Mechanism of 1,4-dioxane microbial degradation revealed by 16S rRNA and metatranscriptomic analyses

Xiangyu Guan, Fei Liu, Jing Wang, Caoxiang Li and Xiaoxuan Zheng

ABSTRACT

1,4-Dioxane (dioxane), a probable human carcinogen, often exists in industrial wastewater and domestic sewage. In this study, we applied 16S rRNA and metatranscriptomic methods to analyze the dioxane biodegradation mechanism by activated sludge. Tetrahydrofuran (THF) was added as an essential co-metabolite to promote the degradation of dioxane. We found the dioxane removal ratio increased with increasing THF concentrations. When the THF concentration increased from 60.0 to 200.0 mg/L, the dioxane degradation rate was stable. Three additions of ~60.0 mg/L THF resulted in better dioxane degradation than one addition of 200 mg/L THF. Ammonia-oxidizing and denitrifying bacteria with methane monooxygenases (MOs) and ammonia MOs played the most important roles during the degradation of dioxane. Kyoto Encyclopedia of Genes and Genomes metabolic pathway and functional genes analyses showed that the activated sludge system was complex and stable when dioxane was added. In future studies, primers should be designed to identify specific bacteria and functional MO genes, which would help reveal the function of various bacteria and their MOs during dioxane degradation.

Key words | 1,4-dioxane, biodegradation, metatranscriptome, microbial community, tetrahydrofuran

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INTRODUCTION

1,4-Dioxane ($C_4H_8O_2$, dioxane, ), which can be inhaled into the human body in the form of a gas, is a probable human carcinogen (IARC 1999). The World Health Organization established acceptable limits of dioxane in drinking water in 2003. Dioxane is used as a solvent and stabilizer, and is present in polyester manufacturing byproducts; it often exists in industrial wastewater, which makes it a potential source of environmental contamination (Zenker *et al.* 2003; Mohr *et al.* 2010). Dioxane is also used as a surfactant in foods, cosmetics, and detergents and, as a result, it ultimately contaminates domestic sewage (Black *et al.* 2001). Dioxane is highly soluble, mobile, and stable in water, and it is difficult to degrade biologically (Mohr *et al.* 2010). Because of these characteristics, dioxane is very difficult to remove from wastewater, which greatly increases the possibility of surface water and groundwater pollution (Sei *et al.* 2010).

To date, conventional wastewater treatment techniques (including coagulation–precipitation and carbon adsorption) remove dioxane inefficiently. Hydrogen peroxide, ozone, and/or ultraviolet light treatments remove dioxane efficiently but cause secondary environmental pollution (Suh & Mohseni 2004; Coleman *et al.* 2007). Bioremediation processes that are cost-effective and do not result in secondary pollution have become popular treatments for pollutants. *Pseudonocardia*, *Mycobacterium*, and *Acinetobacter* have been reported to directly and aerobically mineralize dioxane as a sole energy and carbon source (Parales *et al.* 1994; Kim *et al.* 2009; Sei *et al.* 2013; Huang *et al.* 2014). Another major way to degrade dioxane is via co-metabolism. *Pseudonocardia* sp. strains K1 and ENV478 were isolated from wastewater and tetrahydrofuran (THF, )-habituated cultures, and they were capable of degrading 1,4-dioxane after the THF was completely degraded (Kohlweyer *et al.* 2000; Vainberg *et al.* 2006). A high dioxane degradation rate occurs following bacterial growth in THF. *Pseudonocardia* sp. ENV478 mediated dioxane degradation resulted in the accumulation of the intermediate product 2-hydroxyethoxyacetic acid (HEAA) (Vainberg *et al.* 2006). It was speculated

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that the inability of strain ENV478 and some other THF-degrading bacteria to efficiently metabolize the intermediate product HEAA was the major reason for their failure to completely degrade dioxane. *Flavobacterium* was isolated from contaminated groundwater, and the rate of dioxane degradation increased with increasing THF concentrations (Sun *et al.* 2011). River, soil, activated sludge, and Arctic groundwater samples have been collected to evaluate the biodegradation potential of dioxane, as well as for biostimulation and bioaugmentation tests (Li *et al.* 2010; Sei *et al.* 2010). In a previous study, the abundance of THF/dioxane monooxygenase (MO) genes (*thmA*/*dxmA*) was determined, and a primer/probe set was developed to assess the dioxane degradation activity by bacterial communities in aquifers (Li *et al.* 2014).

The kinetics of dioxane degradation were examined in a previous study, and 13 of 20 bacteria could degrade dioxane. There are six forms of MOs, and three MOs (particulate methane MO (MMO), single chain tryptophan 2-MO, and toluene *o*-xylene MO) were not able to catalyze the degradation of dioxane (Mahendra & Alvarez-Cohen 2006). Triple quadrupole-mass spectrometry and Fourier transform ion cyclotron resonance mass spectrometry were used to analyze the pathway of 1,4-dioxane degradation (Mahendra *et al.* 2007). Differential gene expression in *Pseudonocardia dioxanivorans* strain CB1190 growing in the presence of 1,4-dioxane, glycolate (a previously identified intermediate of dioxane degradation), or pyruvate as carbon sources was detected by a microarray, and it was shown that only one of the eight MO gene clusters in the CB1190 genome (the one located on plasmid pPSED02) upregulated the pathway for converting dioxane to pyruvate (Sales *et al.* 2013). Glyoxylate carboligase activity in cell extracts from cells pregrown in the presence of dioxane further demonstrated that glyoxylate metabolism plays a key role in dioxane degradation (Grostern *et al.* 2012).

Domestic sewage is one of the most important potential sources of dioxane, and the activated sludge process is the most common method for wastewater treatment. The mechanisms of microbial dioxane degradation using activated sludge are not well understood. To better understand the biodegradation mechanisms, in this study we used 16S rRNA and metatranscriptomic assays to analyze the degradation system. We aimed to address the following four issues: (1) the ability of activated sludge to degrade dioxane; (2) the impact of adding different THF concentrations by different methods on dioxane degradation; (3) the changes in bacterial community composition and diversity during the dioxane degradation process; and (4) the changes in bacterial community function and key enzymes during dioxane degradation.

MATERIALS AND METHODS

Experimental setup

Activated sludge was sampled from the Qinghe sewage treatment site located in Qinghe, north of Beijing, China. It uses the A^2/O treatment process to treat the domestic sewage. The site can treat 0.2 million m^3/d of sewage, and the rate of sewage discharge is 5,000 m^3/d. The mixed-liquor suspended solids concentration is 200–300 mg/L; the chemical oxygen demand is 350–450 mg/L; the 5-day biological oxygen demand is 180–220 mg/L; the pH is 6.8–7.0; and the temperature is 18 °C. To analyze the effects of the THF concentration, as well as the method by which THF is added, on dioxane degradation, one control system (with 1,000 mg/L NaN_3 to inhibit the activities of bacteria) and three different experiment groups, including 10 degradation systems, were employed (Table 1). All enrichments were performed with basal salts medium (BSM). One liter of BSM contained 100 ml of BSM buffer stock (3.24 g of K_2HPO_4, 1.0 g of $NaH_2PO_4{\cdot}H_2O$, and 2.0 g of NH_4Cl) and 100 ml of BSM trace metal stock. The BSM trace metal solution contained (per liter): 1.23 g of nitrilotriacetic acid (disodium salt), 2.0 g of $MgSO_4{\cdot}7H_2O$, 0.12 g of $FeSO_4{\cdot}H_2O$, 0.03 g of $MnSO_4{\cdot}H_2O$, 0.03 g of $ZnSO_4{\cdot}H_2O$, and 0.01 g of $CoCl_2{\cdot}6H_2O$. Five hundred milliliters of BSM medium in 580 ml serum bottles was sterilized at 121 °C and 0.1 MPa for 25 min, and then dioxane and THF were added. All the experimental systems were cultured at 28 °C with shaking at 150 rpm. Two milliliters of liquid was removed at various time points during the degradation process to test the dioxane and THF concentrations.

Analytical methods

The dioxane and THF concentrations were monitored using a GC-2010 plus gas chromatograph (Shimadzu®, Kyoto, Japan) equipped with a flame ionization detector and a split/splitless injection port. An RTX-5 (Shimadzu®) column (60 m × 0.25 mm × 0.25 μm) was used. The gas chromatograph flow rates were 1.22 ml/min N_2, and the flows of the gases used with the flame ionization detector system were 30 ml/min N_2, 50 ml/min H_2, and 50 ml/min air. A constant flow rate was used in the gas chromatograph experiments. The split ratio was set at 1:20 after 3 min of

Table 1 | The treatment in the degradation systems

Groups	Systems no.	Activated sludge (ml)	1,4-Dioxane (mg/L)	BSM	THF (mg/L)	NaN_3
Control	C	150.0	87.0	+	–	+
Experiment Group 1	N1	150.0	91.5	–	–	–
	N2	150.0	96.2	–	90.0	–
	N3	150.0	85.0	+	–	–
	N4	150.0	83.0	+	90.0	–
Experiment Group 2	N5–N10	150.0	100.0	100	10.0 (N5), 20.0 (N6), 60.0 (N7), 100.0 (N8), 150.0 (N9), 200.0 (N10)	–
	G2-C	150.0	100.0	100	–	–
Experiment Group 3	N11	150.0	100.0	100	Adding three times and ~60 mg/L per time	–

'+' indicated adding BSM or NaN_3; '–' indicated no BSM or NaN_3. Triplicate batch-scale parallel control experiments were carried out for each system.

splitless conditions. The temperature programming consisted of an initial temperature of 60 °C for 5 min, which was increased at 20 °C/min to 130 °C. The detection limits of dioxane and THF were 0.8 mg/l.

Total DNA and RNA extraction

We chose three samples (N4-I, the initial time; N4-II, after 5 days (the time at which THF was almost completely degraded and dioxane began to degrade); and N4-III, after 21 days, the time at which dioxane was no longer degraded) in the N4 system to conduct the microbial analysis. For each sample, 60 ml of mixed culture in incubation bottles was transferred to a centrifuge tube and centrifuged at $10{,}800 \times g$ for 10 min. Subsequently, the supernatant was discarded and the samples were stored at −80 °C for DNA and RNA extraction. The stored samples (0.5 g and 2.5 g, respectively) were extracted by the PowerSoil DNA Extraction Kit (MoBio® Laboratories, Carlsbad, CA, USA) to isolate DNA and the E.Z.N.A Soil RNA Kit (Omega Bio-Tek®, Norcross, GA, USA) to isolate RNA, and then they were stored at −20 °C for further use and at −80 °C for permanent preservation. The quantity and quality of the isolated DNA and RNA were evaluated using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific®, Waltham, MA, USA) and agarose gel electrophoresis (Bio-Rad®, Hercules, CA, USA), respectively.

Processing of high-throughput sequencing and statistical analyses

Polymerase chain reactions (PCRs) were performed with the primers 341F and 518R to amplify the V3 region of the 16S rRNA gene (Bartram *et al.* 2011). For each sample, approximately 3 μg of total RNA was first treated with the Ribo-Zero rRNA Removal Kit (Bacteria) (Epicentre, Illumina®, San Diego, CA, USA) to remove ribosomal RNAs. Then, the resulting mRNAs were sheared into fragments of ~180 bp in length, and sequencing libraries were constructed according to a standard protocol provided by Illumina. Library quantification was performed using a Qubit Fluorometer (Invitrogen, Life Technologies®, Grand Island, NY, USA) and a Stratagene Mx3000P Real-time PCR Cycler (Agilent, Santa Clara, CA, USA) prior to cluster generation in a c-Bot automated sequencing system (Illumina®). Both the 16S amplicons and RNA libraries were sequenced using an Illumina HiSeq 2000 high-throughput sequencing instrument with 2×100 bp paired-end sequencing.

High-quality reads were assigned to each sample according to its unique barcode. All the 16S rRNA V3 sequences were analyzed using the QIIME software package (Caporaso *et al.* 2010). Briefly, the reads were filtered initially by QIIME quality filters. Sequences with ≥97% similarity were assigned to the same operational taxonomic units (OTUs). We picked representative sequences for each OTU and used the Ribosomal Database Project classifier to annotate the taxonomic information for each representative sequence (Cole *et al.* 2014).

Metatranscriptomic reads were assembled using SOAPdenovo2 v2.04 (Luo *et al.* 2012), and certain assembled contigs were further scaffolded using PGA (Zhao *et al.* 2008). The MetaGeneMark v2.8 gene prediction tool (Zhu *et al.* 2010) was used to predict genes from assembled contigs. The predicted proteins were compared against the non-redundant database at the National Center for Biotechnology Information using the protein Basic Local Alignment Search Tool (BLAST) with an E-score cutoff value of $<10^{-2}$, and they were taxonomically and functionally annotated

using a combination of lowest common ancestor and consensus approaches (Wang *et al.* 2013). Functional categories and genes in Kyoto Encyclopedia of Genes and Genomes pathways were counted in each sample for further functional analysis. After normalizing the sequence counts of each taxonomic and functional group by the total number of reads, a statistical analysis was performed based on the relative abundance of bacterial taxa and functions. Alpha diversity was calculated using taxonomic and functional metrics (Simpson's Diversity Index, 1/D).

The sequencing data were deposited in the Sequence Read Archive database under the project accession numbers SRR2125738, SRR2125741, SRR2125758, and SRR2125759 (16S rRNA), and SRR2125760, SRR2125784, and SRR2125785 (metatranscriptome).

RESULTS

Dioxane degradation in different pollution systems

Activated sludge was subcultured for 15 days after adding dioxane to the degradation systems, and dioxane began to degrade in system N4 in BSM medium after 3 days (Figure 1(a)). Dioxane began to degrade in system N2 after 5 days. The dioxane degradation rate for system N4 was close to 40%, and the rate for system N2 was 29.7%. With or without BSM medium, dioxane degradation occurred when THF was degraded to a certain concentration. THF in system N2 was degraded to below the detection limit after 7 days, while it was degraded to below the detection limit after 5 days in system N4. Dioxane degradation was greater in system N4 than in system N2. When THF was degraded to below the detection limit, dioxane was no longer degraded after 2 weeks.

We added different concentrations of THF, i.e., 10.0, 20.0, 60.0, 100.0, 150.0, and 200.0 mg/l, to the activated sludge and assessed the effects on dioxane degradation (Figure 1(b)). The dioxane degradation adaptation period increased with increasing THF concentrations. Dioxane began to degrade after 3 days at THF concentrations of 10.0, 20.0, and 60.0 mg/l. Dioxane degraded after 5 days at THF concentrations of 100.0 and 150.0 mg/l, while dioxane degradation occurred after 8 days at a THF concentration of 200.0 mg/l. In system N2, THF degraded to below the detection limit. As the THF concentration increased, the dioxane removal rate increased. Dioxane removal was highest (62%) at THF concentrations of 100.0 and 200.0 mg/l. The dioxane degradation rate increased when the THF concentration increased from 10 to 60.0 mg/l. When the THF

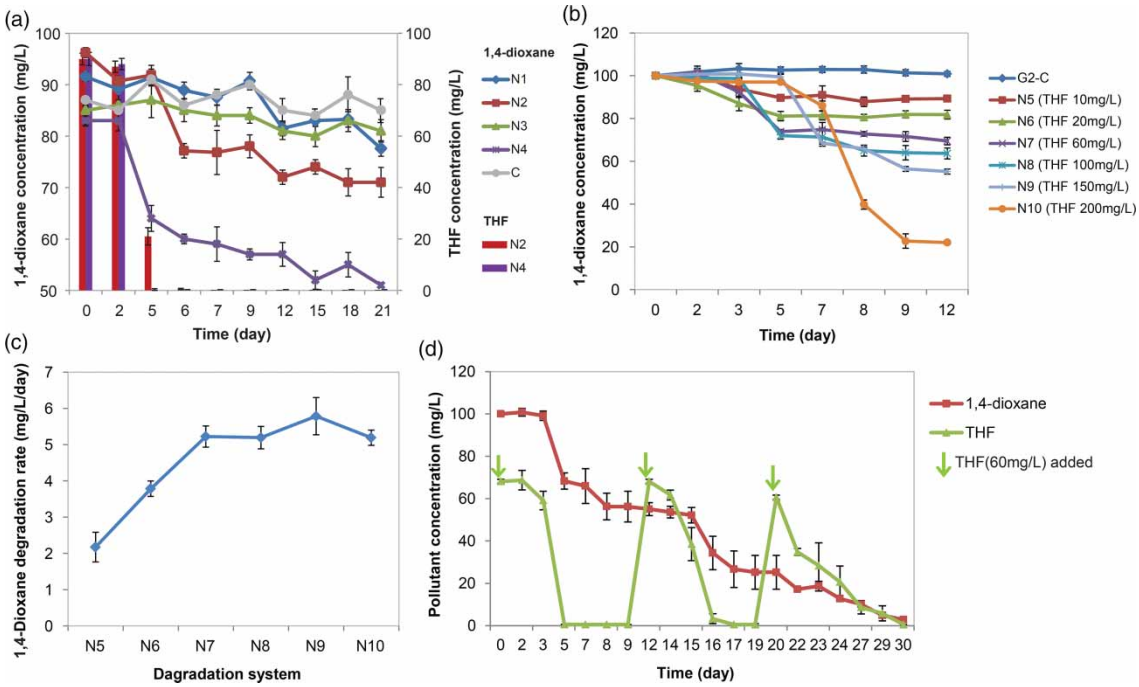

Figure 1 | Changes of dioxane and THF concentrations. (a) Changes of the dioxane and THF concentrations in the N1–N4 degradation systems. (b) Changes in the dioxane concentration under different THF concentrations in the N5–N10 degradation systems. (c) Effect of different THF concentrations on the dioxane degradation rate in the N5–N10 degradation systems. (d) Dioxane degradation under the condition of continuously added THF (N11).

concentration was 60 mg/l or greater, the rate of dioxane degradation stabilized at approximately 5.22 mg/l/day (Figure 1(c)). At THF concentrations of 60.0 mg/l or greater, the dioxane degradation rate was only slightly affected.

Dioxane degraded to below the detection limit after three consecutive additions of 100 mg/l THF (Figure 1(d)). We also compared the impact of subculturing on dioxane degradation in system N11. In the first two degradation processes, the THF degradation rates were very similar, and the detection limit of dioxane degradation was reached after approximately 7 days. After the third addition of THF, THF and dioxane were degraded to below their detection limits after approximately 12 days.

Bacterial community structure analysis based on 16S rRNA genes

Sequencing of three samples (N4-I, -II, and -III) resulted in a total of 283,280 16S rRNA V3 region sequences after quality screening 334,727 raw paired reads, and an average of 1,864 OTUs were obtained at a 97% cutoff. In the N4-I, -II, and -III samples, the most abundant phyla were the Proteobacteria (59.3%, 43.2%, and 47.4%, respectively), the Bacteroidetes (21.4%, 34.8%, and 28.1%, respectively), and the Chloroflexi (35.0%, 55.6%, and 53.7%, respectively). Other highly abundant phyla in the N4-I, -II, and -III samples were the Nitrospirae (19.4%, 16.6%, and 32.5%, respectively), the Verrucomicrobia (18.0%, 15.4%, and 16.7%, respectively), the Acidobacteria (12.3%, 24.7%, and 31.5%, respectively), and the Chlorobi (12.3%, 16.9%, and 20.4%, respectively). The top 20 genera included *Nitrospira*, *Dokdonella*, and *Thauera* (Figure S1, available with the online version of this paper). Bacterial genera that were previously reported to directly metabolize or co-metabolize dioxane exhibited low abundances in this study. The abundance of *Rhodococcus* in the system showed an increasing trend. *Flavobacterium* and *Pseudomonas*, which were previously reported to degrade dioxane (Mahendra & Alvarez-Cohen 2006; Sun *et al.* 2011), showed a decreasing trend from N4-I to N4-III.

Metatranscriptomic analysis of the bacterial community structure

Sequencing of the three samples (N4-I, -II, and -III) resulted in a total number of 336 million paired reads. The paired reads were merged into 61 million long sequences (160 to ~197 bp each), and these paired-end-merged sequences were compared against the non-redundant protein database at the National Center for Biotechnology Information by BLASTX. After normalizing the sequence counts for each taxonomic group by the total number of reads, a statistical analysis was performed on the bacterial composition and abundance at the phylum and genus levels.

The most abundant phyla in the N4-I, -II, and -III samples were the Proteobacteria (84.2%, 46.8%, and 26.1%, respectively), the Bacteroidetes (4.8% 29.7% and 33.1%, respectively), the Spirochaetes (1.87% in N4-I), and the Nitrospirae (12.0% and 11.1%, respectively, in N4-II and N4-III); other phyla included the Firmicutes, the Actinobacteria, the Acidobacteria, the Cyanobacteria, the Chloroflexi, and the Ignavibacteriae. Bacterial genera that were previously reported to directly or co-directly metabolize dioxane were also detected, such as *Flavobacterium* in the phylum Bacteroidetes, *Mycobacterium* and *Rhodococcus* in the phylum Actinobacteria, and *Burkholderia*, *Pseudomonas*, and *Ralstonia* in the phylum Proteobacteria; only *Flavobacterium* and *Pseudomonas* were among the 20 most abundant genera (Bernhardt & Diekmann 1991; Kim *et al.* 2009; Sun *et al.* 2011).

The genus-level analysis showed that *Thauera*, *Candidatus Accumulibacter*, and *Flavobacterium* were among the 20 most abundant genera that commonly exist in activated sludge (Shin *et al.* 2010). Other genera, such as *Nitrospira*, *Nitrosomonas*, *Haliangium*, and *Fluviicola*, have different nutritional needs for growth in natural environments (Figure S2, available with the online version of this paper). We found that the abundance of bacteria involved in the degradation of THF and dioxane increased from N4-I to N4-III (Table 2). *Flavobacterium* was 12-fold more abundant in N4-II than in N4-I. The abundances of *Ralstonia* and *Rhodococcus* also increased from N4-I to N4-II. Although the abundances of *Rhodococcus* and *Pseudonocardia* were low, they increased significantly from N4-I to N4-II. We suggest that these dioxane-degrading bacteria became the most

Table 2 | Relative abundance of genera involving 1,4-dioxane degradation in system N4 based on metatranscriptomic analyses

Genus	N4-I	N4-II	N4-III
Flavobacterium	0.18%	2.23%	1.08%
Pseudomonas	1.56%	0.77%	0.43%
Burkholderia	0.60%	0.62%	0.76%
Ralstonia	0.20%	0.37%	0.08%
Rhodococcus	0.03%	0.15%	0.09%
Mycobacterium	0.10%	0.09%	–
Pseudonocardia	–	0.12%	0.03%

active genera because of the addition of the co-metabolic substrate THF. In system N4-III (in which the concentration of dioxane did not change), the bacterial community composition also exhibited greater changes. The relative abundance of genera involved in dioxane degradation, such as *Flavobacterium*, *Burkholderia*, *Rhodococcus*, and *Pseudonocardia*, in N4-II was greater than that of N4-I.

Bacterial community diversity

As shown in Table 3, the taxonomic richness (based on 16S rRNA and metatranscriptomic data) decreased in the N4-II samples; from system N4-I to N4-II and N4-III, the total abundance of taxa (based on 16S rRNA and metatranscriptomic data) decreased, and the functional genes (based on metatranscriptomic data) decreased substantially. Simpson's Diversity Index of taxa (based on 16S rRNAs) and functional genes decreased, while that of taxa (based on metatranscriptome) increased.

Changes in metabolic pathways and enzymes in the N4 degradation system

We found that common metabolic pathways of carbohydrate metabolism, energy metabolism, xenobiotic biodegradation and metabolism, amino acid metabolism, and other pathways existed in all three samples. Meanwhile, the relative abundances of several important functional genes that maintain essential activities and the energy metabolism of the organisms were high (Figure S3, available with the online version of this paper).

Table 3 | Diversity indexes three samples collected fromN4 degradation system

	N4-I	N4-II	N4-III
Richness			
16S rRNA taxonomy	507	457	455
Metatranscriptomic taxonomy	1,049	895	769
Functional genes	2,930	2,237	2,151
Total abundance			
16S rRNA taxonomy	399,751	399,799	399,799
Metatranscriptomic taxonomy	99,515	99,547	99,642
Functional genes	190,579	109,929	72,545
Simpson Diversity Index, 1/D			
16S rRNA taxonomy	7.46	3.90	4.28
Metatranscriptomic taxonomy	4.62	20.75	27.37
Functional genes	373.02	292.83	263.61

The relative abundance of genes involved in carbohydrate metabolism was highest (27.73%) in N4-II, while the relative abundance of genes involved in energy metabolism was highest (25.12%) in N4-III (Figure S3). In the carbohydrate metabolism pathway, the abundance of genes in the pentose phosphate metabolic pathway increased the most (the relative abundance increased from 0.41% in N4-I to 1.52% in N4-II to 2.35% in system N4-III), followed by the citrate cycle genes (the relative abundance increased from 1.84% in N4-I to 5.11% in N4-II, and then decreased to 2.17% in N4-III). As shown in Table 4, the energy metabolism pathways mainly included methane metabolism, nitrogen metabolism, oxidative phosphorylation, and other metabolic pathways. The carbon fixation pathway genes involved in energy metabolic pathways showed the largest change (the relative abundance of genes increased from 4.31% in N4-I to 7.07% in N4-II, and then decreased to 4.78% in N4-III), followed by sulfur metabolism genes (the relative abundance increased from 2.13% in system N4-I to 2.73% in system N4-II, and then increased to 3.33% in N4-III). Moreover, the abundances of genes involved in nitrotoluene degradation and styrene degradation, which are xenobiotic degradation pathways, changed greatly (Table 4).

The metabolic pathway analysis showed that the relative abundance of oxidoreductases was predominant, and it changed the most among the six enzymes. It decreased from 17.69% in N4-I to 15.31% in N4-II, and then increased to 20.26% in N4-III (Figure 2(a)). This was followed by transferases (the relative abundance of which decreased from 9.81% in N4-I to 9.09% in N4-II, and then increased to 11.20% in N4-III) (Figure 2(a)). The functional MO genes were extracted and analyzed, and they included MMO, ammonia MO (AMO), phenylalanine-4-MO, kynurenine MO-3, THF MO, and other MOs (Figure 2(b)). The most abundant MOs detected were AMOs, followed by MMOs, both of which increased from N4-I to N4-III. Kynurenine-3-MO and THF MO were not detected in N4-I and N4-II, but they were found in N4-III (Figure 2(b)).

DISCUSSION

In this study, we analyzed the feasibility and effectiveness of microbiological methods to remove dioxane. The concentration and manner of addition of the co-metabolized substrate THF affected the removal of dioxane. The 16S rRNA and metatranscriptomic analyses of the microbial communities showed that the co-metabolic degradation of

Table 4 | Relative abundance of the metabolic pathways in the N4 degradation system

Metabolism	Metabolic pathways	N4-I (reads)	N4-II (reads)	N4-III (reads)
Global and overview maps	Carbon metabolism	14.55%	14.62%	14.53%
Energy metabolism	Methane metabolism	9.31%	8.62%	9.66%
Xenobiotics biodegradation and metabolism	Nitrotoluene degradation	3.57%	6.60%	6.60%
Energy metabolism	Carbon fixation	4.31%	7.07%	4.78%
Carbohydrate metabolism	Pyruvate metabolism	5.23%	6.28%	4.71%
Carbohydrate metabolism	Propanoate metabolism	5.17%	5.71%	4.65%
Carbohydrate metabolism	Butanoate metabolism	4.53%	5.09%	4.02%
Metabolism of cofactors and vitamins	Thiamine metabolism	2.79%	4.40%	5.33%
Global and overview maps	Biosynthesis of amino acids	6.54%	3.95%	5.05%
Carbohydrate metabolism	Citrate cycle	1.84%	5.11%	2.17%
Energy metabolism	Nitrogen metabolism	5.29%	3.14%	4.81%
Energy metabolism	Sulfur metabolism	2.13%	2.73%	3.32%
Carbohydrate metabolism	Gluconeogenesis	1.43%	2.91%	1.78%
Amino acid metabolism	Valine, leucine and isoleucine degradation	3.65%	2.71%	1.40%
Amino acid metabolism	Cysteine and methionine metabolism	3.49%	1.46%	2.77%
Amino acid metabolism	Alanine, aspartate and glutamate metabolism	1.80%	1.45%	3.00%
Energy metabolism	Oxidative phosphorylation	3.26%	1.11%	2.56%
Carbohydrate metabolism	Pentose phosphate pathway	0.41%	1.52%	2.35%
Xenobiotics biodegradation and metabolism	Styrene degradation	1.05%	1.04%	2.80%
Amino acid metabolism	Tyrosine metabolism	0.82%	0.90%	2.80%
Others	Others	18.82%	13.56%	10.91%

dioxane resulted from the joint action of a group of bacteria and their MOs, and that the structure of the activated sludge was also stable during the degradation of dioxane.

Activated sludge was used as the source of microbes for the dioxane degradation experiments, and the presence of THF was required for dioxane degradation. THF is more likely to be utilized by microorganisms than dioxane. Although THF is an environmental pollutant, activated sludge can completely degrade it. It has been reported that microbes can completely mineralize THF (Kohlweyer *et al.* 2000; Chen *et al.* 2010). Thus, the addition of THF will stimulate the microbial degradation of dioxane, but it will not affect the environment. Dioxane degradation occurred in the presence of THF, and after the THF was degraded, dioxane was no longer degraded. This phenomenon was previously observed for either microbial communities or single species (Vainberg *et al.* 2006; Sun *et al.* 2011). Therefore, different THF concentrations and methods of THF addition are very important for the treatment of dioxane. Furthermore, we applied 16S rRNA and metatranscriptomic methods to explore the changes in the structure and function of the microbial community in the activated sludge during dioxane degradation.

The addition of BSM medium promoted dioxane degradation primarily due to the increases in the abundance of ammonia-oxidizing and denitrifying bacteria, which reduced the length of the adaptation period. In previous studies, dioxane degradation required a long (3-month) period of adaptation in mixed cultures during the co-metabolic degradation of dioxane (Zenker *et al.* 2000). It was reported that a test group of bacteria from dioxane contaminated soil continued to degrade dioxane for 9 months in the presence of THF (Sun *et al.* 2011). The adaptation period was 3 months for the bioaugmentation degradation of dioxane at 4 °C (Li *et al.* 2010). Seven-, 14-, and 21-day adaptation periods were observed using different environmental samples as bacterial sources for dioxane degradation (Sei *et al.* 2010). A previous study compared the combined impacts of 1-butanol and 1-butanol in BSM medium on dioxane degradation (Li *et al.* 2010). The results demonstrated that proteins in the culture increased greatly, and the removal of dioxane was high, which is similar to our results.

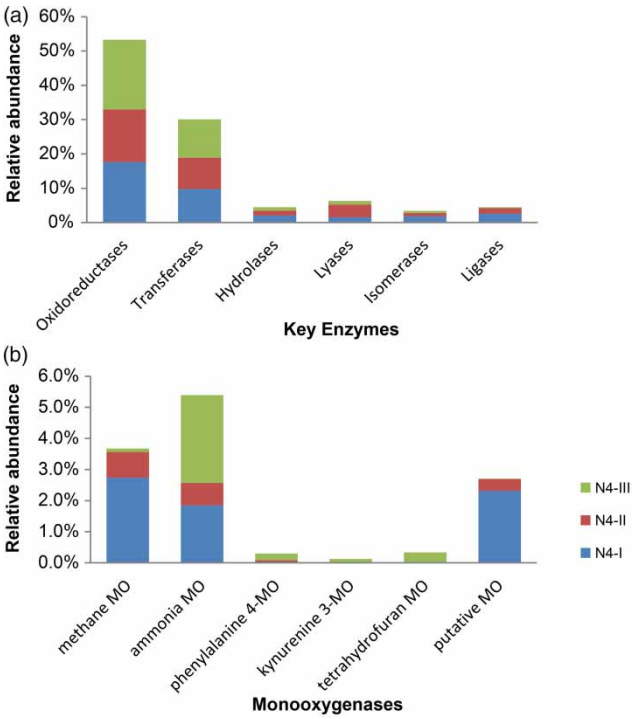

Figure 2 | Relative abundances of key enzymes and MO in N4-I, -II, and III. N4-I, day 0, the initial time sample in the N4 degradation system; N4-II, day 5, the sample collected from the N4 degradation system when THF was almost completely degraded and dioxane was beginning to degrade; and N4-III, day 21, the sample collected from the N4 degradation system when dioxane was no longer degraded.

From point of view in the chemical structure, THF is simpler than dioxane and easier to degrade. Under the pressure of THF, the expression of monooxygenase increased in ammonia-oxidizing and denitrifying bacteria, and then promoted the degradation of dioxane. An analysis of the ability of a single bacterial species to degrade dioxane at different THF concentrations showed that the dioxane degradation rate increased with increasing THF concentrations (Sun *et al.* 2011). However, microorganisms exist in the environment as microbial communities. We found that as the THF concentration increased, microbial communities needed longer adaptation periods before they degraded dioxane. THF had little effect on the dioxane degradation rate at concentrations ranging from 60.0 to 200.0 mg/L, but the dioxane degradation ratio increased with increasing THF concentrations. The degradation rate in system N10 (in which there were three additions, ~60.0 mg/L each, of THF) was higher than that in system N9 (one addition of 200.0 mg/L THF). The reason why dioxane was not degraded after the degradation of THF was nearly complete was due to the accumulation of toxic intermediates (e.g., acetylene) during the metabolic process (Mahendra & Alvarez-Cohen 2006). However, the most likely reason is that the THF cofactor was exhausted during the degradation process, thus preventing further dioxane degradation. Alternatively, enzymes involved in dioxane degradation rapidly lost their activity (Zenker *et al.* 2000). For example, the enzyme cofactor NADH was exhausted during the degradation of trichloroethylene when methane was used as the growth substrate (Alvarez-Cohen & McCarty 1991; Chang & Alvarez-Cohen 1995). We suggest that multiple additions of low concentrations of THF aided the recovery of the auxiliary factor (e.g., NADH) and decreased the intermediate byproduct in the culture system. During the remediation of dioxane-contaminated environments, we should select the appropriate THF concentration and method of addition to best degrade this pollutant.

Because of the complexity of microbial communities in activated sludge systems, we applied 16S rRNA and metatranscriptomic methods to understand the changes in the composition and function of the microbial community in response to dioxane. In the initial stage, the phylum Nitrospirae increased in abundance in response to the addition of nitrogen. The addition of the co-metabolized substrate THF to dioxane changed the microbial community composition. According to the 16S rRNA V3 region analysis, the relative abundance of dioxane-degrading bacteria was low and showed different tendencies. However, the abundance of the dioxane-degrading bacteria found in the samples of this study increased significantly, except for the genus *Pseudomonas*. This indicates that the dioxane-degrading bacteria not only became more abundant, but also more active. *Pseudomonas* spp. are capable of degrading many pollutants (Ma *et al.* 2012; Khan *et al.* 2015). Although *Pseudomonas* spp. degrade dioxane, they are not sensitive to it. When THF, which served as the carbon source and energy source, was exhausted, the abundance and metabolic activity of the dioxane-degrading bacteria decreased. When enzyme cofactors decrease and enzyme activity is lost, target pollutants are no longer degraded (Alvarez-Cohen & McCarty 1991; Chang & Alvarez-Cohen 1995; Zenker *et al.* 2000).

The diversity and composition of the bacterial communities changed in response to the THF concentration. The dioxane-degrading bacteria, which were previously reported to increase in the reaction system, did not become dominant in our study. The abundance of common bacteria, including nitrogen-removing bacteria, in the wastewater treatment plant remained high. Furthermore, oxygen consumption and the lack of microbial available carbon and other substances cause the accumulation of toxic metabolic substances, which reduce the abundance and the Simpson's

Diversity Index of the bacterial communities. The diversity of the 16S rRNA taxa decreased, but the diversity of the metatranscriptomic taxonomy increased. This indicates that the activity of low-abundance species in system N4-I increased. Moreover, it suggests that the transcriptional activity of dioxane-degrading bacteria in systems N4-II and N4-III increased. The diversity of metatranscriptomic taxonomy increased and functional diversity decreased, indicating that the transcriptional activity of the dioxane-degrading bacteria in the community increased, but that they possibly transcribed a functionally similar transcriptome, which tended to increase the abundance of transcripts from genes encoding MOs.

The analyses of gene composition and diversity showed that the relative abundances of metabolized substances, as well as the genes involved in energy metabolism, in N4-II and N4-III were high. Carbohydrate and energy metabolism are the basic and primary metabolic pathways for the growth and reproduction of bacterial communities. In the activated sludge system, the pathway of xenobiotic biodegradation and metabolism was more active than the other pathways, which aids the biodegradation of pollutants. In this study, both carbohydrate metabolism and xenobiotic biodegradation and metabolism in N4-II were more active than those in N4-I and III, which indicates that the abundances of bacteria and degraded enzymes (MMOs are known to oxidize over 300 compounds (Hazen 2010)) are likely to increase greatly under the selective pressure of THF and dioxane. Although the abundance and diversity of the N4-I, -II, and -III samples fluctuated, they did not change significantly. This demonstrates that the microbial communities in the activated sludge system were complex and stable compared with other degradation systems with low-diversity microbial communities.

The analysis of dioxane-degrading genes showed that the abundance of oxidoreductase genes was the highest and that it changed with time. Mahendra *et al.* (2007) studied the dioxane degradation kinetics of bacterial MOs, including THF MO and propane MO. Their results showed that toluene 2-MO, toluene 3-MO, and toluene 4-MO, as well as soluble MMO, played the main roles in dioxane degradation. A comparison of the MOs in the three samples (N4-I, -II, and -III) in this study showed that the most abundant genes encoded AMOs, followed by MMOs, while THF MO was detected in system N4-III. AMO and MMO catalyze the oxidation of a broad range of organic compounds, with oxygen serving as a terminal electron acceptor (Pornwongthong *et al.* 2014). In this study, they were the most dominant MOs that co-metabolically degraded dioxane. AMO and MMO were more abundant than the other MOs that were associated with the bacterial community. Nitrifying, ammonia-oxidizing, and denitrifying bacteria were the dominant genera because the activated sludge mainly dealt with nitrogen removal. In this study, *Nitrosomonas* was a dominant ammonia-oxidizing bacterium, and it is likely to express the AMO for dioxane degradation. *Thauera* and *Nitrospira* were the dominant denitrifying bacteria in the degradation system, and they greatly contribute to nitrogen removal in wastewater treatment plants and a denitrifying, quinoline-removal bioreactor (Fischer & Majewsky 2014; Langone *et al.* 2014). MMOs are widely expressed by nitrifying, anaerobic ammonium oxidation (anammox), and denitrifying bacteria (Langone *et al.* 2014). Not only do ammonia-oxidizing bacteria express AMOs, two *Nitrospira* species do so as well. The sequences of genes encoding MMOs have been reported to be highly similar to those encoding AMOs (Santoro 2016). We suggest that ammonia-oxidizing and denitrifying bacteria with MMOs and AMOs played the most important roles in this study.

Moreover, the diversity and stability of the bacterial community composition and function remained high when sewage sludge was used for the dioxane treatment. The relationships and influences between genera are the key to revealing the mechanism of dioxane degradation. Although we identified the dominant bacterial groups and MOs in this study, we hypothesize that the degradation of dioxane is the result of a variety of bacteria and that it is associated with various enzymes in the dioxane degradation pathway in the complex, activated sludge medium. Based on our results, in future studies we will design several quantitative PCR primers to identify specific bacteria and functional genes, which would aid the quantitative characterization of various dioxane-degrading bacteria and their functional enzymes during dioxane degradation.

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