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Microbial community functional structure in response to antibiotics in pharmaceutical wastewater treatment systems

Yu Zhang [a], Jianping Xie [b,c], Miaomiao Liu [a], Zhe Tian [a], Zhili He [b], Joy D. van Nostrand [b], Liren Ren [d], Jizhong Zhou [b,e,f], Min Yang [a,*]

[a] State Key Laboratory of Environmental Aquatic Chemistry, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, P.O. Box 2871, Beijing 100085, China
[b] Institute for Environmental Genomics, Department of Microbiology and Plant Biology, University of Oklahoma, Norman, OK 73019, USA
[c] School of Mineral Processing and Bioengineering, Central South University, Changsha, Hunan 410083, China
[d] Institute of Environmental Science and Technology, North China Pharmaceutical Group Corporation, Hebei, China
[e] Earth Science Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94270, USA
[f] State Key Joint Laboratory of Environment Simulation and Pollution Control, School of Environment, Tsinghua University, Beijing 100084, China

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ABSTRACT

It is widely demonstrated that antibiotics in the environment affect microbial community structure. However, direct evidence regarding the impacts of antibiotics on microbial functional structures in wastewater treatment systems is limited. Herein, a high-throughput functional gene array (GeoChip 3.0) in combination with quantitative PCR and clone libraries were used to evaluate the microbial functional structures in two biological wastewater treatment systems, which treat antibiotic production wastewater mainly containing oxytetracycline. Despite the bacteriostatic effects of antibiotics, the GeoChip detected almost all key functional gene categories, including carbon cycling, nitrogen cycling, etc., suggesting that these microbial communities were functionally diverse. Totally 749 carbon-degrading genes belonging to 40 groups (24 from bacteria and 16 from fungi) were detected. The abundance of several fungal carbon-degrading genes (e.g., glyoxal oxidase (*glx*), lignin peroxidase or ligninase (*lip*), manganese peroxidase (*mnp*), endochitinase, exoglucanase_genes) was significantly correlated with antibiotic concentrations (Mantel test; $P < 0.05$), showing that the fungal functional genes have been enhanced by the presence of antibiotics. However, from the fact that the majority of carbon-degrading genes were derived from bacteria and diverse antibiotic resistance genes were detected in bacteria, it was assumed that many bacteria could survive in the environment by acquiring antibiotic resistance and may have maintained the position as a main player in nutrient removal. Variance partitioning analysis showed that antibiotics could explain 24.4% of variations in microbial functional structure of the treatment systems. This study provides insights into the impacts of antibiotics on microbial functional

* *Corresponding author*. 18 Shuangqing Road, Haidian District, Beijing 100085, China. Tel.: +86 6292 3475; fax: +86 6292 3541.
E-mail address: yangmin@rcees.ac.cn (M. Yang).
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structure of a unique system receiving antibiotic production wastewater, and reveals the potential importance of the cooperation between fungi and bacteria with antibiotic resistance in maintaining the stability and performance of the systems.

1. Introduction

Since the introduction of penicillin, antibiotic production and application for the treatment of bacterial infections and diseases have continued to increase (Kümmerer, 2003). Along with the fermentative production of antibiotics, large volumes of antibiotic production wastewater containing antibiotics of up to several mg L^{-1} are routinely generated (Larsson et al., 2007; Li et al., 2008a), which are much higher than previously reported in other environments (Kümmerer, 2009).

Activated sludge processes, a biological approach utilizing bacterial metabolic functions for the removal of organic nutrients (Seviour and Nielsen, 2010; Wagner and Loy, 2002) have been widely applied for the treatment of antibiotic production wastewater. As antibiotics are designed to target bacteria, there is a concern that high concentrations of antibiotics in wastewater may seriously inhibit bacterial growth, leading to deterioration in bacterial functions for the removal of organic nutrients. A reduction in the number of bacteria and alterations in microbial populations were observed in model sewage purification systems when different antibiotics were added with concentrations equivalent to those in hospital wastewater (Al-Ahmad et al., 1999; Kümmerer and Al-Ahmad, 2000). Our previous studies using clone libraries and quantitative PCR demonstrated that under high antibiotic levels (mainly with streptomycin), aerobic wastewater treatment communities may maintain system stability through adjusting bacterial and eukaryal compositions (Deng et al., 2012). Fungi, which are able to utilize organic nutrients in wastewater (Adav et al., 2007), were abundant with *Ascomycota* being the dominant phyla.

At the same time, the high antibiotic concentrations and bacterial densities in biological antibiotic production wastewater treatment systems make it easy for bacteria to acquire resistance and for antibiotic resistance genes to proliferate (Kim et al., 2007; Tenover, 2006). Our previous studies have consistently shown that the high antibiotic levels in antibiotic production wastewater could lead to the occurrence of abundant antibiotic resistance in bacterial isolates from antibiotic production wastewater treatment systems (Li et al., 2009, 2010). So it is possible that bacteria possessing antibiotic resistance may play an important role for nutrient removal. However, little has been done to evaluate the impacts of residual antibiotics in antibiotic production wastewater on the functions of microbial communities in biological treatment systems from a holistic view.

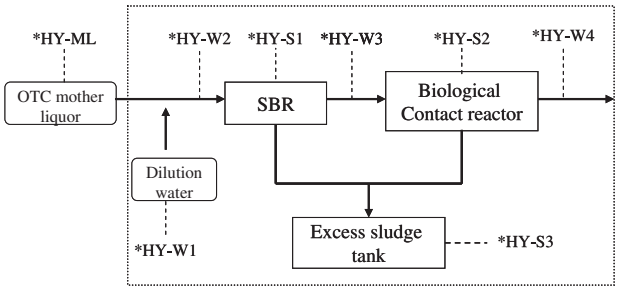
The rapid development of high throughput metagenomic approaches like GeoChip (He et al., 2010a) has made it possible to easily examine microbial functional diversity, composition and structure in a rapid fashion. GeoChip 3.0, which contains more than 57,000 gene variants from 292 functional gene families, including antibiotic resistance genes (He et al., 2010a), has been extensively employed to analyze the functional gene structure of microbial communities in different environments (He et al., 2010b; Trivedi et al., 2012; Wang et al., 2009). It is, therefore, a suitable tool for examining the impact of antibiotic residues on microbial communities with regard to microbial functional gene structures as well as the occurrence of antibiotic resistance in biological antibiotic production wastewater treatment systems.

This study aimed to reveal how the microbial functional communities in biological antibiotic production wastewater treatment systems respond to the high concentrations of residual antibiotics and maintain their nutrient removal functions. To answer this question, the functional gene structures of microbial communities in two biological antibiotic production wastewater treatment plants mainly receiving oxytetracycline-containing wastewater were analyzed with GeoChip 3.0 in combination with bacterial 16S rRNA gene clone library and quantitative PCR (for bacterial 16S rRNA genes, fungal 18S rRNA genes, and tetracycline resistance genes (*tet* genes)). The results of this study will provide useful information for the establishment of a sound process and operational strategy for successful antibiotic production wastewater treatment.

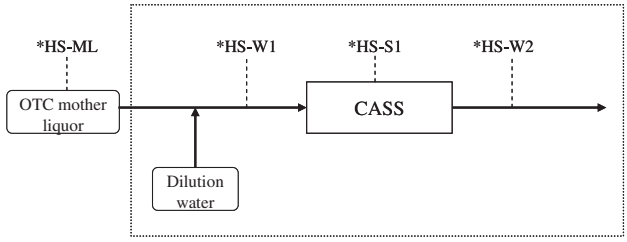
2. Material and methods

2.1. Study facilities, sampling sites and characterization of samples

Worldwide annual output of the tetracycline class of antibiotics has reached more than 20,000 tons. Over 1000 and 6000 tons of oxytetracycline are produced every year in the two studied antibiotic manufacturing plants – North China Pharmaceutical Group Corporation (HY) and Huashu Pharmaceutical Company (HS), respectively, in Shijiazhuang city, north China. These are among the largest oxytetracycline production facilities in the world. Biological antibiotic production wastewater treatment plants in HY and HS have been in use since 2000 and 2001, respectively. The wastewater treatment system in HY receiving oxytetracycline crystal mother liquor (HY-ML) consists of a sequential batch reactor (SBR) (hydraulic retention time, 8 h) and a two-stage submerged biological contact reactor using fiber bundle as the bio-carrier (hydraulic retention time, 8 h for each) (Fig. 1a). The dilution water (HY-W1) composing of the equipment washing water, penicillin G (Penicillin) production wastewater and a small amount of sewage are used to dilute the HY-ML. The total production of excess sludge from HY is approximately 125 tons (dry weight) per year. The final effluent in HY (HY-W4) is discharged to a nearby river. The mixed liquor suspended solids in the SBR is maintained at 4000–5000 mg/L. One cycle of the SBR includes



(a) HY biological antibiotic production wastewater treatment system



(b) HS biological antibiotic production wastewater treatment system

Fig. 1 – The flow chart and sampling sites of (a) HY- and (b) HS- biological antibiotic production wastewater treatment systems. "*": Sampling sites. W-water sample; S-sludge samples. (HS-ML and HY-ML are fresh and old oxytetracycline waste mother liquor, which were sampled from a pipe after 1 h and deposit tank after 12 h of oxytetracycline production, respectively; HY-W1: Dilution water in HY (consists mainly of discharged circulating cooling water and rinse water from the fermentation tanks and plate-and frame filter cloth, as well as wastewater from the penicillin productions). HY-W2: SBR influent; HY-W3: SBR effluent; HY-W4: the final effluent of HY; HY-S1: activated sludge from SBR; HY-S2: activated sludge from biological contact reactor; HY-S3: excess sludge of HY; HS-W1: CASS influent; HS-W2: the final effluent of HS; HS-S1: activated sludge from CASS. Dilution water in HS consists mainly of discharged circulating cooling water and rinse water from the fermentation tanks and plate-and frame filter cloth, as well as wastewater from the avermectin productions).

four operational steps, namely filling (1 h), aeration (5 h), settling (1.5 h), and decanting (0.5 h).

The wastewater treatment system in HS receiving oxytetracycline crystal mother liquor (HS-ML) is a cyclic activated sludge system (CASS; another type of SBR) producing an average flow of 6000 m^3 per day (Fig. 1b). Some other waste streams from the plant are also introduced into the CASS to dilute the HS-ML. The average hydraulic retention time of CASS is approximately 48 h and the mixed liquor suspended solids is maintained at 4000–5000 mg/L. The final effluent of HS (HS-W2) is discharged to the local sewage collection system. The oxytetracycline concentrations in the influents and effluents of the HY and HS systems varied in a range between 1.0 and 12 mg/L and 0.2 and 1.5 mg/L, respectively. As shown in Fig. 1, six water (5 L) and four sludge (100 ml) samples were collected for GeoChip analysis from HS and HY in January 2009. Water samples were collected using automated samplers over three successive days. At the same time, two mother liquor samples were collected from each system, respectively. Grab sludge samples were taken from the respective site during the sampling period. A detailed description of sample collection is provided in the supporting information. No rain event was registered either during the sampling days or the previous week.

Aerobic activated sludge samples from two non-antibiotic (Inosine-S and Ethanol-S) fermentation wastewater treatment plants in Xinxiang City, north China, were collected as controls, because these facilities mainly receive inosine and ethanol production waste streams, respectively. Details of antibiotic analysis and water and sludge characteristics were described previously (Li et al., 2008a, 2008b). All samples were analyzed for chemical properties in triplicate and average values were reported.

2.2. *Microbial community DNA isolation and purification for GeoChip analysis*

Ten ml of each wastewater sample was filtered through 0.22 μm polycarbonate membranes (GTTP, Millipore, Ireland), and biomass on the membranes was collected in 2 ml sterilized tubes for DNA extraction. Sludge samples were centrifuged at 9167 g for 10 min at 4 °C, and 0.25 g (wet weight) of sediment for each sample was used for DNA extraction. Community DNA was extracted using a freeze-grinding method as described previously (Zhou et al., 1996), and purified using a Promega Wizard® DNA Clean-Up System (Madison, WI, USA) according to the manufacturer's directions. DNA quality was evaluated by the absorbance ratios at A260/A280 and A260/A230 using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). Only DNA with A260/280 and A260/230 ratios >1.7 and 1.8, respectively, were used for further GeoChip analysis. DNA was quantified using PicoGreen (1) and a FLUOstar Optima (BMG Labtech, Jena, Germany). Purified DNA was stored at −80 °C until use.

2.3. *Procedure of GeoChip analysis*

Whole community genome amplification was used to generate approximately 3.0 μg of DNA with 50 ng of purified DNA as the template using the TempliPhi Kit (GE Healthcare, Piscataway, NJ, USA) following the manufacturer's instructions. Single-strand binding protein (267 ng/μl) and spermidine (0.1 mM) were also added to the reaction mix to improve the amplification efficiency (Wu et al., 2006). The reactions were incubated at 30 °C for 10 h and stopped by heating the mixtures at 65 °C for 10 min. The DNA (~3 μg) was labeled with Cy5 fluorescent dye (GE Healthcare, Piscataway, NJ, USA) by random priming (van Nostrand et al., 2009; Wu et al., 2008) purified with a QIAquick purification kit (Qiagen, Valencia, CA, USA) and dried in a SpeedVac (45 °C, 45 min; ThermoSavant, Austin, TX, USA). Dried labeled DNA was resuspended in hybridization buffer (50 μl; 40% formamide, 5 × SSC, 0.1% SDS, 0.1 μg/μl Salmon sperm DNA) and denatured at 98 °C for 3 min, and then kept at 65 °C until hybridization. Hybridizations were performed at 42 °C for 10 h using a

MAUI 12-Bay Hybridization System (BioMicro Systems Inc, Salt Lake City, USA). After hybridization, arrays were scanned with a ScanArray 500 microarray scanner (PerkinElmer, Boston, MA, USA) at 633 nm using a laser power of 90% and a photomultiplier tube (PMT) gain of 75%. Scanned images were processed using ImaGene, version 6.1 (BioDiscovery, El Segundo, CA, USA).

Raw data obtained using ImaGene were uploaded to our laboratory's microarray data manager (http://ieg.ou.edu/microarray/) and pre-processed using the data analysis pipeline with the following major steps: (i) Spots flagged as poor-quality by ImaGene 6.1 and with a signal to noise ratio [SNR, SNR = (Signal Intensity-Background)/Standard deviation of background] less than 3.0 were removed; (ii) The normalized intensity of each spot was calculated by dividing the signal intensity of each spot by the mean intensity of the effective spots of the array; (iii) If any of the replicates had (Signal Intensity - Mean Signal Intensity) more than twice the standard deviation, they were removed as outliers. Preprocessed GeoChip data were used for further statistical analysis.

2.4. Clone library analysis and quantitative PCR

After DNA extraction by the FastDNA Spin kit for soil (Qbiogene, Solon, OH) following the kit protocol, various conventional molecular analyses, including construction of bacterial 16S rRNA gene clone libraries, quantitative PCR for bacterial 16S rRNA genes, fungal 18S rRNA genes and tetracycline resistance genes (*tet* genes) were performed for sludge samples from the biological antibiotic production wastewater treatment systems as well as the controls.

The primers used for the amplification of bacterial 16S rRNA genes were 27f and 1492r (Polz et al., 1999). To construct the rRNA gene libraries for each sample, the products of three PCR amplifications were pooled and purified (Fermentas, Canada). The purified products were then ligated into the pMD-18T Cloning System according to manufacturer's instructions (Takara, Japan). The ligation products were transformed into competent cells (Tiangen, China), using IPTG (isopropyl-β-D-thiogalactopyranoside). White colonies were randomly picked and screened directly for inserts by performing colony PCR with primers M13f (−47)/M13r (−48) for the vector. The PCR-amplified products of positive recombinants were digested with the restriction enzyme Hae III (Takara, Japan) at 37 °C for 4 h, electrophoresed in 1.5% agarose gels at 200 V for 20 min, and stained with ethidium bromide. Clones with similar banding patterns were grouped together, and one representative clone from each group was chosen for sequencing. The obtained sequences of ca. 1500 bp bacterial 16S rDNA were compiled and compared to available rDNA sequences in GenBank using the NCBI BLAST program.

Sixteen tet genes (six RPP genes (*tet*(M), *tet*(O), *tet*(Q), *tet*(T), *tet*(W) and *tet*(B/P)), nine EFP genes (*tet*(A), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(K), *tet*(L), *tet*(Z) and *tet*(A/P)) and one inactivating enzyme *tet*(X)) were investigated (Table S1). The PCR assays were conducted in 25 μL volume reactions using an ABI PCR System 9700 (ABI, USA). The PCR products were checked by electrophoresis on a 1% (weight/volume) agarose gel in 1 × TBE buffer. The positive amplicons were further assured by cloning and sequencing. Eleven detected *tet* genes (*tet*(A), *tet*(C), *tet*(E), *tet*(G), *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(Q), *tet*(W), *tet*(X)) were quantified for all samples using SYBR-Green real-time PCR. Standard plasmids carrying target genes were obtained by TA clones and extracted using a TIANpure Mini Plasmid kit (Tiangen, China). To minimize the variance caused by different bacterial abundance, as well as different DNA extraction and quantification efficiencies, the relative abundance of *tet* genes were obtained by normalizing their copy numbers to those of the 16S rRNA genes. Real-time PCR assays were performed for the quantification of bacterial 16S rRNA and fungal 18rRNA genes using primers 341f/534r and FF390/FR1, respectively (Table S1). All samples were run in triplicate. A duplicate tenfold dilution series of standard DNA was used to generate a standard curve. A detailed description is provided in the supporting information with the primers and references listed in Table S1. A detailed description is provided in the supporting information with the primers and references listed in Table S1.

2.5. Statistical analysis

Wastewater and sludge properties (Table S2 and Table S3) were standardized to have comparability prior to further analysis using the formula, $z = (x_i - \overline{x})/s$, where x_i is the sample value, $\overline{x}$ is the mean of all samples, and s is the standard deviation on (Freeman, 1994; Xie et al., 2011). Diversity indices including Shannon-Weaver H and Simpson were calculated using R 2.9.1 (http://www.r-project.org/) with the vegan package to evaluate the microbial functional diversity. Mantel test was used to examine the relationship between the abundance of functional genes and the antibiotic concentrations in ten samples from the biological antibiotic production wastewater treatment systems (Mantel, 1967). The standardized GeoChip data for the gene categories (and families) of each sample was set as the first matrix, and the normalized wastewater and sludge data of each sample was set as the second matrix. Canonical correspondence analysis (CCA) was used to discern possible linkages between microbial community functional structures and water properties in ten samples from the systems, and partial CCA was used to analyze the contributions of different chemical water variables to microbial functional structures. Mantel tests, CCA and partial CCA were performed using R 2.13.1 (http://www.r-project.org/) with the vegan and stats packages. Hierarchical clustering analysis was performed with CLUSTER (http://rana.lbl.gov/EisenSoftware.htm) and visualized by TREEVIEW (http://rana.stanford.edu/) (Eisen et al., 1998). Significance tests were conducted by Monte Carlo permutation (999 times). Generally, in this manuscript, P values <0.05 are regarded as significant. All microarray hybridization data are available at the Institute for Environmental Genomics, University of Oklahoma (http://ieg.ou.edu).

3. Results

3.1. Wastewater and sludge characteristics

Detailed water and sludge characteristics are summarized in Tables S2 and S3 (in Supporting Information), respectively.

The original oxytetracycline crystal mother liquors of plants HS and HY (HY-ML and HS-ML) contained extremely high levels of oxytetracycline residues (844–1077 mg/L). Even after dilution, the oxytetracycline concentrations were 4.25 and 3.66 mg/L in the influents, and 0.49 and 1.19 mg/L in the final effluents of the HY and HS systems, respectively. On the other hand, plant HY also produces penicillin, and the penicillin concentrations were 0.49 mg/L in the SBR influent (HY-W2) and 0.07 mg/L in the final effluent (HY-W4). The oxytetracycline and penicillin concentrations of the four sludge samples (HY-S1, S2, S3 and HS-S1) were 52–1106 μg/g and 0–22.8 μg/g (dry weight), respectively. The effluent chemical oxygen demand (COD) was decreased from 1640 to 3200 mg/L to 222 and 332 mg/L in HS and HY plants, respectively, and the effluent NH_4^+ was also reduced from 130 to 164 to 2.7–5.1 mg/L, showing that the two wastewater treatment systems functioned well in terms of nutrient removal.

3.2. *Overview of microbial functional gene patterns*

According to the GeoChip 3.0 analysis result, a total of 6133 functional genes from all of the nine functional gene categories targeted by the chip were detected (Table S4 in Supporting Information), including antibiotic resistance, carbon cycling (mainly carbon degradation), nitrogen cycling, sulphur cycling, phosphorus cycling, metal resistance and energy processes (He et al., 2010a), showing the presence of diverse functional genes in these systems. A total of 4520 genes were detected among the four sludge samples, while 3945 were detected in the eight wastewater samples. SBR influent in HY (HY-W2: 45.0%) and activated sludge in HS (HS-S1: 43.2%) had more unique genes than the other samples as shown in Table 1 (in bold face). The influent (HS-W1) and effluent (HS-W2) in HS had the most overlapping genes (48.8%), while oxytetracycline mother liquor of HS (HS-ML) and HS-S1 had the fewest (3.9%) (Table 1: in italic). The Shannon-Weaver indices of sludge samples (6.06, 6.93, 6.02 and 7.94 for HY-S1, S2, S3 and HS-S1, respectively) were directly calculated from GeoChip 3.0 data of functional genes within the same range with soil samples (5.98–7.20) (Xiong et al., 2010). Evenness varied in a narrow range (0.86–0.97), indicating an even distribution of functional genes in the antibiotic production wastewater treatment systems. A similar diversity trend was also obtained using a Simpson's reciprocal diversity index (1/D), which varied between 55.02 (HS-ML) and 1827.82 (HS-S1: activated sludge in HS) (Table 1).

Among the 27,812 probes in GeoChip 3.0, phylogenetically, 24,939 probes (89.7%) target 2744 species of bacteria, 886 (3.2%) target 140 species of archaea and 1759 (6.3%) target 262 species of fungi (He et al., 2010a). In this study, 5609 detected genes were derived from *Bacteria*, 154 from *Archaea* (most *Crenarchaeota* and *Euryarchaeota*) and 333 from *Fungi* (mostly *Ascomycota*) (Table S5 in Supporting Information). For bacteria, 1252 genes were from *α-Proteobacteria*, 781 from *β-Proteobacteria*, 1039 from *γ-Proteobacteria*, 613 from *Actinobacteria*, and 398 from *Firmicutes* (Table S5 In Supporting Information).

The clone library results showed that activated sludge in the two systems was mainly affiliated with the *β-Proteobacteria* and *Sphingobacteria*, followed by some other classes including *Flavobacteria*, *α-Proteobacteria*, etc., which was comparable with the 16S rRNA gene clone library result of the control system treating inosine production wastewater (Table S6 in

Table 1 – Overlap, uniqueness, diversity and the total number of detected genes in antibiotic production wastewater samples.

Unique and overlap genes[a]	HY-ML	HY-W1	HY-W2	HY-W3	HY-W4	HY-S1	HY-S2	HY-S3	HS-ML	HS-W1	HS-W2	HS-S1
HY-ML	**72 (11.7)**	*398 (16.5)*	*148 (14.4)*	*368 (23.3)*	*135 (15.3)*	*249 (23.4*	*407 (24.1)*	*234 (22.4)*	*110 (15.0)*	*316 (25.7)*	*313 (26.1)*	*460 (11.7)*
HY-W1		**621 (28.2)**	*198 (7.7)*	*707 (25.0)*	*168 (6.9)*	*392 (15.6)*	*869 (30.9)*	*351 (14.0)*	*105 (4.5)*	*555 (21.5)*	*558 (22.0)*	*1367 (29.6)*
HY-W2			**252 (45.0)**	*141 (8.0)*	*78 (8.8)*	*165 (15.1)*	*169 (9.02)*	*144 (13.4)*	*67 (9.3)*	*179 (13.6)*	*161 (12.43)*	*214 (5.18)*
HY-W3				**187 (14.0)**	*156 (9.9)*	*280 (16.0)*	*863 (44.1)*	*277 (16.1)*	*95 (6.5)*	*391 (20.8)*	*426 (23.6)*	*1022 (25.0)*
HY-W4					**115(28.7)**	*102 (10.2)*	*159 (9.22)*	*74 (7.5)*	*51 (8.8)*	*120 (9.9)*	*105 (8.8)*	*202 (5.1)*
HY-S1						**134 (19.2)**	*397 (22.2)*	*295 (27.7)*	*84 (9.7)*	*384 (30.8)*	*382 (31.5)*	*400 (9.8)*
HY-S2							**96 (6.5)**	*370 (20.8)*	*112 (7.0)*	*582 (31.7)*	*611 (34.6)*	*1206 (29.7)*
HY-S3								**138 (20.8)**	*90 (11.2)*	*340 (27.1)*	*372 (31.3)*	*395 (9.8)*
HS-ML									**32 (14.0)**	*97 (9.1)*	*99 (9.7)*	*151 (3.9)*
HS-W1										**95 (10.9)**	*600 (48.8)*	*584 (14.1)*
HS-W2											**53 (5.9)**	*618 (15.2)*
HS-S1												**1635 (43.2)**
H[b]	5.97	7.11	5.86	6.76	5.44	6.06	6.93	6.02	4.67	6.60	6.39	7.94
Evenness[c]	0.93	0.93	0.93	0.94	0.91	0.93	0.95	0.93	0.86	0.97	0.94	0.96
1/D[d]	215.53	686.72	197.56	454.76	137.19	179.18	514.28	198.11	55.02	481.61	299.97	1827.82
Total genes	615	2199	560	1336	401	699	1483	663	228	933	896	3783

a Italicized values indicate the number of overlapping genes (%) between samples; bolded values indicate the number of unique genes (%) in each sample.
b Shannon–Weaver index, higher number represents higher diversity.
c Shannon–Weaver evenness index.
d Reciprocal of Simpson's index, higher number represents higher diversity.

Supporting Information). Similarly, Li et al. (2011) found that bacterial communities in antibiotic containing water samples shared many common phylogenetic groups with those in the two reference upstream rivers.

Although *Streptomyces rimosus* is used for the production of oxytetracycline in these two facilities, *Firmicutes* were abundant in oxytetracycline mother liquors (HS-ML: 60%; HY-ML: 19%) (Fig. S1 in the Supporting Information), which was in accordance with our clone library results: clones of *Firmicutes* (mostly *Lactobacillus* sp.) accounted for 90% and 35% of clones in HS-ML and HY-ML, respectively (see Table S6 in Supporting Information). It has been reported that some special groups like *Clostridia* and *Bacilli* belonging to *Firmicutes* were associated with antibiotic containing environments (Li et al., 2011).

3.3. Changes in carbon-degrading genes

A total of 987 carbon-cycling functional genes were detected from 5199 genes in the GeoChip, with 749 genes related to carbon degradation belonging to 40 carbon-degrading gene groups (24 from bacteria and 16 from fungi). Among them, 254 genes involved in glyoxylate cycle (isocitrate lyase (*aceA*) and malate synthase (*aceB*)) exhibited the highest abundance, with the highest signal intensities observed in sludge samples. Microbial functional genes related to starch, chitin, cellulose, and hemicellulose degradation were present in high abundance. Several α-amylase (*amyA*) genes for starch degradation from *Lactobacillus* sp. were found with high intensity in oxytetracycline ML samples (HY-ML and HS-ML). About 73–89% (82% in average) carbon-degrading genes in signal intensity were derived from bacteria.

On the other hand, 11–23% (16% in average) carbon-degrading genes in signal intensity of GeoChip were derived from eukaryota (all belonging to fungi). A total of three different patterns were observed as shown in Fig. S2. Cluster 1, the smallest one, is involved only in cellulose and hemicellulose degradation (cellobiase_fungi, *ara*_fungi and xylanase_fungi). Cluster 2 includes nine fungal gene groups, primarily from sludge samples, most of which are involved in starch, chitin, pectin, and lignin degradation. Cluster 3 containing four gene groups, primarily in activated sludge in HS (HS-S1), are mainly involved in glyoxylate cycle (isocitrate lyase (*aceA*_fungi) and malate synthase (*aceB*_fungi)).

Mantel tests were performed to examine the relationship between the abundance of carbon-degrading genes and the antibiotic concentration in the ten samples from the two antibiotic production wastewater treatment systems (the two oxytetracycline mother liquor samples were excluded because the oxytetracycline concentrations were extremely high (844–1077 mg/L) in comparison with the other samples). The relationship between the abundance of all carbon-degrading genes (from bacteria and fungi) and total antibiotic concentrations was not significant ($r = 0.356$, $P = 0.149$). However, five of the fungal carbon-degrading gene groups were significantly correlated with the oxytetracycline concentration (endochitinase_fungi for chitin degradation: $r = 0.672$, $P = 0.005$; exoglucanase_fungi for cellulose degradation: $r = 0.933$, $P = 0.036$; *glx* for lignin degradation: $r = 0.685$, $P = 0.026$; *lip* for lignin degradation: $r = 0.954$, $P = 0.027$; *mnp* for lignin degradation: $r = 0.853$, $P = 0.026$), and one correlated with the penicillin concentration (endochitinase_fungi: $r = 0.623$, $P = 0.033$) (Table 2: in bold face). In bacteria, only the abundance of *aceA* was significantly correlated with the oxytetracycline concentration ($r = 0.757$, $P = 0.038$) (Table 2: in bold face). No significant correlation was observed between the bacterial carbon-degrading gene groups and the penicillin concentration.

3.4. Changes in antibiotic resistance gene category

A total of 559 antibiotic resistance genes from 11 gene families, including five transporter genes (ATP-binding cassette

Table 2 – The relationship between microbial community functional structure for carbon degradation and antibiotics revealed by Mantel test.

Gene category	Oxytetracycline		Penicillin	
	r_M	P	r_M	P
AceA	0.7567	**0.038**	−0.0179	0.406
*AceA*_fungi	0.5198	0.163	−0.2111	0.659
AceB	0.7263	0.071	−0.2372	0.836
*AceB*_fungi	0.4104	0.180	−0.2444	0.795
AssA	0.2925	0.183	−0.2098	0.922
CDH	0.1990	0.139	−0.1790	0.584
acetylglucosaminidase	0.3916	0.090	−0.1396	0.558
amyA	0.6130	0.119	0.5930	0.068
*amyA*_fungi	0.0599	0.149	−0.1395	0.499
ara	0.3755	0.172	−0.1242	0.555
*ara*_fungi	0.1594	0.128	−0.1746	0.759
camDCAB	−0.1260	0.496	−0.1316	0.318
cda	0.9171	0.055	−0.1725	0.567
cellobiase	0.0424	0.321	−0.2485	0.838
*cellobiase*_fungi	−0.0806	0.268	−0.1266	0.458
endochitinase	0.1804	0.192	−0.1727	0.667
*endochitinase*_fungi	0.6717	**0.005**	0.6234	**0.033**
endoglucanase	−0.2073	0.739	−0.0582	0.528
*endoglucanase*_fungi	0.3193	0.135	−0.1841	0.449
exochitinase	0.3921	0.186	−0.2569	0.876
exoglucanase	0.1172	0.139	0.0391	0.218
*exoglucanase*_fungi	0.9327	**0.036**	−0.0634	0.434
glucoamylase	0.0013	0.306	0.5969	0.087
*glucoamylase*_fungi	0.8019	0.095	−0.2157	0.813
*glx*_fungi	0.6849	**0.026**	−0.0259	0.366
*isopullulanase*_fungi	−0.1452	0.505	−0.0363	0.276
limEH	0.5055	0.059	−0.1688	0.720
*lip*_fungi	0.9538	**0.027**	−0.0946	0.365
*mnp*_fungi	0.8530	**0.026**	−0.1045	0.614
nplT	0.1473	0.231	0.4040	0.179
pectinase	0.9656	0.105	−0.1606	0.622
*pectinase*_fungi	0.5160	0.141	−0.1329	0.508
phenol_oxidase	0.1137	0.341	−0.1242	0.629
phenol_oxidase_fungi	0.1755	0.280	−0.2732	0.851
pulA	0.3372	0.220	−0.2032	0.588
vanA	0.2230	0.200	−0.2173	0.739
vdh	0.4224	0.159	−0.2071	0.609
xylA	0.5474	0.092	0.0890	0.318
xylanase	−0.1355	0.487	−0.1244	0.420
*xylanase*_fungi	−0.0370	0.217	0.9374	0.094

The signal intensity of genes among ten samples from biological treatment systems was used as the first matrix; the normalized antibiotic concentrations were used as the second matrix. **Bolded values** indicate significant *P*-values (<0.05).

(ABC), multidrug toxic compound extrusion (MATE), major facilitator superfamily (MFS), multidrug efflux (Mex), and small multidrug resistance efflux pumps (SMR)), showed positive hybridization signals. The final effluents (HY-W4 and HS-W2) in HY an HS and excess sludge in HY (HY-S3) had 31, 84, and 53 antibiotic resistance genes from 11 gene families, respectively (Table S4 In Supporting Information).

GeoChip 3.0 contains 423 probes for four β-lactamase (Classes A-D) genes and tetracycline and vancomycin resistance genes (He et al., 2010a). In total, 132 genes on the GeoChip showed positive hybridization signal (Table S4 In Supporting Information). Among them, 13 genes encoding *tet* resistance were detected, with nine related to ribosomal protection mechanism *tet*(M). The relative abundance of *tet* genes was high in oxytetracycline ML samples (HY-ML and HS-ML), while β-lactamase genes were rich in other water samples (Fig. S3 In Supporting Information). Seven genes involving vancomycin resistance (*van*) were detected and a dominant one derived from *Alkaliphilus oremlandii* was detected across ten samples. A total of 427 transporter gene probes from 1181 gene sequences in GeoChip involved with ABC, MATE, MFS, Mex and SMR from transporter families of multidrug efflux systems were detected (Bolhuis et al., 1997; Poole, 2007), with SMR being dominant (Fig. S3 in supporting information).

Mantel tests were also performed to examine the relationship between the abundance of antibiotic resistance genes and the antibiotic concentrations in the ten samples from the two antibiotic production wastewater treatment systems (the two oxytetracycline ML samples were excluded). Significant correlation ($P < 0.05$) was observed between the abundance of antibiotic resistance genes and total antibiotic concentrations ($r = 0.695$, $P = 0.004$). Among the 11 antibiotic resistance gene covered by GeoChip 3.0, two were positively correlated with the oxytetracycline concentration (β-lactamase_C: $r = 0.770$, $P = 0.039$; MFS: $r = 0.853$, $P = 0.046$), and one was correlated with the penicillin concentration (SMR: $r = 0.631$; $P = 0.035$) (Table S7 In Supporting Information: In Bold Face).

3.5. *Contributions of water characteristics to variations of microbial functional gene structure*

CCA was performed to discern possible linkages between microbial functional structure and chemical and physical water variables in the ten samples from the two wastewater treatment systems (Fig. S4 In Supporting Information). The top four water variables were included in the CCA biplot (penicillin, oxytetracycline, COD and NH_4^+) based on automatic forward selection and variance inflation factors with 999 Monte Carlo permutations. The first axis, which was negatively correlated with penicillin, explained 33.0% of the microbial functional diversity observed, and the second axis, which was positively correlated with COD and NH_4^+, but negatively correlated with oxytetracycline, explained 27.3% of the total variation.

The contributions of antibiotics (A) (the sum of penicillin and oxytetracycline), COD (C), and NH_4^+ (N) on microbial functional community variations were determined with variance partitioning analysis (VPA) using partial CCA. The total variation was partitioned into the pure effects of A, C and N, interactions between any two components (A × C, A × N and N × C), common interactions of all three components (A × N × C) and the unexplained portion (Fig. 2a). A total of 49.1% of the variation was significantly explained ($P = 0.029$) by the three components (Fig. 2b). Antibiotics, COD, and NH_4^+ were able to independently explain 24.4%, 13.2% and 11.5% of the total variations observed, respectively. It should be noted that the VPA results were acquired based on limited sample size (ten samples).

4. Discussion

Since antibiotics are explicitly designed to target bacteria, high antibiotic residues in wastewater likely affect microbes in biological antibiotic production wastewater treatment systems where bacteria normally play a dominant role: bacterial carbon transformation functions may be disturbed; at the same time, bacteria possessing antibiotic resistance could survive in this environment. We used GeoChip, which has been widely applied for dissecting the microbial community functional structure in both natural and contaminated environments (Liang et al., 2011; van Nostrand et al., 2009; Wu

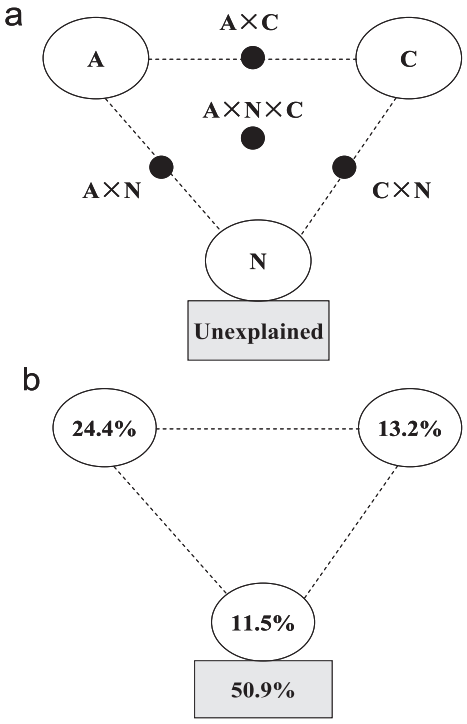


Fig. 2 – Variation partitioning analysis of microbial diversity explained by antibiotics (A), COD (C), and NH_4^+ (N). (a) General outline, (b) All functional genes. Each diagram represents the biological variation partitioned into the relative effects of each variable or a combination of variables, in which geometric areas are proportional to the respective percentages of explained variation. The edges of the triangle represent the variation explained by each variable alone. The sides of the triangles represent interactions of any two variables and the middle of the triangles represents the interaction of all three variables.

et al., 2008; Zhou et al., 2008), to evaluate variations of the functional structure of microbial communities in two full-scale oxytetracycline production wastewater treatment systems (HY and HS) with a long history (over ten years). In contrast to our expectation that the community functional structure may be relatively simple due to the presence of antibiotics, GeoChip data indicated the existence of almost all key functional gene categories covered by the GeoChip, including carbon degradation, nitrogen fixation, and denitrification, etc., suggesting that these microbial communities still maintained their functional diversity.

Fermentative antibiotic production wastewater commonly contains substrate residues (polysaccharide, cellulose, and hemicellulose), the target products (antibiotics), by-products like glyoxylate, malate and isocitrate and extraction solvents (Ayar-Kayali and Tarhan, 2006; Chan and Sim, 1998). Therefore, various carbon-degrading genes such as starch, cellulose/hemicellulose, chitin, lignin degradation genes, *VanA*, *aceB*, and *amyA* from the microbes in activated sludge are required to decompose these organic substances. Although only 6% of the whole microbial community functional genes detected in GeoChip 3.0 were derived from fungi, they represent average 16% in carbon-degrading genes. However, further studies are required to reveal the relevant contribution of fungi to carbon degradation in the systems. As shown in Fig. S2, some key carbon-degrading genes from fungi, such as *glx*, *lip*, *mnp*, endochitinase and exoglucanase genes were observed with high signal intensities in sludge samples. The abundance of these fungal functional genes was found to be significantly correlated with antibiotic concentrations ($P < 0.05$). By comparison, only the abundance of *aceA* from bacteria was significantly correlated with the oxytetracycline concentration ($P < 0.05$). These results showed that the presence of relatively high oxytetracycline concentrations may have enhanced the role of fungal carbon-degrading genes in nutrient removals. The dominant fungal carbon-degrading genes were mainly derived from *Ascomycota* such as *Aspergillus*, which are ubiquitous in natural environments (Schuster et al., 2002) with nutrient degrading abilities (Mannana et al., 2005). Some detected genes were found from *Pichia* and *Candida*, which are capable of degrading diverse organic compounds in wastewater (Adav et al., 2007; Hesham et al., 2006; Lv et al., 2011; Zheng et al., 2002).

At the same time, quantitative PCR results revealed that the copy ratios of fungi/bacteria (3.73×10^{-2} to 1.24) based on specific gene copy numbers in activated sludge samples (HY-S1, HY-S2, and HS-S1) were much higher than the other fermentation wastewater treatment systems (Inosine-S; 1.87×10^{-4} and Ethanol-S, 6.50×10^{-4}) (Table 3), showing the selection of fungi by the presence of antibiotics. This result was in agreement with previous reports that the existence of antibiotics can result in significant population shifts from bacteria to fungi in soil and aquatic media (Bundschuh et al., 2009; Demoling et al., 2009; Thiele-Bruhn and Beck, 2005; Zielezny et al., 2006). One of our previous studies using clone libraries and quantitative PCR techniques also demonstrated that fungi were abundant with the dominance of *Ascomycota* under the presence of antibiotics (mainly streptomycin) (Deng et al., 2012).

However, the majority of carbon-degrading genes were derived from bacteria (average signal intensity: 82%), showing that many bacteria could survive in the environment with a high antibiotic concentration. This could explain why relatively high COD removal was achieved during antibiotic wastewater treatment, as shown in Table S2 (in Supporting Information). So it is speculated that bacteria were playing a main role in nutrient removal, while the carbon-degrading fungal populations may have contributed to the COD removal. At the same time, antibiotic resistance genes and transporters were abundant and diverse in biological antibiotic production wastewater treatment systems, as shown in Table S4 and Fig. S3 (in Supporting Information). *tet*(M)-like subfamily, *tet*(Y) and *tet*(A) genes were detected, which was in accordance with our previous result that 12.2%, 22.2% and 69.3% of bacterial isolates from oxytetracycline wastewater effluent contained *tet*(M), *tet*(Y) and *tet*(A) (Li et al., 2010). The levels of three gene families involved in antibiotic resistance, including β-lactamase-C, MFS, and SMR were significantly correlated with antibiotic concentration ($P < 0.05$) (Table S7 In Supporting Information), which was consistent with previous reports that environmental antibiotic residues might impose selective pressure on bacterial communities to acquire antibiotic resistance (Smith et al., 2004; Pei et al., 2007). On the other hand, nine *tet* genes including *tet*(M) and *tet*(A) were successfully quantified using quantitative PCR for the same samples (Table S8 in Supporting Information). The lower number of *tet* genes detected by Geochip was due to the limited number of *tet* gene probes on GeoChip 3.0. The relative abundance of the total *tet* genes in the samples from HS and HY systems (1.6×10^{-1}–3.7×10^{0}) was higher than that in the

Table 3 – Fungal, bacterial gene copy numbers and the ratio of Fungi and bacteria gene copy numbers (Fungi/Bacteria) of activated sludge samples from antibiotic production wastewater biological treatment systems, an inosine production wastewater treatment system (Inosine-S), and an ethanol production wastewater (Ethanol-S). Standard deviations are shown in the parenthesis.

Sample	Fungal 18S rRNA gene copy numbers (copies per μL DNA)	Bacterial 16S rRNA gene copy numbers (copies per μL DNA)	Fungi/bacteria
HS-S1 (Activated sludge in HS)	1.04×10^{7} (5.94×10^{5})	8.40×10^{6} (1.14×10^{5})	1.24×10^{0} (7.07×10^{-2})
HY-S1 (Activated sludge from SBR in HY)	3.35×10^{6} (1.11×10^{6})	5.99×10^{7} (5.19×10^{6})	5.60×10^{-2} (1.85×10^{-2})
HY-S2 (Activated sludge from biological contact reactor in HY)	1.50×10^{6} (2.24×10^{6})	4.02×10^{7} (3.50×10^{6})	3.73×10^{-2} (1.30×10^{-4})
Inosine-S	3.33×10^{4} (1.08×10^{4})	1.78×10^{8} (2.73×10^{7})	1.87×10^{-4} (6.05×10^{-5})
Ethanol-S	4.90×10^{4} (7.51×10^{3})	7.44×10^{7} (2.57×10^{6})	6.59×10^{-4} (1.10×10^{-4})

control systems (1.7×10^{-2}–3.1×10^{-2}) and sewage treatment plants (Zhang and Zhang, 2011; Zhang et al., 2009), showing that bacteria survived in the environment by possessing antibiotic resistance and the discharge of antibiotic resistance genes from the antibiotic production wastewater treatment systems is worthy of attention (Liu et al., 2012).

Understanding the factors that influence microbial functional structure is an important goal in microbial ecology. VPA showed that antibiotics contributed 24.4% variations in microbial community functional genes (Fig. 2b), indicating that antibiotics were one of the factors shaping microbial functional structure in the antibiotic production wastewater treatment systems. This is understandable since antibiotics are designed to target bacteria. More extensive studies with a focus on more antibiotic categories are currently underway to examine the impact of antibiotics on microbial functional structures in different biological antibiotic production wastewater treatment systems.

Knowledge on microbial community functional structure is useful in establishing a sound process and operational strategy for successful antibiotic production wastewater treatment. As discussed above, treating antibiotic production wastewater with conventional biological processes leads to the production and discharge of abundant antibiotic resistance genes in bacteria into the environment, which represents a potential risk. So new strategies are needed for the efficient treatment of antibiotic production wastewater with a focus on controlling the production and discharge of antibiotic resistance gene. Since many key functional genes of fungi involved in carbon degradation were abundant in the presence of antibiotics, it may be possible to employ the fungal role in antibiotic production wastewater treatment. Since fungi are normally not the target of antibiotics, another advantage for the use of fungi is that it is able to maintain the stability of biological treatment systems in response to shock antibiotic loads.

It should be noted that the DNA-based GeoChip analysis may only detect functional potentials of microbial communities. To validate the results from this study, additional in-depth analyses including metagenome sequencing and functional activity assays are needed.

5. Conclusions

Despite the bacteriostatic effects of antibiotics, the microbial structures from two biological wastewater treatment systems treating antibiotic production wastewater were proven to be functionally diverse. The abundance of several fungal carbon-degrading genes (e.g., *glx*, *lip*, *mnp*, endochitinase, exoglucanase_genes) was significantly correlated with antibiotic concentrations (Mantel test; $P < 0.05$). Diverse antibiotic resistance genes were detected in bacteria, and the abundance of these genes was significantly correlated with antibiotic concentrations ($P < 0.05$). Considering the fact that abundant bacterial carbon-degrading genes were detected in samples from the antibiotic production wastewater treatment systems, it is speculated that bacteria could maintain their carbon-degrading functions by acquiring antibiotic resistance even under the relatively high antibiotic concentration, while the carbon-degrading fungal populations may have also contributed to the COD removal.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2013.08.003.

REFERENCES

Adav, S.S., Chen, M.Y., Lee, D.J., Ren, N.Q., 2007. Degradation of phenol by aerobic granules and isolated yeast *Candida tropicalis*. Biotechnology and Bioengineering 96 (5), 844–852.

Al-Ahmad, A., Daschner, F.D., Kümmerer, K., 1999. Biodegradability of cefotiam, ciprofloxacin, meropenem, penicillin G and sulfamethoxazole and inhibition of waste water bacteria. Archives of Environmental Contamination and Toxicology 37 (2), 158–163.

Ayar-Kayali, H., Tarhan, L., 2006. Vancomycin antibiotic production and TCA-glyoxalate pathways depending on the glucose concentration in *Amycolatopsis orientalis*. Enzyme and Microbial Technology 38 (6), 727–734.

Bolhuis, H., van Veen, H.W., Poolman, B., Driessen, A.J., Konings, W.N., 1997. Mechanisms of multidrug transporters. FEMS Microbiology Reviews 21 (1), 55–84.

Bundschuh, M., Hann, T., Gessner, M.O., Schulz, R., 2009. Antibiotics as a chemical stressor affecting an aquatic decomposer-detritivore system. Environmental Toxicology and Chemistry 28 (1), 197–203.

Chan, M., Sim, T.S., 1998. Malate synthase from *Streptomyces clavuligerus* NRRL3585: cloning, molecular characterization and its control by acetate. Microbiology 144 (11), 3229–3237.

Demoling, L.A., Baath, E., Greve, G., Wouterse, M., Schmitt, H., 2009. Effects of sulfamethoxazole on soil microbial communities after adding substrate. Soil Biology & Biochemistry 41 (4), 840–848.

Deng, Y.Q., Zhang, Y., Gao, Y.X., Li, D., Liu, R.Y., Liu, M.M., Zhang, H.F., Hu, B., Yu, Tao, Yang, M., 2012. Microbial community compositional analysis for series reactors treating high level antibiotic wastewater. Environmental Science & Technology 46 (2), 795–801.

Eisen, M.B., Spellman, P.T., Brown, P.O., Botstein, D., 1998. Cluster analysis and display of genome-wide expression patterns. Proceedings of the National Academy of Sciences of the United States of America 95 (25), 14863–14868.

Freeman, W.H., 1994. In: Sokal, R.R., Rohlf, F.J. (Eds.), Biometry: the Principles and Practices of Statistics in Biological Research, third ed. New York, USA.

He, Z., Deng, Y., van Nostrand, J.D., Tu, Q.C., Xu, M.Y., Hemme, C.L., Li, X.Y., Wu, L.Y., Gentry, T.J., Yin, Y.F., Liebich, J., Hazen, T.C., Zhou, J.Z., 2010a. GeoChip 3.0 as a high-throughput tool for analyzing microbial community composition, structure and functional activity. ISME Journal 4, 1167–1179.

He, Z., Xu, M., Deng, Y., Kang, S., Kellogg, L., Wu, L.Y., van Nostrand, J.D., Hobbie, S.E., Reich, P.B., Zhou, J.Z., 2010b. Metagenomic analysis reveals a marked divergence in the structure of belowground microbial communities at elevated CO_2. Ecology Letters 13 (5), 564–575.

Hesham, A.E.L., Sardar, K., Liu, X.C., Zhang, Y., Wang, Z.Y., Yang, M., 2006. Application of PCR-DGGE to analyze the yeast population dynamics in slurry reactors during degradation of polycyclic aromatic hydrocarbons in weathered oil. Yeast 23 (12), 879–887.

Kim, S., Jensen, J.N., Aga, D.S., Weber, A.S., 2007. Tetracycline as selector for resistant bacteria in activated sludge. Chemosphere 66 (9), 1643–1651.

Kümmerer, K., 2003. Significance of antibiotics in the environment. Journal of Antimicrobial Chemotherapy 52 (1), 5–7.

Kümmerer, K., 2009. Antibiotics in the aquatic environment – a review – part I. Chemosphere 75 (4), 417–434.

Kümmerer, K., Al-Ahmad, A., 2000. Mersch–Sundermann, V. Biodegradability of some antibiotics, elimination of the genotoxicity and affection of wastewater bacteria in a simple test. Chemosphere 40 (7), 701–710.

Larsson, D.G., de Pedro, C., Paxeus, N., 2007. Effluents from drug manufactures contains extremely high levels of pharmaceuticals. Journal of Hazardous Materials 148 (3), 751–755.

Li, D., Qi, R., Yang, M., Zhang, Y., Yu, T., 2011. Bacterial community characteristics under long-term antibiotic selection pressures. Water Research 45 (18), 6063–6073.

Li, D., Yang, M., Hu, J.Y., Ren, L.R., Zhang, Y., Li, K.X., 2008a. Determination and fate of oxytetracycline and related compounds in oxytetracycline production wastewater and the receiving river. Environmental Toxicology and Chemistry 27 (1), 80–86.

Li, D., Yang, M., Hu, J.Y., Zhang, Y., Chang, H., Jin, F., 2008b. Determination of penicillin G and its degradation products in a penicillin production wastewater treatment plant and the receiving river. Water Research 42 (1–2), 307–317.

Li, D., Yang, M., Hu, J.Y., Zhang, J., Liu, R.Y., Gu, X., Zhang, Y., Wang, Z.Y., 2009. Antibiotic-resistance profile in environmental bacteria isolated from penicillin production wastewater treatment plant and the receiving river. Environmental Microbiology 11 (6), 1506–1517.

Li, D., Yu, T., Zhang, Y., Yang, M., Li, Z., Liu, M.M., Qi, R., 2010. Antibiotic resistance characteristics of environmental bacteria from an oxytetracycline production wastewater treatment plant and the receiving river. Applied and Environmental Microbiology 76 (11), 3444–3451.

Liang, Y., van Nostrand, J.D., Deng, Y., He, Z.L., Wu, L.Y., Zhang, X., Li, G.H., Zhou, J.Z., 2011. Functional gene diversity of soil microbial communities from five oil-contaminated fields in China. ISME Journal 5, 403–413.

Liu, M.M., Zhang, Y., Yang, M., Tian, Z., Ren, L.R., Zhang, S.J., 2012. Abundance and distribution of tetracycline resistance genes and mobile elements in an oxytetracycline production wastewater treatment system. Environmental Science & Technology 46 (14), 7551–7557.

Lv, W.Z., Hesham, A.E.L., Zhang, Y., Liu, X.C., Yang, M., 2011. Impacts of cell surface characteristics on population dynamics in a sequencing batch yeast reactor treating vegetable oil-containing wastewater. Applied Microbiology and Biotechnology 90 (5), 1785–1793.

Mannana, S., Fakhru'l-Razia, A., Alam, M.Z., 2005. Use of fungi to improve bioconversion of activated sludge. Water Research 39 (13), 2935–2943.

Mantel, N., 1967. The detection of disease clustering and a generalized regression approach. Cancer Research 27, 209–220.

Pei, R., Cha, J., Carlson, K., Pruden, A., 2007. Response of antibiotic resistance genes (ARG) to biological treatment in dairy lagoon water. Environmental Science & Technology 41 (14), 5108–5113.

Polz, M.F., Harbison, C., Cavanaugh, C.M., 1999. Diversity and heterogeneity of epibiotic bacterial communities on the marine nematode *Eubostrichus dianae*. Applied and Environmental Microbiology 65 (9), 4271–4275.

Poole, K., 2007. Efflux pumps as antimicrobial resistance mechanism. Annals of Medicine 39 (3), 162–176.

Schuster, E., Dunn-Coleman, N., Frisvad, J.C., van Dijck, P., 2002. On the safety of Aspergillus niger – a review. Applied Microbiology and Biotechnology 59 (4–5), 426–435.

Seviour, R., Nielsen, P.H. (Eds.), 2010. Microbial Ecology of Activated Sludge. IWA Publishing.

Smith, M.S., Yang, R.K., Knapp, C.W., Niu, Y.F., Peak, N., Hanfelt, M.M., Galland, J.C., Graham, D.W., 2004. Quantification of tetracycline resistance genes in feedlot lagoons by quantitative PCR. Applied and Environmental Microbiology 70 (12), 7372–7377.

Tenover, F.C., 2006. Mechanisms of antimicrobial resistance in bacteria. The American Journal of Medicine 119 (6), 3–10.

Thiele-Bruhn, S., Beck, I.C., 2005. Effects of sulfonamide and tetracycline antibiotics on soil microbial activity and microbial biomass. Chemosphere 59 (4), 457–465.

Trivedi, P., He, Z., van Nostrand, J.D., Albrigo, G., Zhou, J.Z., Wang, N., 2012. Huanglongbing alters the structure and functional diversity of microbial communities associated with citrus rhizosphere. ISME Journal 6, 363–383.

van Nostrand, J.D., Wu, W., Wu, L., Deng, Y., Carley, J., Carroll, S., He, Z.L., Gu, B.H., Luo, J., Criddle, C.S., Watson, D.B., Jardine, P.M., Marsh, T.L., Tiedje, J.M., Hazen, T.C., Zhou, J.Z., 2009. GeoChip-based analysis of functional microbial communities during the reoxidation of a bioreduced uranium-contaminated aquifer. Environmental Microbiology 11 (10), 2611–2626.

Wagner, M., Loy, A., 2002. Bacterial community composition and function in sewage treatment systems. Current Opinion in Biotechnology 13 (3), 218–227.

Wang, F., Zhou, H., Meng, J., Peng, X.T., Jiang, L.J., Sun, P., Zhang, C.L., van Nostrand, J.D., Deng, Y., He, Z.L., Wu, L.Y., Zhou, J.Z., Xiao, X., 2009. GeoChip-based analysis of metabolic diversity of microbial communities at the Juan de Fuca Ridge hydrothermal vent. Proceedings of the National Academy of Sciences of the United States of America 106 (12), 4840–4845.

Wu, L., Kellogg, L., Devol, A.H., Tiedje, J.M., Zhou, J.Z., 2008. Microarray-based characterization of microbial community functional structure and heterogeneity in marine sediments from the gulf of Mexico. Applied and Environmental Microbiology 74 (14), 4516–4529.

Wu, L., Liu, X., Schadt, C.W., Zhou, J.Z., 2006. Microarray-based analysis of subnanogram quantities of microbial community DNAs by using whole-community genome amplification. Applied and Environmental Microbiology 72 (7), 4931–4941.

Xie, J., He, Z., Liu, X.X., Liu, X.D., van Nostrand, J.D., Deng, Y., Wu, L.Y., Zhou, J.Z., Qiu, G.Z., 2011. GeoChip-based analysis of the function gene diversity and metabolic potential of microbial communities in acid mine drainage. Applied and Environmental Microbiology 77 (3), 991–999.

Xiong, J., Wu, L., Tu, S., van Nostrand, J.D., He, Z.L., Zhou, J.Z., Wang, G.J., 2010. Microbial communities and functional genes associated with soil arsenic contamination and the rhizosphere of the arsenic-hyperaccumulating plant *Pteris vittata* L. Applied and Environmental Microbiology 76 (21), 7277–7284.

Zhang, T., Zhang, M., Zhang, X.X., Fang, H.H., 2009. Tetracycline resistance genes and tetracycline resistant lactose-fermenting Enterobacteriaceae in activated sludge of sewage treatment plants. Environmental Science & Technology 43 (10), 3455–3460.

Zhang, X., Zhang, T., 2011. Occurrence, abundance, and diversity of tetracycline resistance genes in 15 sewage treatment plants across China and other global locations. Environmental Science & Technology 45 (7), 2598–2604.

Zheng, S.K., Yang, M., Li, P., 2002. Seed yeast cultivation for salad oil manufacturing wastewater treatment. Journal of Environmental Sciences 14 (1), 39–43.

Zhou, J., Bruns, M.A., Tiedje, J.M., 1996. DNA recovery from soils of diverse composition. Applied and Environmental Microbiology 62 (2), 316–322.

Zhou, J., Kang, S., Schadt, C.W., Garten Jr., C.T., 2008. Spatial scaling of functional gene diversity across various microbial taxa. Proceedings of the National Academy of Sciences of the United States of America 105 (22), 7768–7773.

Zielezny, Y., Groeneweg, J., Vereecken, H., Tappe, W., 2006. Impact of sulfadiazine and chlorotetracycline on soil bacterial community structure and respiratory activity. Soil Biology & Biochemistry 38 (8), 2372–2380.