team, A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria, 2018. http://www.r-project.org.
[28] H. Liu, L. Wang, M. Lei, Positive impact of biochar amendment on thermal balance during swine manure composting at relatively low ambient temperature, Bioresour. Technol., 273 (2019) 25–33.
[29] Y. Bin Ho, M.P. Zakaria, P.A. Latif, N. Saari, Degradation of veterinary antibiotics and hormone during broiler manure composting, Bioresour. Technol., 131 (2013) 476–484.

[30] L. Wang, G. Chen, G. Owens, J. Zhang, Enhanced antibiotic removal by the addition of bamboo charcoal during pig manure composting, RSC Adv., 6 (2016) 27575–27583.
[31] D.W. Kim, T.M. Heinze, B.S. Kim, L.K. Schnackenberg, K.A. Woodling, J.B. Sutherland, Modification of norfloxacin by a *Microbacterium* sp. strain isolated from a wastewater treatment plant, Appl. Environ. Microbiol., 77 (2011) 6100–6108.
[32] I.A. Parshikov, J.B. Sutherland, Microbial transformations of antimicrobial quinolones and related drugs, J. Ind. Microbiol. Biotechnol., 39 (2012) 1731–1740.
[33] M. Chen, W. Chu, Photocatalytic degradation and decomposition mechanism of fluoroquinolones norfloxacin over bismuth tungstate: experiment and mathematic model, Appl. Catal. B, 168–169 (2015) 175–182.
[34] Y. Ji, C. Ferronato, A. Salvador, X. Yang, J.-M. Chovelon, Degradation of ciprofloxacin and sulfamethoxazole by ferrous-activated persulfate: implications for remediation of groundwater contaminated by antibiotics, Sci. Total Environ., 472 (2014) 800–808.
[35] L. Zhu, B. Santiago-Schübel, H. Xiao, H. Hollert, S. Kueppers, Electrochemical oxidation of fluoroquinolone antibiotics: mechanism, residual antibacterial activity and toxicity change, Water Res., 102 (2016) 52–62.
[36] P. Widsten, A. Kandelbauer, Laccase applications in the forest products industry: a review, Enzyme Microb. Technol., 42 (2008) 293–307.
[37] M. Tuomela, M. Vikman, A. Hatakka, M. Itävaara, Biodegradation of lignin in a compost environment: a review, Bioresour. Technol., 72 (2000) 169–183.
[38] A. Hatakka, Lignin-modifying enzymes from selected white-rot fungi: production and role from in lignin degradation, FEMS Microbiol. Rev., 13 (1994) 125–135.
[39] F. Meng, X. He, Effects of naturally occurring grit on the reactor performance and microbial community structure of membrane bioreactors, J. Membr. Sci., 496 (2015) 284–292.
[40] Z. Xu, L. Qin, M. Cai, W. Hua, M. Jin, Biodegradation of kraft lignin by newly isolated *Klebsiella pneumoniae*, *Pseudomonas putida*, and *Ochrobactrum tritici* strains, Environ. Sci. Pollut. Res., 25 (2018) 14171–14181.
[41] M. Rusch, A. Kauschat, A. Spielmeyer, A. Römpp, H. Hausmann, H. Zorn, G. Hamscher, Biotransformation of the antibiotic Danofloxacin by *Xylaria longipes* leads to an efficient reduction of Its antibacterial activity, J. Agric. Food Chem., 63 (2015) 6897–6904.