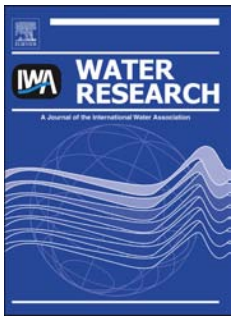

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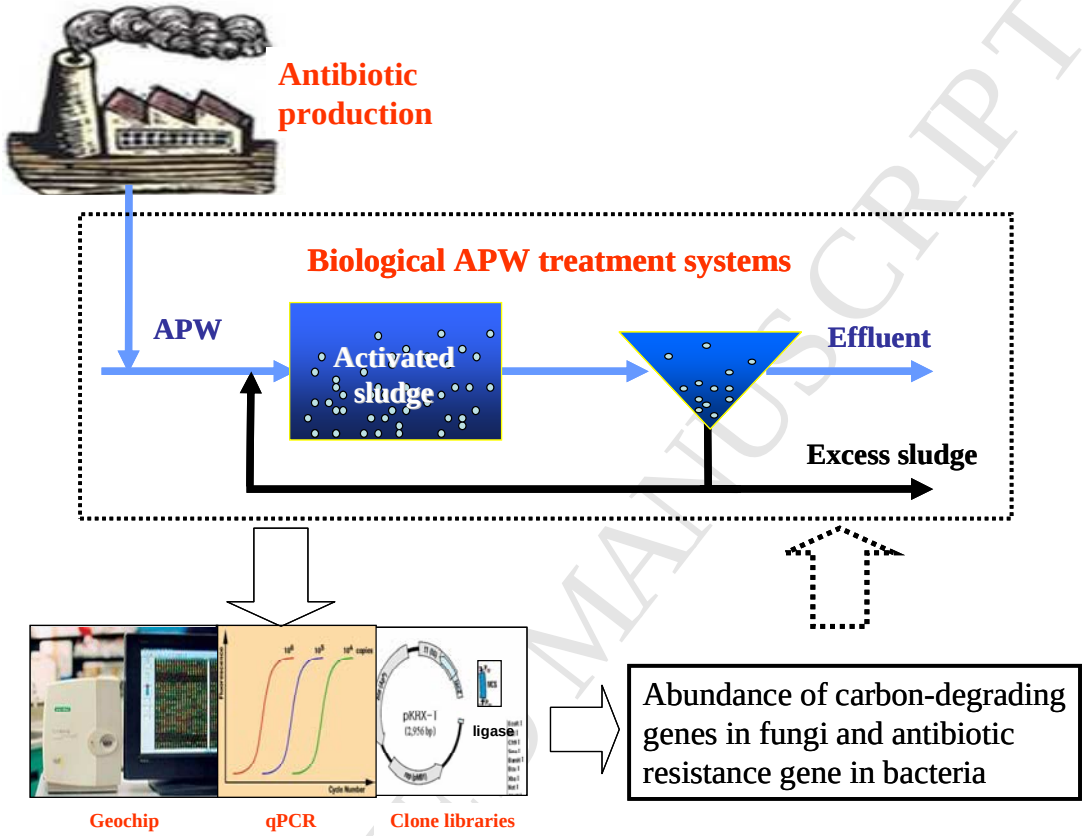
1 # Graphical abstract

2 

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

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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 ligniase (*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 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.

Keywords: Antibiotic production wastewater; Antibiotic resistance; Fungi; Geochip.

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 et al., 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., 2011). 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 2012, 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 GeoChip3.0 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 1,000 and 6,000 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, 8h) 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 4,000-5,000 mg/liter. One cycle of the SBR includes 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 6,000 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 4,000-5,000 mg/liter. 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/liter and 0.2 and 1.5 mg/liter, respectively. As shown in Fig. 1, six water (5 liter) 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 9,167 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

hours 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 hours 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

198 inactivating enzyme *tet*(X)) were investigated (Table S1). The PCR assays were conducted in
199 25 μL volume reactions using an ABI PCR System 9700 (ABI, USA). The PCR products were
200 checked by electrophoresis on a 1% (weight/volume) agarose gel in 1×TBE buffer. The
201 positive amplicons were further assured by cloning and sequencing. Eleven detected *tet* genes
202 (*tet*(A), *tet*(C), *tet*(E), *tet*(G), *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(Q), *tet*(W), *tet*(X)) were
203 quantified for all samples using SYBR-Green real-time PCR. Standard plasmids carrying
204 target genes were obtained by TA clones and extracted using a TIANpure Mini Plasmid kit
205 (Tiangen, China). To minimize the variance caused by different bacterial abundance, as well
206 as different DNA extraction and quantification efficiencies, the relative abundance of *tet*
207 genes were obtained by normalizing their copy numbers to those of the 16S rRNA genes.
208 Real-time PCR assays were performed for the quantification of bacterial 16S rRNA and
209 fungal 18rRNA genes using primers 341f/534r and FF390/FR1, respectively (Table S1). All
210 samples were run in triplicate. A duplicate tenfold dilution series of standard DNA was used
211 to generate a standard curve. A detailed description is provided in the supporting information
212 with the primers and references listed in Table S1. A detailed description is provided in the
213 supporting information with the primers and references listed in Table S1.

214 *2.5. Statistical analysis*

215 Wastewater and sludge properties (Table S2 and Table S3) were standardized to have
216 comparability prior to further analysis using the formula, $z = (x_i - \bar{x})/s$, where x_i is the
217 sample value, $\bar{x}$ is the mean of all samples, and s is the standard deviation on (Freeman et
218 al., 1994; Xie et al., 2011). Diversity indices including Shannon-Weaver H and Simpson were
219 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 Table S2 and Table 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 (844-1,077 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 52-1,106 μg/g and 0-22.8 μg/g (dry weight), respectively. The effluent chemical oxygen demand (COD) was decreased from 1,640 and 3,200 mg/L to 222 and 332 mg/L in HS and HY plants, respectively, and the effluent NH_4^+ was also reduced from 130-164 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 6,133 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 4,520 genes were detected among the four sludge samples, while 3,945 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 2,744 species of bacteria, 886 (3.2%) target 140 species of archaea and 1,759 (6.3%) target 262 species of fungi (He et al., 2010a). In this study, 5,609 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, 1,252 genes were from *α-Proteobacteria*, 781 from *β-Proteobacteria*, 1,039 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 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 5,199 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 (844-1,077 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 (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 1,181 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. 2.b). 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 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 et al., 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., 2011).

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 GeoChip3.0. 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 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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Figure and Scheme Captions:

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.)

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.

Titles and legends to Tables

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

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

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.

Highlights

- Microbial communities treating antibiotic wastewater were functionally diverse.
- The role of fungal functional genes was enhanced by antibiotics.
- Antibiotic resistant bacteria played a main role in carbon degradation.

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	2,199	560	1,336	401	699	1,483	663	228	933	896	3,783

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.

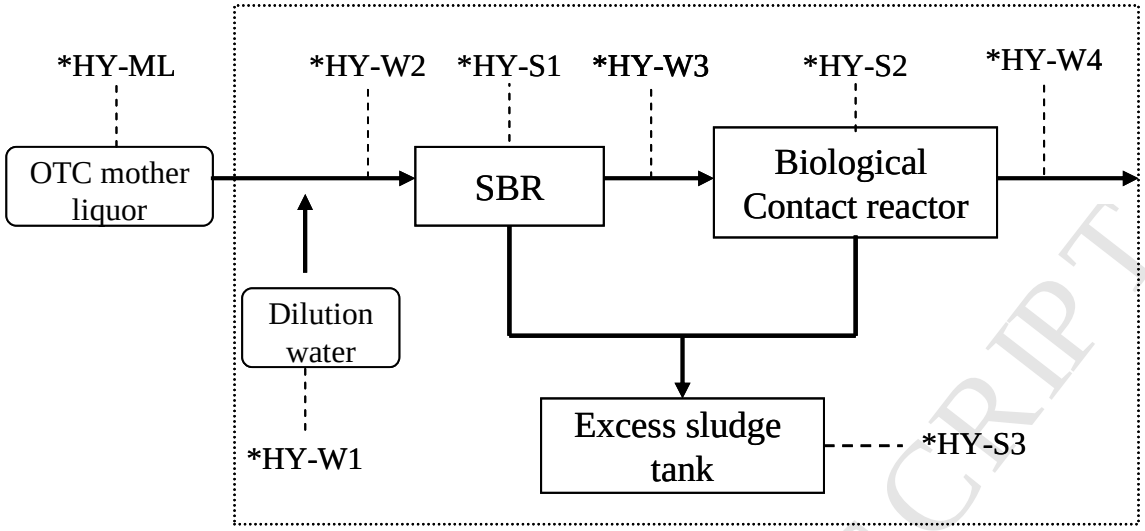
Table 2–The relationship between microbial community functional structure for carbon degradation and antibiotics revealed by Mantel test.

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

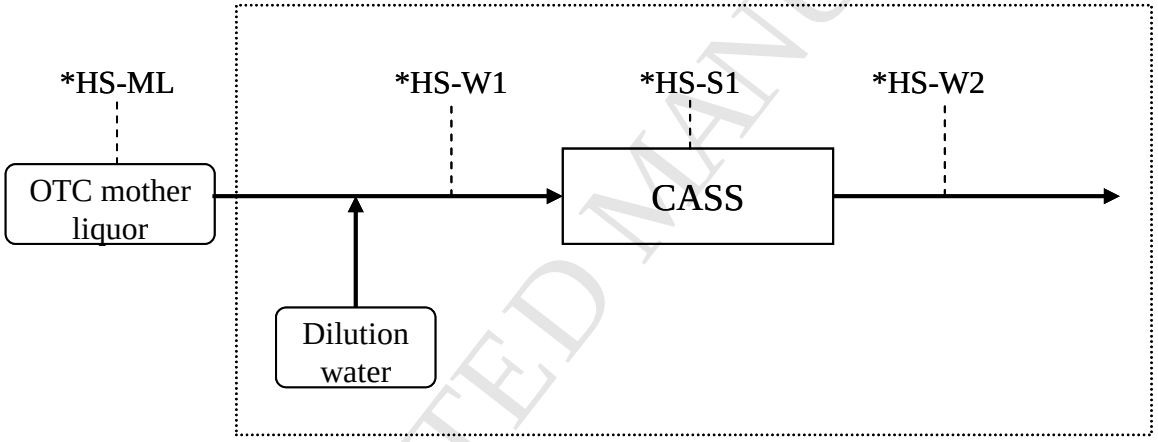
a. The signal intensity of genes among ten samples from biological treatment systems was used as the first matrix; the normalized antibiotic concentrations were used as the second matrix. **Bolded values** indicate significant *P*-values (<0.05).

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})



(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.)

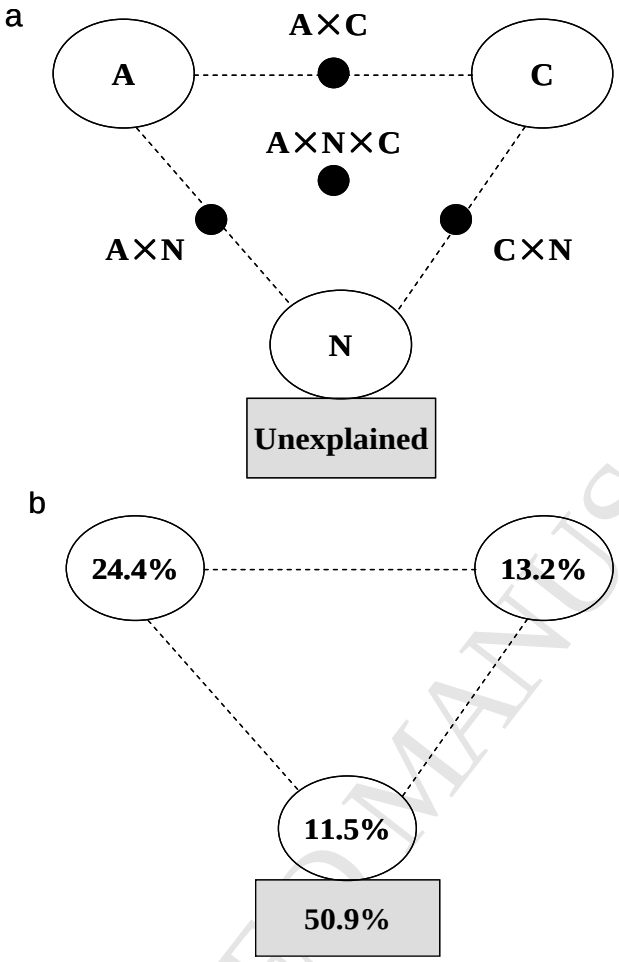


Fig. 2–Variation partitioning analysis of microbial diversity explained by antibiotics (A), COD (C), and NH_4^+ (N). (a) General outline, (b) All functional genes. Each diagram represents the biological variation partitioned into the relative effects of each variable or a combination of variables, in 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 thre

Supporting information

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

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A list of abbreviations

glx: glyoxal oxidase
lip: lignin peroxidase or ligniase
mnp: manganese peroxidase
*ara*_fungi: fungal arabinofuranosidase
tet genes: tetracycline resistance genes
aceA: isocitrate lyase
*aceB*_fungi: malate synthase
COD: chemical oxygen demand
HY: North China Pharmaceutical Group Corporation
HS: Huashu Pharmaceutical company
SBR: sequential batch reactor
CASS: cyclic activated sludge system
HY-S1: Activated sludge from SBR in HY
HY-S2: Activated sludge from biological contact reactor in HY
HY-S3: Excess sludge in HY
HS-S1: Activated sludge in HS
HY-ML: Oxytetracycline mother liquor in HY
HY-W1: Dilution water in HY
HY-W2: SBR influent in HY
HY-W3: SBR effluent in HY
HY-W4: Final effluent of HY
HS-ML: Oxytetracycline mother liquor in HS
HS-W1: Influent in HS
HS-W2: Effluent in HS
Inosine-S: Activated sludge for treating inosine fermentation production wastewater
Ethanol-S: Activated sludge for treating ethanol fermentation production wastewater
ABC: ATP-binding cassette
MATE: multidrug toxic compound extrusion
MFS: major facilitator superfamily
Mex: multidrug efflux
SMR: small multidrug resistance efflux pumps

Materials and methods

Description of sample collection: All wastewater samples were taken as flow-proportional composite samples using automated samplers that collected defined volumes every 4 h over the three days period, and were stored on ice for transporting back to the laboratory. Activated sludge and excess sludge after dewatering by belt-compress were collected from respective site. Amber glass bottles used for sample collection were pre-rinsed with ultra-pure water. All activated sludge and excess sludge samples were stored on ice for transporting back to the laboratory, and prepared for microbial community analysis. A total of 4 liter of the original water samples was used for chemical analysis. A portion (100 ml and 20 ml of water and sludge samples, respectively) were centrifuged at 9,167 and 2,291 g, for water and sludge samples, respectively, for 10 min at 4°C, and then stored at -80°C until used for DNA extraction. COD_{Cr}, BOD_5, NH_3-N, TN and TP were analyzed for the influent and effluent by spectra-photometric method (Shimadzu UV-160), and SS was analyzed by filtration and 48 h-incubation under 105^{o}C. All the above determinations were carried out in triplicate according to the national standard method of China (GB/T 19923 2005), and the average results were calculated. The pollutant concentrations (antibiotic concentrations and chemical oxygen demand (COD) concentrations) in the influents and effluents of the two systems were relatively stable according to our investigations over the last five years. Aerobic activated sludge samples from two non-antibiotic (Inosine-S and Ethanol-S) fermentation wastewater treatment plants were collected as controls, because these facilities mainly receive inosine and ethanol production waste streams, respectively.

Determination of antibiotic concentrations: Concentrations of oxytetracycline and its three reported hydrolysates (4-epi-oxytetracycline (EOTC), α-Apo-oxytetracycline (α-OTC), β-Apo-oxytetracycline (β-OTC)) and penicillin G were determined using high performance liquid chromatography and mass/mass spectrometry (UPLC-MS-MS). Standards of these antibiotics with purities higher than 95% were purchased from Sigma Aldrich. Stock solutions (1 mg/ml) were prepared in methanol and diluted to the range of 0.02-1 mg/liter to obtain the standard curves. Water samples were filtered through 0.22 μm polycarbonate membrane and diluted with ultrapure water (Millipore, USA) to obtain readings in the middle of the standard

curve. Antibiotics in sludge were extracted by acetonitrile and methanol combined with ultrasonic after freeze-drying. Extract solutions were combined and evaporated to dryness by nitrogen gas. The residues were dissolved in a 2-ml solution of 90% acetonitrile and 10% ammonium acetate (100 mM in water) and filtered through 0.22 μm polycarbonate filters. Samples were diluted to the appropriate concentrations for determination. The dry weight of sludge was obtained by incubating 5 g of sample at 105^{o}C for 24-48 h until constant weight was reached. The concentrations of the analytes in sludge samples were modified with dry weight. Conditions for the liquid chromatography and mass spectrum were exactly the same as described before (Li *et al*, 2008; 2009). Determination was carried out in duplicate and the average results were calculated.

PCR and Real-time PCR Assays: 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 for DNA extracted from the SBR activated sludge (HY-S1) were conducted in 25 μL volume reactions using an ABI PCR System 9700 (ABI, USA). The PCR mixture consisted of 1×Ex Taq buffer, 5 nM dNTP, 0.625 U Ex Taq (Takara, Japan), 400 nM each primer, 0.5 mg mL-1 BSA, and 1-2 μL DNA. Primers targeting the sixteen *tet* genes were selected from published literature with their amplification sizes and conditions listed in Table S1. The temperature program consisted of initial denaturation at 95°C for 5 min, followed by 35 cycles of 30 s at 95°C, 30 s at different annealing temperatures and extension at 72°C for 45 s, and finished with a final extension step at 72°C for 7 min. Sterile water was used as the negative control in each run. 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). Concentrations of the standard plasmids (ng μL^{-1}) were determined with the Nanodrop ND-1000 (Nanodrop, USA). Their copy concentrations (copies μL-1) were then calculated by the following formulation (Pei *et al*.,

2006).

$$\text{copy concentration}(\text{copies } \mu L^{-1}) = \frac{\text{DNA mass concentration (ng } \mu L^{-1})}{\text{DNA molecular weight (g mol}^{-1})} \times 6.02 \times 10^{23} \times 10^{-9}$$

The 25 μL reactions typically contained 1×Sybr Green I, 1×Dye (Takara), 200 nM each primer, 0.5 mg mL-1 BSA, and 2 μL DNA templates. Real-time PCR was run using an ABI7300 apparatus (ABI, USA) by the following program: 95°C for 30s, 40 cycles consisting of: (i) 95°C for 10s, (ii) annealing temperature for 15s, (iii) 72°C for 15s, and (iv) 78°C for 26s to collect the fluorescent signals. The melting process was automatically generated by the ABI7300 software. Triplicate real-time PCR assays were performed for the decimally diluted standard plasmids to obtain the standard curves. Duplicate real-time assays were performed for all samples and negative controls. To prevent the inhibition of the sample matrix, 10-100 fold diluted samples were used for quantification.

The following requirements were satisfied to obtain reliable quantification: R^2 higher than 0.99 for standard curves over 5 orders of magnitude and amplification efficiencies based on slopes between 90% and 110%. The specificity was assured by the melting curves and gel electrophoresis. 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). Thermal cycling conditions consisted of 30s at 95 ^{o}C followed by 40 amplification cycles of 10s at 95 ^{o}C, 15s at an annealing temperature (60 ^{o}C for bacterial genes, and 55 ^{o}C for fungal gene), and 15s at 72 ^{o}C. A melt curve profile was obtained by heating the mixture to 95 ^{o}C, cooling to 65 ^{o}C (15s), and slowly heating to 95 ^{o}C at 0.1 ^{o}C s^{-1} with continuous measurement of fluorescence. PCR quality water was used as negative control. All samples were run in triplicate. A duplicate tenfold dilution series of standard DNA was used to generate a standard curve. The standard curves for bacterial and fungal genes had R^2 values of 0.95-0.99 and the amplification efficiencies were 90-110%.

GenBank Accession number: Nucleotide sequences of the bacterial 16S rRNA genes were deposited in the GenBank database under the Accession No. JN245629-JN245879,

KC311355-KC311361, and KC352321- KC352355.

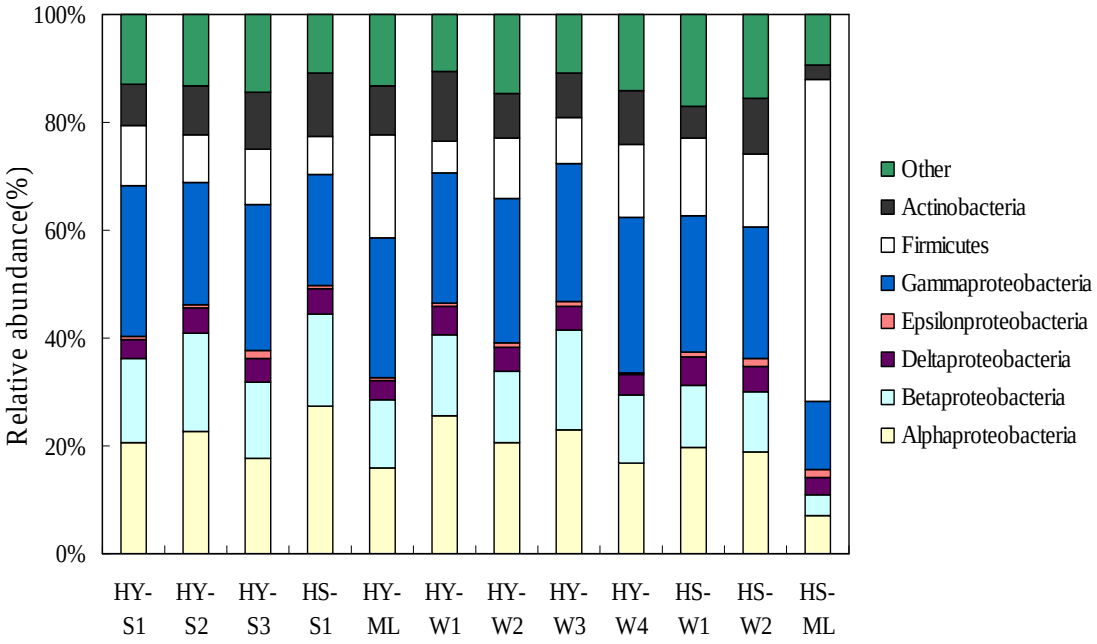

Figure S1. Relative abundance (represents the total signal intensity of genes) of host bacteria based on measurement from the GeoChip hybridization.

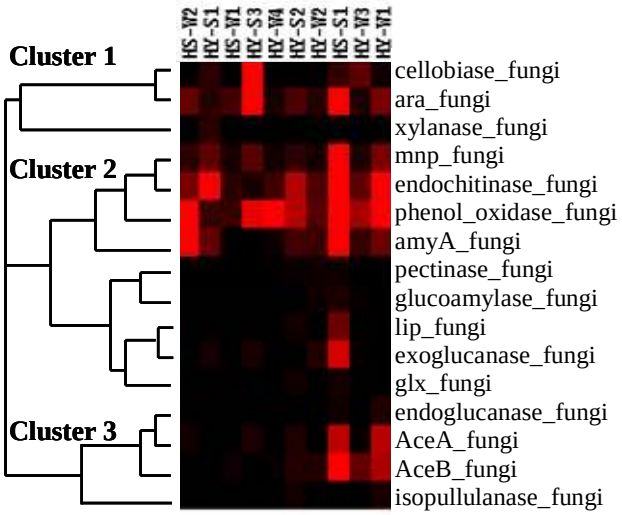

Figure S2 Clustering analysis of fungal gene groups involved in carbon degradation. The gene group name is presented. Heat maps were generated in CLUSTER and visualized using TREEVIEW. Red indicated signal intensities above background, whereas black indicates signal intensities below intensities. Brighter red coloring indicates higher signal intensities.

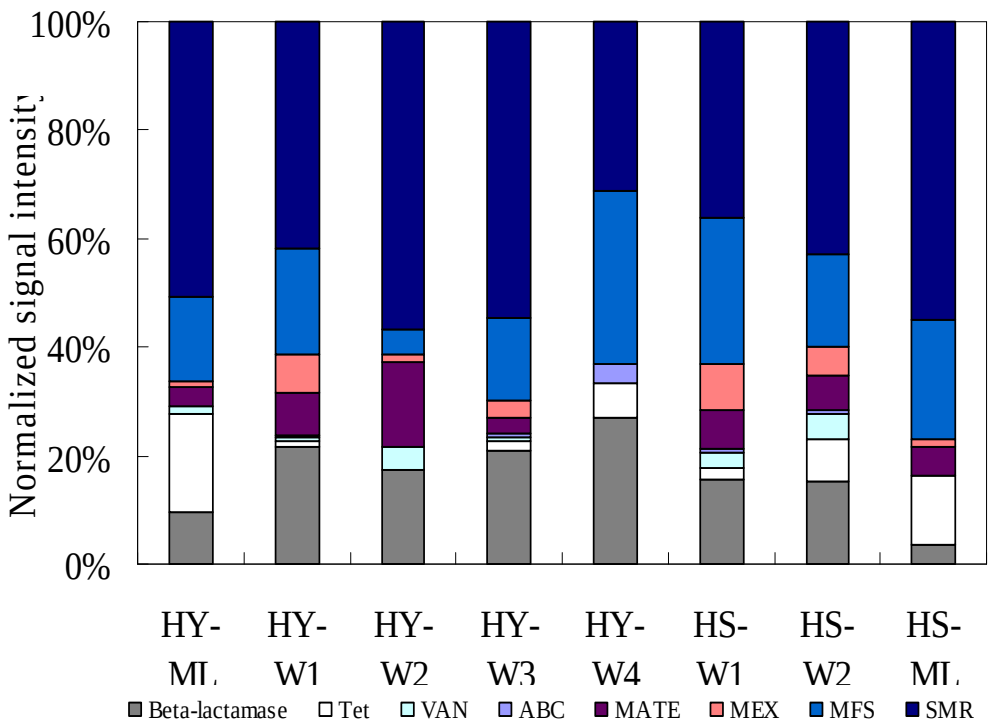

Figure S3 Relative abundance (represents the total signal intensity of genes) of antibiotic resistance genes and transporters based on measurement from the GeoChip hybridization.

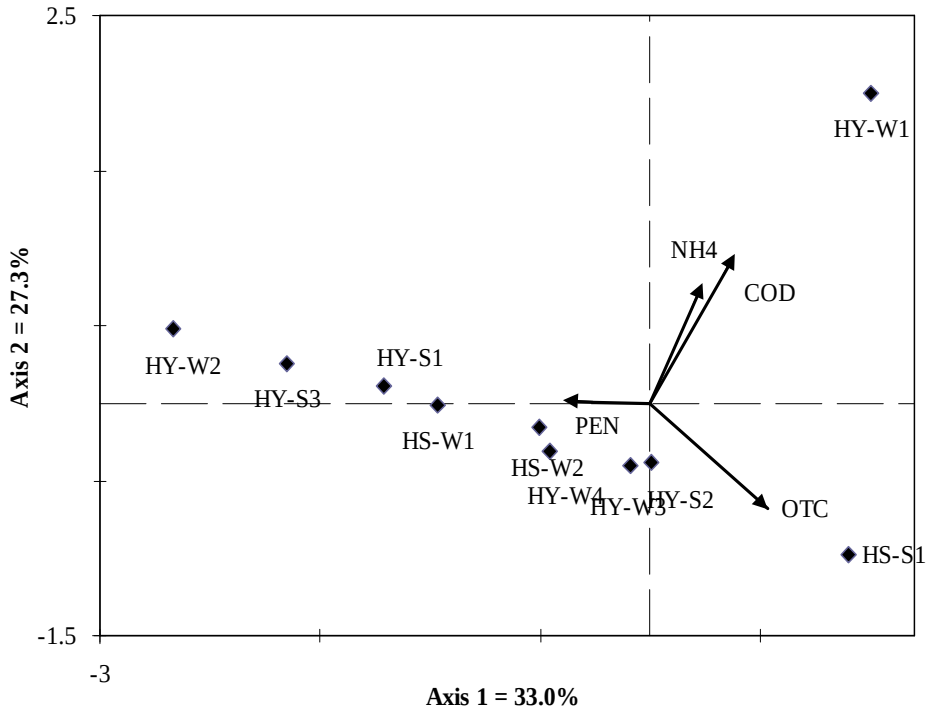

Figure S4 Canonical correspondence analysis (CCA) of GeoChip hybridization signal intensities and wastewater chemical data that were significantly related to microbial community variations in APW biological treatment systems. The percentages of variation explained by each axis are shown.

Table S1 PCR primers used in this study for real-time PCR and clone library.

Primers	Specificity	Sequence (5'to 3')	Reference
tet(A)	*tet* gene (real-time PCR)	GCTACATCCTGCTTGCCTTC	Ng *et al*., 2001
		CATAGATCGCCGTGAAGAGG	
tet(C)	*tet* gene (real-time PCR)	CTTGAGAGCCTTCAACCCAG	
		ATGGTCGTCATCTACCTGCC	
tet(E)	*tet* gene (real-time PCR)	AAACCACATCCTCCATACGC	
		AAATAGGCCACAACCGTCAG	
tet(G)	*tet* gene (real-time PCR)	GCTCGGTGGTATCTCTGCTC	
		AGCAACAGAATCGGGAACAC	
tet(L)	*tet* gene (real-time PCR)	TCGTTAGCGTGCTGTCATTC	
		GTATCCCACCAATGTAGCCG	
tet(O)	*tet* gene (real-time PCR)	AACTTAGGCATTCTGGCTCAC	
		TCCCACTGTTCCATATCGTCA	
tet(X)	*tet* gene (real-time PCR)	CAATAATTGGTGGTGGACCC	
		TTCTTACCTTGGACATCCCG	
tet(A/P)	*tet* gene (real-time PCR)	CTTGGATTGCGGAAGAAGAG	
		ATATGCCCATTTAACCACGC	
tet(M)	*tet* gene (real-time PCR)	ACAGAAAGCTTATTATATAAC	Aminov *et al*., 2001
		TGGCGTGTCTATGATGTTCAC	
tet(Q)	*tet* gene (real-time PCR)	AGAATCTGCTGTTTGCCAGTG	
		CGGAGTGTCAATGATATTGCA	
tet(W)	*tet* gene (real-time PCR)	GAGAGCCTGCTATATGCCAGC	
		GGGCGTATCCACAATGTTAAC	
tet(T)	*tet* gene (real-time PCR)	AAGGTTTATTATATAAAAGTG	
		AGGTGTATCTATGATATTTAC	
tet(B/P)	*tet* gene (real-time PCR)	AAAACTTATTATATTATAGTG	
		TGGAGTATCAATAATATTCAC	
tet(D)	*tet* gene (real-time PCR)	GGAATATCTCCCGGAAGCGG	Aminov *et al*., 2002
		GGAATATCTCCCGGAAGCGG	
tet(K)	*tet* gene (real-time PCR)	TCGATAGGAACAGCAGTA	
		CAGCAGATCCTACTCCTT	
tet(Z)	*tet* gene (real-time PCR)	CCTTCTCGACCAGGTCGG	
		ACCCACAGCGTGTCCGTC	
341f	Bacterial 16S rRNA	CCTACGGGAGGCAGCAG	Koike et al., 2007
534r	gene (real-time PCR)	TTACCGCGGCTGCTGGCAC	
FF390	Fungal 18S rRNA gene	AICCATTCAATCGGTAIT	Nicolas et al., 2011
FR1	(real-time PCR)	CGATAACGAACGAGACCT	
27f	Bacterial 16S rRNA	AGAGTTTGATC(A/C)TGGCTCAG	Polz et al., 1999
1492r	gene (Clone library)	GGTTACCTTGTTACGACTT	

Table S2 Chemical and physical characteristics of wastewater samples.

Plant	Sample	COD (mg/liter)	NH_4^+ (mg/liter)	NO_3^- (mg/liter)	SO_4^{2-} (mg/liter)	oxytetracycline (mg/liter)	Penicillin (mg/liter)	T °C	pH
HY	HY-ML: Oxytetracycline mother liquor	11,000	1,620	214	1,010	844	0.00	22	4.8
	HY-W1: Dilution water	19,000	283	33.6	8,180	0.00	0.57	85	3.8
	HY-W2: SBR influent	3,200	164	2.00	624	4.25	0.49	22	6.8
	HY-W3: SBR effluent	310	19.0	35.6	993	2.05	0.16	22	6.8
	HY-W4: Final effluent of HY	332	5.07	49.0	926	0.49	0.07	22	6.8
HS	HS-ML: oxytetracycline mother liquor	17,300	1,940	109	1,370	1,077	0.00	24	4.2
	HS-W1: CASS influent	1,640	130	0.35	152	3.66	0.00	22	7.5
	HS-W2: Final effluent of HS	222	2.72	16.2	218	1.19	0.00	24	7.4

Table S3 Chemical characteristics of sludge samples

Samples	water (%)	Oxytetracycline (μg/g)	Penicillin G (μg/g)
HY-S1: Activated sludge from SBR in HY	98.38	173	22.8
HY-S2: Activated sludge from biological contact reactor in HY	98.51	131	7.8
HY-S3: Excess sludge in HY	86.48	52	0.0
HS-S1: Activated sludge from CASS in HS	97.07	1106	0.0

Table S4 Number of functional genes detected by GeoChip 3.0 grouped by functional process.

			Sample, no.											
Gene category		**Gene no.[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**
Antibiotic resistance	**Total**	**559**	**79**	**226**	**44**	**175**	**31**	**60**	**190**	**52**	**23**	**87**	**84**	**423**
	Transporters	427	59	167	28	129	22	44	147	43	18	67	62	325
	β-lactamases	112	15	52	13	40	6	11	34	6	3	17	17	85
	Tet	13	4	4	2	5	3	4	7	2	2	2	4	8
	Van	7	3	3	1	0	1	2	1	5	1	1	0	1
Carbon cycling	**Total**	**987**	**90**	**315**	**86**	**183**	**64**	**116**	**184**	**109**	**37**	**129**	**142**	**548**
	Carbon degradation	749	78	242	63	134	52	91	144	81	29	99	108	416
	Carbon fixation	175	8	61	15	36	10	19	34	18	5	22	27	112
	Methanogenesis	17	2	5	5	6	0	2	5	4	2	6	5	13
	Methane oxidation	23	0	6	2	3	1	1	0	2	0	2	1	4
Nitrogen cycling	**Total**	**703**	**63**	**240**	**67**	**146**	**56**	**85**	**148**	**83**	**26**	**107**	**104**	**409**
	N fixation	230	25	82	30	51	23	37	56	36	10	60	54	127
	Nitrification	12	0	3	2	2	0	0	1	1	1	0	1	3
	Denitirfication	276	21	96	17	56	22	31	52	26	10	28	29	169
	Anaerobic ammonium oxidation	8	1	1	2	2	0	0	1	1	0	2	1	4
Sulphur cycling	**Total**	**370**	**46**	**157**	**33**	**96**	**38**	**30**	**105**	**39**	**13**	**59**	**51**	**235**
	S reduction	242	28	98	23	58	22	23	59	30	10	41	35	140
Organic Remediation	**Total**	**2210**	**196**	**826**	**176**	**435**	**110**	**243**	**539**	**216**	**70**	**317**	**302**	**1472**
Phosphorus cycling	**Total**	**126**	**13**	**43**	**13**	**25**	**7**	**18**	**29**	**18**	**8**	**17**	**21**	**62**
Metal resistance	**Total**	**948**	**98**	**310**	**112**	**225**	**70**	**117**	**233**	**116**	**37**	**173**	**146**	**523**
Energy process	**Total**	**106**	**11**	**42**	**9**	**21**	**5**	**8**	**21**	**9**	**1**	**13**	**12**	**63**
other category	**Total**	**122**	**17**	**38**	**18**	**28**	**18**	**20**	**32**	**19**	**11**	**29**	**32**	**46**
Total genes		6133	615	2199	560	1336	401	699	1483	663	228	933	896	3783

a. Genes detected in at least one sample were used for gene number calculation.

Table S5 Phylogenetic groups of antibiotic production wastewater treatment systems detected by GeoChip 3.0 (detected numbers).

Phylogenetic groups	**HY-ML**	**HY-W1**	**HY-W2**	**HY-W3**	**HY-W4**	**HY-S1**	**HY-S2**	**HY-S3**	**HS-ML**	**HS-W1**	**HS-W2**	**HS-S1**	**Total**
Bacteria	**551**	**2040**	**508**	**1229**	**359**	**637**	**1382**	**603**	**211**	**852**	**810**	**3537**	**5609**
Alphaproteobacteria	91	470	83	277	58	115	317	101	30	162	164	872	1252
Betaproteobacteria	58	275	53	193	41	64	197	61	15	85	76	539	781
Gammaproteobacteria	131	383	105	238	80	143	260	122	43	172	165	633	1039
Deltaproteobacteria	27	98	29	57	16	26	61	25	15	38	38	151	242
Epsilonproteobacteria	4	9	7	7	1	4	8	7	6	5	9	16	31
Actinobacteria	37	222	37	91	35	55	120	50	8	55	58	402	613
Firmicutes	76	118	59	88	30	71	105	71	40	92	87	194	398
Cyanobacteria	1	5	2	3	2	2	3	1	1	2	3	7	103
Bacteroidetes	6	30	7	17	2	12	17	7	5	16	14	43	93
Chloroflexi	10	25	6	20	6	6	22	11	3	14	13	38	71
Chlorobi	8	18	6	8	3	5	8	7	0	8	6	22	35
Deinococcus-Thermus	7	15	6	5	4	9	9	7	3	10	6	20	32
Planctomycetes	3	10	3	7	1	6	10	7	1	8	7	15	26
Acidobacteria	0	8	2	3	1	3	4	2	0	2	1	17	22
Verrucomicrobia	4	6	2	8	3	1	7	2	1	5	4	9	13
Spirochaetes	1	3	1	2	1	2	1	2	1	5	3	1	7
Tenericutes	1	3	0	3	1	1	2	2	0	2	2	3	5
Thermotogae	3	4	2	0	2	2	1	2	1	3	1	3	5
Chlamydiae	1	1	1	0	0	2	2	1	1	1	1	2	4
Aquificae	1	1	2	1	0	1	2	1	0	2	2	4	4
Thermodesulfobacteria	1	1	2	1	1	0	1	0	1	1	0	2	4
Nitrospirae	0	0	0	0	1	0	0	0	0	0	0	0	1
Umclassified	117	565	132	294	106	165	349	166	44	221	209	963	828
Archaea	**14**	**49**	**13**	**35**	**13**	**22**	**37**	**16**	**4**	**37**	**32**	**69**	**154**
Crenarchaeota	5	11	4	10	5	5	11	4	2	12	8	17	
Euryarchaeota	7	24	4	18	6	15	19	9	2	20	19	43	
Umclassified	2	14	5	7	2	2	7	3	0	5	5	9	
Eukaryota	**48**	**95**	**33**	**64**	**23**	**35**	**57**	**40**	**11**	**38**	**49**	**154**	**333**
Ascomycota	46	84	26	51	16	30	47	30	10	33	38	121	
Basidiomycota	1	7	6	8	3	4	7	7	0	4	9	23	
Umclassified	1	4	1	5	4	1	3	3	1	1	2	10	

*Genes were grouped based on phylogenetic markers on the GeoChip 3.0.

Table S6 Phylogenetic affiliation of bacterial sequences of the 16S rRNA gene clone libraries.

Classification		Percentages of bacterial populations (%)							
phylum	Class	HY-ML	HY-W2	HY-W4	HY-S1	HY-S2	HS-ML	HS-S1	Inosine-S
Proteobacteria	*Alpha*	17.6	4.1	6.6	3.7		-	17	3.5
	Beta	17.6	1.4	44.3	42.7	39.3	-	18.7	53.0
	Gamma	5.9	16.4	8.2	1.9		-	3.4	18.8
	Delta	-	-	-			-	3.4	
	Epsilon	-	-	-	9.3	1.8	-		
Firmicutes	*Bacilli*	17.6	11.0	1.6			86.6	-	
	Clostridia	11.8	27.4	3.3			6.7	1.7	3.5
	others	-	1.4	-			-	-	8.8
Bacteroidetes	*Bacteroidetes*	-	21.9	1.6			6.7		
	Flavobacteria	-	-	3.3	5.6	3.6	-	5.1	
	Sphingobacteria	-	-	-	25.9	35.7	-	6.8	
	others				5.6	1.8			3.5
Actinobacteria	*Actinobacterida*	11.8	-	1.6	-	-	-	-	
Chloroflexi	*Anaerolineae*	-	4.1	-	-	-	-	3.4	
Nitrospirae	*Nitrospira*	-	-	-	-	-	-	3.4	
Acidobacteria	Unclassified		-	-	-	-	-	16.9	
Planctomycetes	Unclassified	-	-	-	-	-	-	5.1	
Unclassified			5.1	24.6	5.3	17.8		15.1	8.9
NO. of OTUs (NO of Clones)		12(17)	32(68)	31(58)	25(54)	34(56)	3(30)	26(59)	11(67)

Table S7 The relationship between microbial community functional structure for antibiotic resistance and antibiotics revealed by Mantel test.

Gene category	Oxytetracycline		Penicillin	
	r_M	*P*	r_M	*P*
Tet	-0.0196	0.332	0.1030	0.223
Van	0.0363	0.364	-0.2334	0.733
β_lactamase_A	0.5799	0.097	-0.2952	0.833
β_lactamase_B	0.5597	0.112	-0.2118	0.687
β_lactamase_C	0.7695	**0.039**	-0.1237	0.481
β_lactamase_D	0.3528	0.129	0.1564	0.225
*MATE*_antibiotic_transporter	0.4398	0.146	-0.1196	0.475
*ABC*_antibiotic_transporter	0.2953	0.108	0.1195	0.210
*MFS*_antibiotic_transporter	0.8531	**0.046**	-0.2189	0.783
*Mex*_antibiotic_transporter	0.1569	0.14	-0.1476	0.519
*SMR*_antibiotic_transporter	0.2629	0.157	0.6313	**0.035**

a. 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).

Table S8 Concentrations of *tet* genes normalized to the 16s rRNA genes during the sampling processes (Average value of five samples over four years). The standard deviations were all less than 10% of the mean values, and so they are not shown in the table.)

Sample	*tet* (A)	*tet* (C)	*tet* (G)	*tet* (L)	*tet* (M)	*tet* (O)	*tet* (Q)	*tet* (W)	*tet* (X)	Total *tet*
HY-ML	3.6×10^{-2}	1.4×10^{-3}	UD	7.9×10^{-2}	3.1×10^{-2}	1.6×10^{-2}	3.6×10^{-2}	2.2×10^{-2}	3.2×10^{-4}	2.2×10^{-1}
HY-W2	1.4×10^{-1}	3.2×10^{-2}	5.1×10^{-2}	6.1×10^{-2}	1.1×10^{-2}	1.0×10^{0}	1.8×10^{0}	1.6×10^{-1}	3.7×10^{-3}	3.2×10^{0}
HY-W3	1.8×10^{-1}	2.7×10^{-1}	1.2×10^{-1}	1.8×10^{-2}	6.3×10^{-3}	2.1×10^{-2}	2.5×10^{-1}	1.2×10^{-2}	9.6×10^{-3}	8.8×10^{-1}
HY-W4	9.2×10^{-1}	2.0×10^{-1}	2.2×10^{-1}	6.4×10^{-3}	2.8×10^{-3}	1.3×10^{-3}	2.6×10^{-2}	3.3×10^{-3}	2.9×10^{-2}	1.4×10^{0}
HY-S1	7.0×10^{-1}	4.5×10^{-1}	1.9×10^{-1}	5.9×10^{-2}	9.3×10^{-3}	4.2×10^{-3}	4.4×10^{-2}	3.2×10^{-3}	9.2×10^{-2}	1.6×10^{0}
HY-S2	7.2×10^{-1}	5.4×10^{-1}	2.7×10^{-1}	1.2×10^{-2}	5.5×10^{-3}	1.4×10^{-3}	9.6×10^{-3}	2.4×10^{-3}	1.0×10^{-1}	1.7×10^{0}
HY-S3	9.7×10^{-2}	5.5×10^{-2}	-	2.0×10^{-5}	2.9×10^{-3}	8.1×10^{-5}	3.1×10^{-4}	2.7×10^{-4}	4.8×10^{-4}	1.6×10^{-1}
HS-ML	5.7×10^{-2}	2.2×10^{-3}	-	2.8×10^{-3}	2.9×10^{-1}	1.5×10^{-1}	4.5×10^{-1}	6.8×10^{-2}	3.4×10^{-4}	1.0×10^{0}
HS-W1	5.4×10^{-2}	6.9×10^{-2}	-	4.5×10^{-4}	3.9×10^{-2}	9.9×10^{-3}	1.5×10^{-1}	1.2×10^{-1}	1.5×10^{-3}	4.4×10^{-1}
HS-W2	4.0×10^{-1}	6.8×10^{-2}	-	1.3×10^{-4}	1.34×10^{-1}	8.7×10^{-3}	4.8×10^{-2}	1.4×10^{0}	2.2×10^{-3}	2.1×10^{0}
HS-S1	3.1×10^{0}	6.0×10^{-1}	-	6.6×10^{-5}	5.1×10^{-5}	1.2×10^{-3}	1.2×10^{-3}	2.2×10^{-2}	6.9×10^{-3}	3.7×10^{0}
Control	9.2×10^{-4}	3.0×10^{-3}	4.1×10^{-3}	UD	UD	2.8×10^{-4}	1.1×10^{-4}	1.6×10^{-4}	1.0×10^{-2}	1.7×10^{-2}
	-2.2×10^{-3}	-1.6×10^{-2}	-1.5×10^{-2}	-7.3×10^{-6}		-7.3×10^{-4}	-9.2×10^{-4}	-5.7×10^{-4}	-1.3×10^{-2}	-3.1×10^{-2}

'-': No available data. 'UD': Undetectable. Control system: Inosine-S and ethanol-S; The concentration of Tet(E) and Tet(K) were below the detection limits.

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