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Microbial population shift caused by sulfamethoxazole in engineered-Soil Aquifer Treatment (e-SAT) system

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Abstract The engineered-Soil Aquifer Treatment (e-SAT) system was exploited for the biological degradation of Sulfamethoxazole (SMX) which is known to bioaccumulate in the environment. The fate of SMX in soil column was studied through laboratory simulation for a period of 90 days. About 20 ppm SMX concentration could be removed in four consecutive cycles in e-SAT. To understand the microbial community change and biological degradation of SMX in e-SAT system, metagenomic analysis was performed for the soil samples before (A-EBD) and after SMX exposure (B-EBD) in the e-SAT. Four bacterial phyla were found to be present in both the samples, with sample B-EBD showing increased abundance for Actinobacteria, Bacteroidetes, Firmicutes and decreased Proteobacterial abundance compared to A-EBD. The unclassified bacteria were found to be abundant in B-EBD compared to A-EBD. At class level, classes such as Bacilli, Negativicutes, Deltaproteobacteria, and Bacteroidia emerged in sample B-EBD owing to SMX treatment, while Burkholderiales and Nitrosomonadales appeared to be dominant at order level after SMX treatment. Furthermore, in response to SMX treatment, the family Nitrosomonadaceae appeared to be dominant. *Pseudomonas* was the most dominating bacterial genus in A-EBD whereas *Cupriavidus* dominated in sample B-EBD. Additionally, the sulfur oxidizing bacteria were enriched in the B-EBD sample, signifying efficient electron transfer and hence organic molecule degradation in the e-SAT system. Results of this study offer new insights into understanding of microbial community shift during the biodegradation of SMX.

Keywords Sulfamethoxazole · Metagenome · e-SAT · Wastewater · Illumina sequencing

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Introduction

In the last few decades, the uses of antibacterial agents have improved quality of life and public health worldwide. After consumption of pharmaceuticals, a substantial part of the original substance or its metabolites are excreted in urine or faeces, resulting in the release of large quantities of pharmaceutical agents into municipal wastewater (Rivas et al. 2012). Trace amounts of antibiotics have been detected in wastewater treatment plants, effluents, and surface waters. Further, these residues remain bio-available in the soil when it is irrigated using sewage wastewater, or contaminated surface waters (Bruce et al. 2010; Kummerer and Henninger 2003). The occurrence of antibiotics in the environmental ecosystems is a matter of great public health concern (Nakada et al. 2010). Some group of pharmaceuticals is not only effect and directly pressure on microbial communities, but also the risk of universal spreading of antibiotic-resistant bacterial genes (Yan et al. 2013). Chronic exposures to antimicrobial agents in trace-level (ng–μg/l) results in the development of antibiotic resistance in microbial population, which poses a major threat to public health and quality of life (Andreozzi et al. 2005; Szczepanowski et al. 2009; Avisar et al. 2010; Wei et al. 2015).

Sulfamethoxazole (SMX) is one of the sulfonamide group of antimicrobial drugs used for the treatment of

number of bacterial infections in humans and animals (Larcher and Yargeau 2011; Jiang et al. 2012). This drug is most frequently found in the aquatic environment and is only partially removed in municipal wastewater treatment plant (WWTP) (Gao et al. 2014). It has been detected in the range of 0.21 to 2.8 µg/L concentrations in wastewaters, 5.597 µg/L concentrations in raw wastewaters from sewage treatment plant and 12 ng/L concentrations in source water (Batt et al. 2007; Peng et al. 2008; Benotti et al. 2008). SMX is difficult to degrade and therefore persists in the environment particularly in the aquatic streams for long period of time (Bouju et al. 2012). SMX has become a point of interest because of its occurrence in wastewaters at concentrations related to bacterial resistance (Jiang et al. 2014). Hence the removal of this drug from the effluent of wastewater treatment plant is necessary before discharge into the environment.

To overcome the threat of such antibiotic occurrence in natural ecosystems, advanced technologies are required that can prevent its release into the environment. The physical and chemical treatment methods generally cause the formation of possibly toxic intermediates. For this reason, biodegradation is likely to be an effective method for the removal of SMX (Onesios and Bouwer 2012). The possibility of complete degradation of SMX by biological means is based on the fact that it serves as a carbon and nitrogen source to microbes in nutrient-limiting conditions (Herzog et al. 2013; Muller et al. 2016). The most common biological methods employed for SMX degradation are activated sludge process (Drillia et al. 2005; Collado et al. 2013; Reis et al. 2014; Zhang et al. 2016) and microbial fuel cell reactor (Wang et al. 2016).

According to previous reports, about 67% sulfonamides are detected in effluent despite conventional treatment process (Peng et al. 2011). Therefore, additional treatment process is required after the treatments in WWTPs for the improvement of water quality. Recently, Soil Aquifer Treatment (SAT) has been employed to treat WWTP effluents via artificial water recharging. SAT can remove pharmaceutical and personal care products (PPCPs) from wastewater and serves as one of the oldest and cost effective methods (Onesios and Bouwer 2012). SAT has benefits such as comparatively easy operation, low operational cost, higher success and stable performance, through which water quality is improved by means of combined functions of physical and biochemical processes (Zhang et al. 2016; Onesios and Bouwer 2012). Wastewater treatment by SAT depends on the natural ability of soil to regenerate water resources (Guizani et al. 2011).

He et al. (2016) studied the effects of different operating conditions such as packing material, hydraulic retention time and vadose condition on the removal of PPCPs in SAT. Many researchers studied the fate and removal rate

of SMX in soil, but SMX adsorption is very low in most soils (Schaffer et al. 2015). Therefore, it is hypothesized that sorption into the aquifer is usually insignificant, and the observed reduction of SMX concentration in the environmental compartment is mostly due to degradation. Soil column experiment reported aerobic degradation of SMX when supplied with surface water (Baumgarten et al. 2011). In addition, based on laboratory studies also indicated that SMX degradation occurs biologically and preferentially under anaerobic condition (Valhondo et al. 2015). Recently, Rodriguez-Escales and Sanchez-Vila (2016) formulated a model for the degradation pathway of SMX under different redox conditions (aerobic condition, denitrification, and iron-reducing conditions) in groundwater. However, reports on the environmental fate of SMX and particularly its biodegradation by the e-SAT system are limited. The aim of this study is to find out the degradation potential of laboratory e-SAT systems for SMX and further, to understand the shift in the microbial community of the e-SAT system after SMX treatment using metagenomic approach.

Materials and methods

Chemicals

All chemicals and solvents used were either molecular grade or HPLC grade. Sulfamethoxazole was purchased from Sigma-Aldrich (USA). Magnesium sulphate ($MgSO_4$), ammonium sulphate (NH_4SO_4), di-potassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), sodium chloride (NaCl), tri-sodium citrate ($Na_3C_6H_5O_7$), acetonitrile and sodium dihydrogen phosphate (NaH_2PO_4) were purchased from Merck (India).

Sampling

A sulfamethoxazole manufacturing industry located in the state of Andhra Pradesh, India served as the sampling site in this study. Soil samples were collected from five different wastewater spillage sites within the industry. The collected samples were stored at 4 °C until further use.

Soil samples from selected sites were collected from depths ranging between 0 and 75 cm. The specified depth ranges were 0–25 cm (layer-1), 25–50 cm (layer-2) and 50–75 cm (layer-3). The soil samples were homogenized, sieved and gravel fractions of >1.7 mm were reused. The samples were then mixed and used for setting up e-SAT column. The physicochemical parameters such as pH, electrical conductivity, cation exchange capacity (CEC) were estimated for all the soil samples as described by Jackson (1973). Bulk density was determined as per the method of Blake (1965). Soil moisture content was determined using

gravimetric method (Famiglietti et al. 1998). Total organic carbon (TOC) content was analyzed using TOC analyzer (SHIMADZU-TOC-V$_{CPH}$). Estimation of soil elements was carried out using microwave digestion followed by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) (Yafa and Farmer 2006).

Fabrication and packing of e-SAT system

Two e-SAT systems were fabricated using acrylic material (Inner diameter 4.5 cm and length 62 cm) comprising of inlet, outlet and three sampling ports (placed uniformly at 11 cm distances). The experimental e-SAT was packed uniformly using collected soil samples consisting of different particle sizes whereas the control e-SAT system was packed with the similar collected soil but the soil was sterilized (autoclaved at 121 °C for 20 min) before packing (Lian et al. 2013). The top most 11 cm of the column comprised of fine soil, followed by 11 cm of coarse soil and the last layer of 9 cm was packed with gravels. The three sections were separated by 2 cm soil sand and lowest part of the column was packed with glass wool (2 cm). The schematics of the e-SAT system are depicted in Fig. 1.

e-SAT column operation and determination of SMX concentration

Two e-SAT columns were operated (experimental and control) in order to understand the influence of microbial community in the removal of SMX. Prior to column operation, the experimental e-SAT system was acclimated for 10 days with the minimal medium in order to stabilize the rate of infiltration and enriched the microbial growth within e-SAT system. In case of control e-SAT system, the column was acclimated with distilled water for stabilization and homogeneous condition within the column (Guizani et al. 2011). Further, both e-SAT systems were fed with SMX solution (20 ppm SMX prepared in distil water) through the inlet of e-SAT column using a peristaltic pump at a flow rate of 0.3 ml/min and at a linear flow rate of 0.19 mm/min. The e-SAT system operated under gravity flow condition. The infiltrated liquid collected from outlet again fed into e-SAT system. This cycle was repeated until maximum removal of SMX in e-SAT achieved. The column was run for 90 days and total 19 to 20 cycles were carried out in e-SAT system. The e-SAT column experiment and metagenomic data presented in the manuscript are from a single experiment on e-SAT system.

SMX concentration (in the inlet and outlet fraction) was monitored using High-Performance Liquid Chromatography (HPLC) as described by Rodayan et al. (2010) with slight modification. HPLC system was equipped with Waters 2998 photodiode array (PDA) detector at a wavelength of 265 nm. The mobile phase consisted of 20 mM sodium dihydrogen phosphate (prepared in Milli-Q):acetonitrile (1:1, v/v). Chromatographic separation was obtained by isocratic mode of elution using symmetry C-18 column (3.5 μm, 4.6×75 mm) at a flow rate of 1 ml/min. All samples were filtered using syringe filter (0.2 μ) before injection.

### Post run residual SMX concentration in e-SAT system

The residual SMX concentration of soil in both experimental and control e-SAT was determined after 90 days of column operation. The soil was removed from e-SAT system and weighed. Acetonitrile was used for SMX extraction and extraction using soil-liquid extraction method (Aga 2007). The soil was mixed with organic solvent in 1:2 ratio and subjected to acoustic vibration with frequencies above 20 kHz for 10 min in sonication bath followed by mechanical agitation for 15 min using agitator (Remi, India). This cycle was repeated ten times to obtain maximum extraction of the SMX. The sample was then centrifuged and supernatant was collected. The supernatant was concentrated by using vacuum rotary evaporator (Kumar, India) and SMX in the extract was quantified using HPLC.

Extraction of metagenomic DNA

The initial soil sample (before SMX treatment) was collected from e-SAT system, referred as A-EBD and soil sample collected (after SMX treatment) at the end of 90th day was considered as B-EBD. Metagenomic DNA was isolated from composite soil samples (A-EBD and B-EBD) using Fast spin soil DNA isolation Kit (MP Biomedicals) as per the manufacture's protocol. Metagenomic DNA was isolated from composite soil sample (prepared by mixing subsample of soil collected across the e-SAT system) to acquire maximum bacterial population across the e-SAT system. The isolated DNA was visualized using 1% agarose gel electrophoresis and the quantity and purity of DNA was determined using NanoDrop 1000 spectrophotometer (Thermo-Scientific, USA).

Metagenome sequence analysis

Metagenomic analysis of A-EBD and B-EBD was performed using Illumina sequencing platforms. TruSeq Nano DNA HT Library Preparation Kit (Illumina, USA) was used to prepare the paired-end sequencing library. 200 ng gDNA was fragmented by Covaris to generate a mean fragment distribution of 550 bp. The fragments were then subjected to end-repair. Indexing adapters were ligated to the ends of the DNA fragments, preparing them for hybridization onto a flow cell. The ligated products

Fig. 1 e-SAT system setup
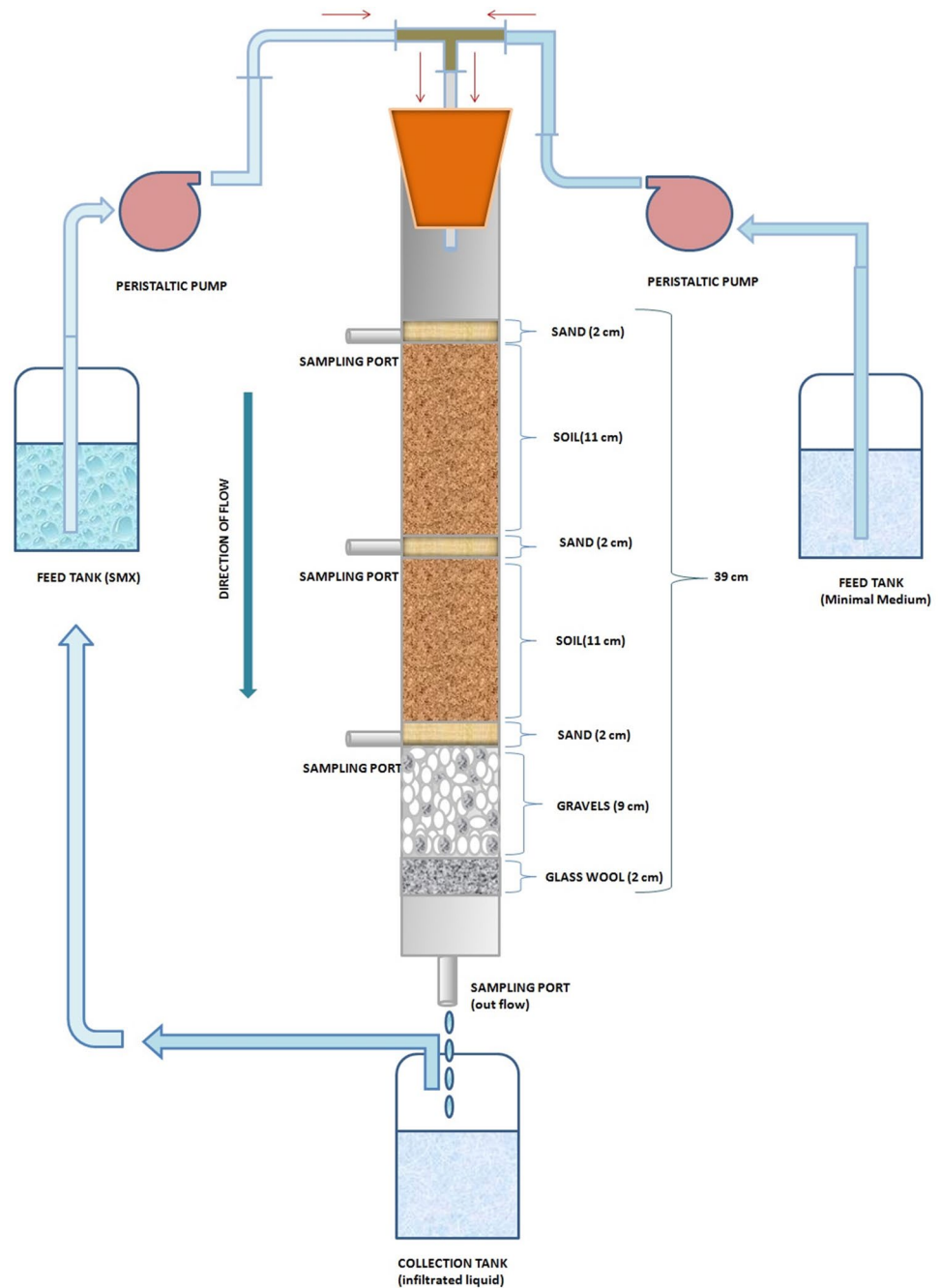
were purified using SP beads supplied in the kit and the size-selected product was PCR amplified as per kit protocol. The amplified library was analyzed in Bioanalyzer 2100 (Agilent Technologies) using High Sensitivity (HS) DNA chip as per manufacturer's instructions. The Qubit concentration for the library and the mean peak size from Bioanalyzer profile were observed before the library was loaded onto NextSeq for cluster generation and paired-end sequencing. De novo assembly of high quality paired-end reads was accomplished using CLC Genomics Workbench 6.0 at default parameters (Yadav et al. 2015).

The steps involved in the analysis of high quality reads are enlisted in Fig. S1.

Bioinformatics analysis

The contigs, from both the A-EBD and B-EBD samples, which do not have any rRNA were subjected to ORF prediction with Prodigal (v2.6.1) and the predicted ORFs along with the rRNA containing contigs were then uploaded to MEGAN (Huson et al. 2007) and Metagenome Rapid Annotation using Subsystem Technology MG-RAST

Table 1 Physico-chemical characteristics of collected soil samples used to set up e-SAT system

Sr. no.	Soil samples	pH	Moisture (%)	Bulk density (g/cm^3)	Electrical conductivity (EC) (uS/cm)	TOC (ppm)	CEC Meq/100 g
1	1st layer	7.22	3.44	0.9844	570	3.88	7.6
2	2nd layer	7.15	3.89	1.1531	430	3.47	7.28
3	3rd layer	7.37	3.94	1.064	600	3.38	7.0

Table 2 Physical properties and volume of packing material used for packing of e-SAT system

| Name of sample	Bulk density (gm/cm^3)	Porosity (%)	Length (cm)	Volume (cm^3)	Void space (ml)
Fine soil	1.34	52.37	11	215.87	113
Fine sand	1.61	38.34	6	117.75	45
Medium coarse soil	2.61	58.87	11	215.87	127
Marble stone	1.73	43.75	9	176.62	77

web server (Meyer et al. 2008) for automated annotation and analysis. It is a widely used online tool for metagenome sequence analysis (More et al. 2014). The data was compared to M5 non-redundant protein database (M5NR) using a maximum identity of 90% and an e-value of 1e-5. To obtain sample specific bacterial population at each phylogenetic level, a Venn diagram was generated using online Venny tool (Oliveros 2009). Genus level phylogenetic tree was constructed and colored distinctly at order level as annotated by MG-RAST-Lowest Common Ancestor (LCA) (Yu and Zhang 2012) algorithm at 90% identity cut-off value. Statistical Analysis of Metagenomic Profiles (STAMP) tool (Parks et al. 2014) was used to compare the statistically significant differential abundance of microbial diversity across two metagenome samples at various phylogenetic levels.

Metagenome data was submitted to National Center for Biotechnology Information (NCBI)-Sequence Read Archive (SRA) repository and can be accessible under accession nos. SRP081059 and SRP081061.

Results

Physico-chemical parameter of soil

The physicochemical characteristics of collected soil samples used for e-SAT column setup from three layers are tabulated in Table 1. 15 elements were identified in the soil samples by ICP-OES analysis and their respective concentrations are enlisted in Table S1. Physical properties and volume of packing material used for packing of e-SAT system are presented in Table 2.
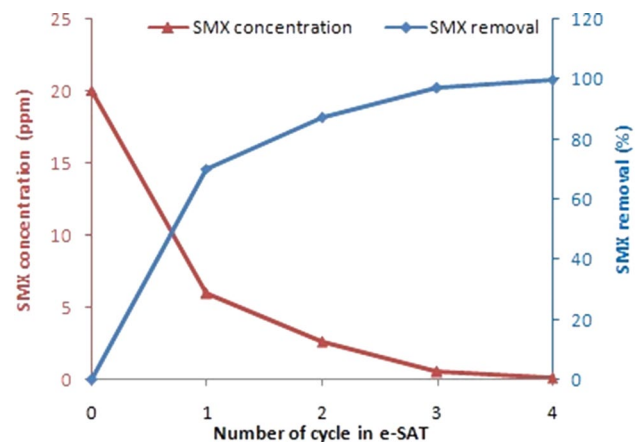
Fig. 2 Plot of SMX removal during each cycle in e-SAT system

SMX removal and post run residual SMX concentration in e-SAT system

Two litres volume of 20 ppm SMX solution was fed into the e-SAT at a constant flow rate of 0.3 ml/min. It took 111 h (4.625 days) for one cycle. Figure 2 represents the SMX removal in each cycle in experimental e-SAT system and chromatogram of SMX removal during each cycle as shown in Fig. S2. About 14.08 ppm (70.15%) SMX was removed after first cycle, while at the end of second cycle 3.38 ppm (87.02%) SMX was removed. Subsequently, in third and fourth cycles 2.03 ppm (97.17%) and 0.48 ppm (99.61%) of SMX was removed respectively. Total 19.97 ppm (99.61%) of SMX was removed in e-SAT system at the end of fourth cycle. Only 0.078 ppm of SMX was detected in the outlet sample at the end of fourth cycle compared to initial 20 ppm SMX. In control e-SAT system, SMX removal was very low, only 12.64 ppm of SMX was

removed and 7.56 ppm was detected in outlet sample at end of fourth cycle.

Following 90th days of column operation, the existence of any residual SMX concentration in soil was analyzed using HPLC in both experimental and control e-SAT system. HPLC analysis of soil extract revealed very low residual SMX concentration (0.73 ppm) in the soil of experimental e-SAT system compared to control e-SAT system (10.44 ppm).

Metagenome sequencing

The libraries were prepared from the samples A-EBD and B-EBD metagenomic DNA using TruSeq Nano DNA HT Library Sample Preparation Kit. The mean sizes of the libraries were 699 and 688 bp respectively. The libraries were sequenced on NextSeq 500 using 2×150 bp chemistry to generate 1.27 Gb and 1.12 Gb of data for the two samples. A total of 63 rRNAs in A-EBD and 91 rRNAs in B-EBD sample were predicted with WebMGA web server using hmm-rRNA algorithm. A total of 54,976 and 46,143 ORFs for sample A-EBD and sample B-EBD respectively were generated through Prodigal tool, which was subject to quality check after which 52,624 and 44,111 sequences were obtained for the respective datasets. The difference in the number of total sequences was addressed by appropriate normalization of both the datasets. The data statistics for the two metagenome samples are presented in Table S2. The MG-RAST ID of the two datasets A-EBD and B-EBD are 4636878.3 and 4636879.3 respectively.

Microbial diversity in e-SAT system

The whole metagenome analysis revealed the diverse microbial population before and after SMX exposure in e-SAT system. At domain level, the microbial biomass was dominated by 90% bacterial population and the proportion of sequences assigned to various domains and statistical difference between the proportions of domains

is indicated in Fig. 3. The bacterial reads were almost equal for both the datasets.

To analyze the microbial population shift after SMX treatment, normalized abundance at phyla and class level were calculated. At phyla level a total of five groups were annotated and their normalized abundance was calculated as given in Table 3. Four bacterial phyla along with unclassified bacteria were present in both the samples, with increased abundance for Bacteroidetes and Firmicutes in sample B-EBD.

Top five dominating bacterial groups at class, order and family level were plotted along with Venn diagrams depicting the number of unique and common groups in each sample at each phylogenetic level (Fig. 4a–c). Total nine bacterial classes were present in sample A-EBD of which class Clostridia disappeared after SMX exposure. At class level, greater diversity was observed in sample B-EBD. Total eight bacterial classes were common in both A-EBD and B-EBD. In addition, classes such as Bacilli, Negativicutes, Deltaproteobacteria, and Bacteroidia appeared to be dominating in sample B-EBD exclusively after the SMX exposure (Table 4). As shown in Fig. 4a betaproteobacteria remained the dominating bacterial class in both the samples, but at a lower abundance value in sample B-EBD. About 61.5% bacterial populations at a class level were common in both samples. The Alphaproteobacteria and Gammaproteobacteria were the other dominating bacterial classes in both the samples.

Table 3 Phyla level classification

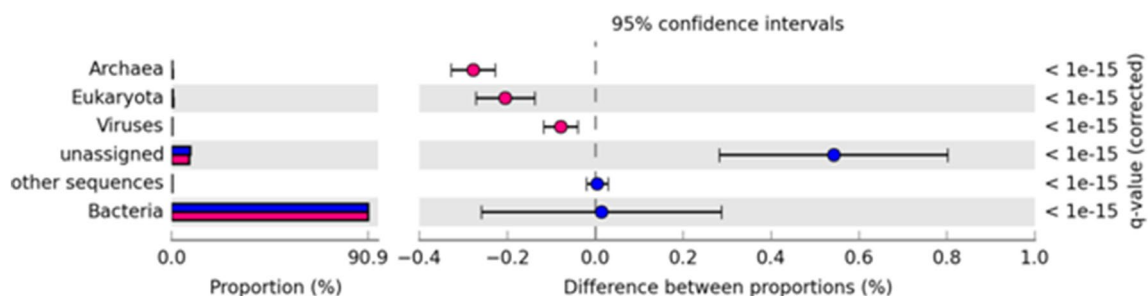| Phylum	Sample A-EBD	Sample B-EBD
Proteobacteria	30.53169656	4.488676294
Unclassified (derived from bacteria)	0.108315597	0.156423568
Actinobacteria	0.032304652	0.034005123
Bacteroidetes	0.005700821	0.013602049
Firmicutes	0.001900274	0.054408198

Fig. 3 Domain level comparison for the metagenome sample A-EBD and B-EBD using STAMP tool, where the statistically significant proportions of each domain in sample A-EBD and B-EBD samples are plot. *Pink color* indicates sample A-EBD and the *blue color* indicates sample B-EBD. (Color figure online)

Fig. 4 Normalized abundance of bacterial population at (**a**) class level (**b**) order and (**c**) family level. The mutual exclusive and common features are also depicted in respective Venn diagrams
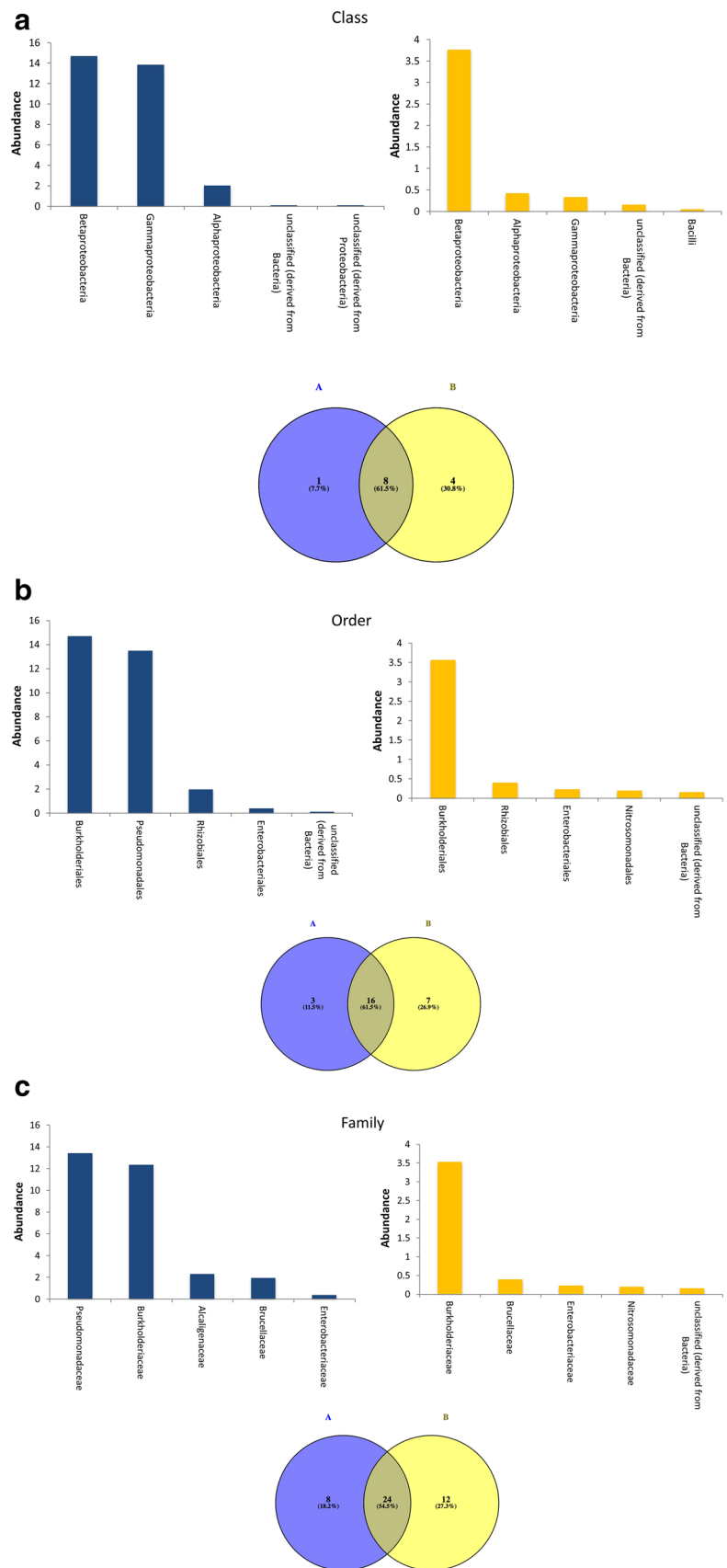
Table 4 The elements of Venn diagram (Fig. 4a) common and exclusive in datasets A-EBD and B-EBD

One elements exclusively in A-EBD	Four elements included exclusively in B-EBD	Eight common elements in A-EBD and B-EBD
Clostridia	Bacilli	Betaproteobacteria
	Negativicutes	Gammaproteobacteria
	Deltaproteobacteria	Alphaproteobacteria
	Bacteroidia	Unclassified (derived from bacteria)
		Unclassified (derived from proteobacteria)
		Actinobacteria (class)
		Sphingobacteria
		Flavobacteria

Sample A-EBD was dominated almost equally by the orders Burkholderiales and Pseudomonadales. The latter diminished in sample B-EBD and was replaced by Nitrosomonadales (Fig. 4b). At order level comparison of the top five bacterial orders revealed the dominance of Burkholderiales, Rhizobiales and Enterobacteriales in both the samples (Fig. 4b). At order level, 61.5% of bacterial populations were common in A-EBD and B-EBD. Furthermore, seven bacterial orders were observed exclusively to sample B-EBD, which included Bacillales, Lactobacillales, Bacteroidales, Cardiobacteriales, Selenomonadales, Desulfobacterales and Oceanospirillales (Table 5). At family level, Pseudomonadaceae and Burkholderiaceae shared dominance in sample A-EBD. The families Pseudomonadaceae and Brucellaceae diminished in sample B-EBD in response to SMX exposure. About 54.5% population was conserved at family level in sample B after SMX exposure (Fig. 4c). The families Shewanellaceae, Gordoniaceae, Microbacteriaceae, Bifidobacteriaceae, Sphingobacteriaceae and Bradyrhizobiaceae were absent in sample B-EBD, in response to SMX (Table 6).

Figure 5a represents the classification of bacterial genera colored at order level using MG-RAST at 90% identity cut-off. The stacked bars indicate the abundance of the genera in sample A-EBD (marked by green color) and sample B-EBD (marked by red color). Before SMX exposure bacterial genera such as *Stenotrophomonas, Gallibacterium, Morganella, Shewanella, Nitrosomonas, Roseateles, Pelomonas, Zymomonas, Diaphorobacter, Pandoraea, Rhizobium, Bradyrhizobium, Shingobacterium, Bifidobacterium, Gordonia and Microbacterium* were abundant in sample A-EBD as marked by green color. The SMX exposure led to disappearance of these genera in the sample B-EBD. Additionally, few bacterial genera such as *Arthrobacter, Aliivibrio, Bacteroides, Halomonas Raoultella*, etc increased in abundant in

Table 5 The elements of Venn diagram (Fig. 4b) common and exclusive in datasets A-EBD and B-EBD

Three elements included exclusively in A-EBD	Seven elements included exclusively in B-EBD	Sixteen common elements in A-EBD and B-EBD
Alteromonadales	Bacillales	Burkholderiales
Bifidobacteriales	Lactobacillales	Pseudomonadales
Clostridiales	Bacteroidales	Rhizobiales
	Cardiobacteriales	Enterobacteriales
	Selenomonadales	Unclassified (derived from bacteria)
	Desulfobacterales	Unclassified (derived from proteobacteria)
	Oceanospirillales	Sphingomonadales
		Actinomycetales
		Aeromonadales
		Vibrionales
		Pasteurellales
		Xanthomonadales
		Sphingobacteriales
		Nitrosomonadales
		Flavobacteriales
		Rhodocyclales

Table 6 The elements of Venn diagram (Fig. 4c) common and exclusive in datasets A-EBD and B-EBD

Eight elements included exclusively in A-EBD	Twelve elements included exclusively in B-EBD	Twenty-four common elements in A-EBD and B-EBD
Shewanellaceae	Staphylococcaceae	Pseudomonadaceae
Gordoniaceae	Bacillaceae	Burkholderiaceae
Microbacteriaceae	Enterococcaceae	Alcaligenaceae
Bifidobacteriaceae	Bacteroidaceae	Brucellaceae
Sphingobacteriaceae	Listeriaceae	Enterobacteriaceae
Unclassified (derived from clostridiales)	Streptococcaceae	Unclassified (derived from bacteria)
Bradyrhizobiaceae	Lactobacillaceae	Moraxellaceae
Unclassified (derived from rhizobiales)	Phyllobacteriaceae	Unclassified (derived from proteobacteria)
	Cardiobacteriaceae	Comamonadaceae
	Veillonellaceae	Sphingomonadaceae
	Desulfobacteraceae	Aeromonadaceae
	Halomonadaceae	Vibrionaceae
		Corynebacteriaceae
		Pasteurellaceae
		Rhizobiaceae
		Xanthomonadaceae
		Mycobacteriaceae
		Nitrosomonadaceae
		Actinomycetaceae
		Micrococcaceae
		Flavobacteriaceae
		Unclassified (derived from sphingobacteriales)
		Unclassified (derived from burkholderiales)
		Rhodocyclaceae

sample B-EBD compared to sample A-EBD. The LCA analysis of the bacterial genera in the two samples was compared to analyze the effect of SMX on microbial community. *Pseudomonas* was the most dominating bacterial genus in A-EBD whereas *Cupriavidus* dominated in sample B-EBD. The top 20 abundant bacterial genera in samples A-EBD and B-EBD are given in Fig. 5b.

Based on the metagenomic analysis of A-EBD and B-EBD, several bacterial genera known for sulfur oxidation were analyzed (Table 7). Nine different bacterial families containing diverse bacterial genera capable of oxidizing sulfur. Sulfur-oxidizing bacteria such as *Thiobacillus, Desulfuromonas, Allochromatium, Nitrococcus, Halothiobacillus, Desulfobulbus, and Desulfurivibrio* were abundant in A-EBD, while *Thiomicrospora, Dethiobacter, Nitrosococcus, Alkalimnicola, Halorhodospira, Thioalkalivibrio* and *Dethiosulfovibrio*, were found to be enriched in sample B-EBD. The bacterial genera *Dethiobacter, Halorhodospira* and *Dethiosulfovibrio* were absent in A-EBD and exclusively present in sample B-EBD.

Discussion

The presence of SMX in the environment has an adverse effect on ecosystem. It is essential to remove such trace pollutants from the environment and also study their effect in the environment. SAT system has been employed for removal of trace organics and contaminants from wastewater (Alidina et al. 2014). Hence, this study was focused on the removal of SMX in e-SAT system and to analyze microbial community shift in response to SMX using metagenomic approach. The role of microbial community shift in the removal of SMX was also elucidated.

In the present study, the e-SAT column could effectively remove 20 ppm SMX. Removal of SMX was more in the experimental e-SAT system compared to the control e-SAT system. In the experimental e-SAT system, presence of microbial biomass was considered to be responsible for higher removal. In case of control e-SAT system only physical and chemical characteristics of soil was responsible for SMX removal with adsorption on soil as the main mechanism. A recent report on the removal of antibiotic
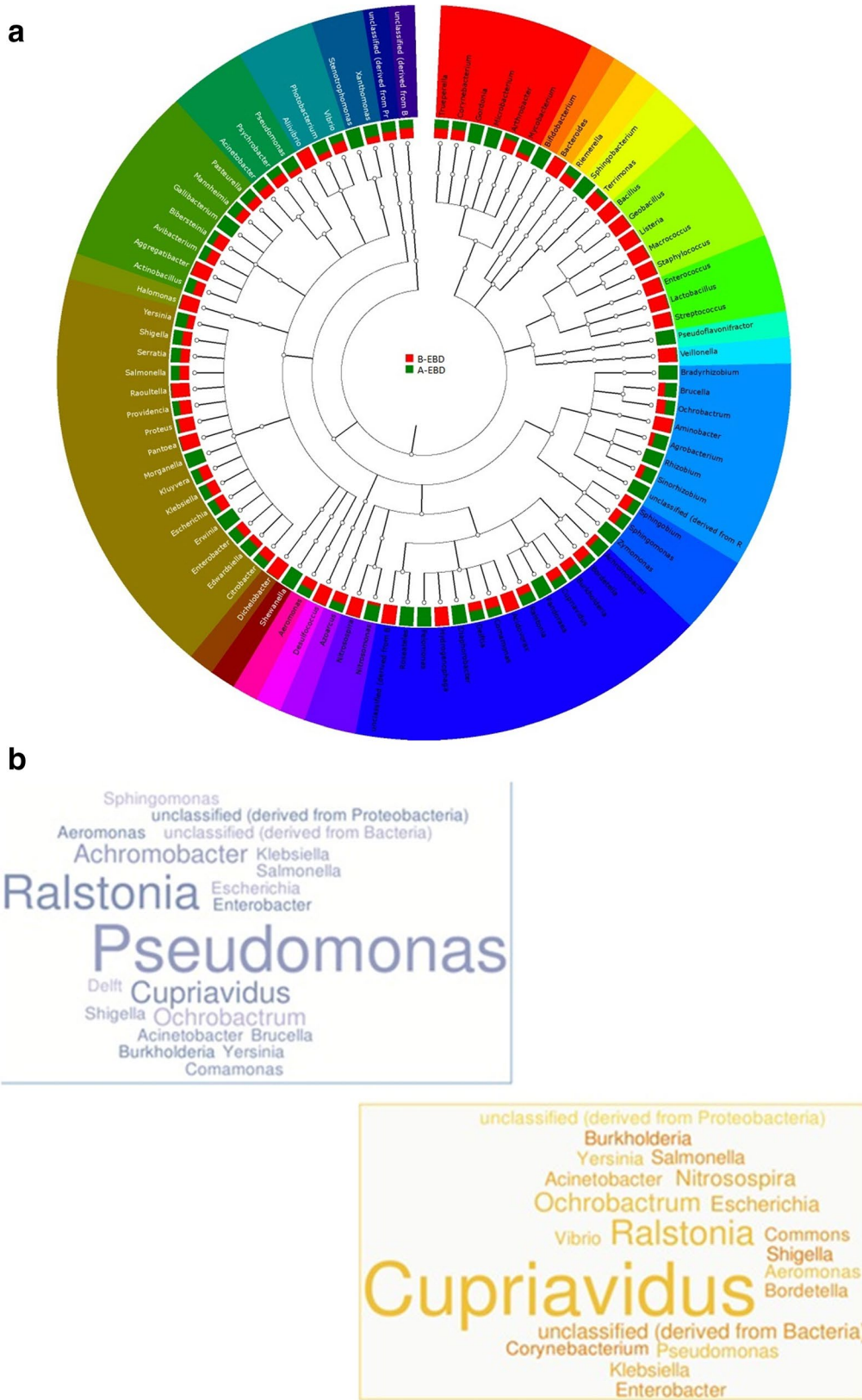
Fig. 5 a Genus level phylogenetic tree which is colored distinctly at order level, using MG-RAST at 90% identity cut off. **b** The top 20 abundant bacterial genera in the two metagenomes are depicted by word cloud

Table 7 Abundance of purple sulphur bacterial genera in the two metagenome samples, with the normalized percentage abundance values and a heat map color scale is given to the values to visualize varying abundances across the two samples

Bacterial family	Bacterial genera	Normalized abundance (%) A-EBD	Normalized abundance (%) B-EBD
Hydrogenophilales	*Thiobacillus*	1.663586	0.807754
Prisarickettsiaceae	*Thiomicrospora*	0.184843	0.323102
Desulfuromonadaceae	*Desulfuromonas*	1.478743	0
Syntrophomonadaceae	*Dethiobacter*	0	3.231018
Chromatiaceae	*Allochromatium*	0.924214	0.161551
	Nitrosococcus	1.478743	2.423263
Ectothiorhodospiraceae	*Alkalimnicola*	0.739372	4.361874
	Halorhodospira	0	0.807754
	Nitrococcus	3.327172	0.969305
	Thioalkalivibrio	1.2939	4.038772
Halothiobacillaceae	*Halothiobacillus*	0.369686	0
Synergestaceae	*Dethiosulfovibrio*	0	0.161551
Desolfobulbaceae	*Desulfobulbus*	0.369686	0.323102
	Desulfurivibrio	0.739372	0.484653

Red is the lowest abundances and green indicates highest abundance values

in SAT indicated that high TOC and CEC enhanced the sorption and vadose condition contributed to the removal of SMX (He et al. 2016). Rodriguez-Escales and Sanchez-Vila (2016) proposed a mathematical model for SMX degradation under iron-reducing conditions which facilitate the breakdown of SMX molecule through enhancing the abiotic mechanism (oxidation of ferrous iron to ferric iron). In the present study, soil used for the packing of e-SAT contained a high amount of Fe (160–240 ppm) (Table S1). Hence, similar Fe-reducing condition might have facilitated abiotic removal of SMX. As reported in the earlier studies, maximum adsorption of antibacterial or pharmaceutical agents occurs at the uppermost layer of the soil, which acts as a filter to remove most of the organic and inorganic contaminant from the wastewater during natural soil aquifer treatment (Zhang et al. 2016; Wei et al. 2015). The soil extraction and subsequent HPLC analysis of residual SMX concentration in the experimental and control e-SAT system revealed biological removal of SMX in the experimental e-SAT system, thus eliminating the possibility of SMX removal by soil adsorption in the e-SAT system. Therefore microbial shift occurs in the e-SAT system may responsible for SMX degradation.

In order to understand the microbial-mediated degradation of SMX in the e-SAT, metagenomic analysis of the DNA samples was carried out before and after exposure of SMX. A drastic shift was observed at phyla level after

SMX exposure (Table 2). In earlier studies, High-throughput 16S rRNA gene sequencing suggested that Actinobacteria, Proteobacteria and Bacteroidetes were the three most abundant phyla in sludge samples of sequencing batch reactors treating tetracycline (TC) and sulfamethoxazole (SMX) (5–50 ppb and 10 ppm) (Zhang et al. 2016). Gonzalez et al. (2008) identified Proteobacteria as the dominant phylum directly related to the processes involved in biological degradation of sulfamethoxazole compounds in the Sequencing Batch Biofilm Reactor (SBBR). Recent studies of SMX effect on activated sludge bacterial community revealed that Actinobacteria community decreased, while Proteobacteria remained steady (Collado et al. 2013). These are in agreement with current results, where sample A-EBD exhibited the abundance of Proteobacteria, whereas the sample B-EBD showed unclassified bacteria, Actinobacteria, Bacteroidetes and Firmicutes as dominating population in the e-SAT system (Table 2).

Recent studies of SMX effect on activated sludge bacterial community revealed that bacteria belonging to Betaproteobacteria and Gammaproteobacteria classes were dominant (Collado et al. 2013). In the present study, Betaproteobacteria class remained to dominate in both the samples and the class Bacilli appeared to be dominating exclusively after the SMX treatment (Fig. 4a). Gonzalez et al. (2008) showed that at order level Burkholderiales, followed by Bdellovibrionales and Nitrosomonadales were

the most dominating groups directly related to the processes involved in biological degradation of sulfamethoxazole compounds in the Sequencing Batch Biofilm Reactor (SBBR). It was observed that Burkholderiales was aerobic bacteria with high biodegradation potential, while Nitrosomonadales and Rhizobiales were involved in the nitrifying and denitrifying process of nitrogen (Gonzalez et al. 2008). Sample A-EBD was dominated almost equally by orders Burkholderiales and Pseudomonadales, but the latter diminished in sample B-EBD and was replaced by Nitrosomonadales (Fig. 4b). Further, in response to SMX treatment the family Nitrosomonadaceae appeared to be dominant (Fig. 4c).The presence of these bacteria in e-SAT system after SMX exposure may indicate their involvement in the biological degradation of SMX.

Two aerobic *Arthrobacter* bacterial strains (D2 and D4) were reported to degrade sulfadiazine (SDZ), a known sulfonamide drug (Deng et al. 2016). In agreement with the earlier study, *Arthrobacter* was found to be abundant in SMX exposed sample (B-EBD) indicating that SMX may be degraded by *Arthrobacter* strain in the present study. Results demonstrate that at a different taxonomic level more than 50% of bacterial community shared between two metagenomes, suggesting a common role in the removal of SMX. The first possibility is that these common bacterial populations may tolerate the SMX concentration used in the present study and secondly, these are may be involved in the degradation of transforming products of SMX.

Taxonomic analysis revealed the presence of a diverse sulfur-oxidizing bacterial population in both metagenome samples of the e-SAT. Sulfur oxidizing bacteria such as Desulfobulbaceae are significant members of aquifer systems as these filamentous microbes perform long-distance electron transfer (LDET) for sulfur oxidation in contaminated aquifer ecologies (Muller et al. 2016). The presence of sulfur-oxidizing bacteria in oxic-anoxic zones of the soil column suggests the efficient transfer of electrons to 1–2 cm distances. In a recently reported study, the aerobic sulfur oxidation was found to enhance uptake of organic carbon in the oceans (Marshall and Morris 2013). Thus, presence of sulfur-oxidizing bacteria in the B-EBD sample suggested efficient electron transfer which may be involved in degradation of organic molecule in e-SAT system.

Conclusion

The presence of antimicrobial agent in wastewater is a global issue, warranting the development of simple, cheap and effective means to minimize their presence. This study describes the effectiveness of engineered-Soil Aquifer treatment (e-SAT) to remove a commonly used antimicrobial agent [Sulfamethoxazole (SMX)] from wastewater.

Metagenomic study revealed the changes in microbial community composition of the e-SAT system after exposure of SMX. The study demonstrated selective stress of SMX on the metabolic activity of soil microbial community. It illustrated the evolution of microbial community in the soil and revealed that sulfur-oxidizing bacteria were enriched after exposure to SMX, which is a useful finding for further studies at gene/pathway level to understand the molecular mechanism in the biological biodegradation of SMX in the e-SAT system.

Compliance with ethical standards

Conflict of interest   The authors declare no conflict of interest.

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