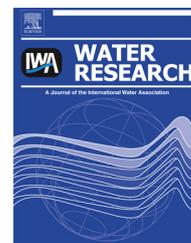


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Linkages between microbial functional potential and wastewater constituents in large-scale membrane bioreactors for municipal wastewater treatment

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ABSTRACT

Large-scale membrane bioreactors (MBRs) have been widely used for the municipal wastewater treatment, whose performance relies on microbial communities of activated sludge. Nevertheless, microbial functional structures in MBRs remain little understood. To gain insight into functional genes and their steering environmental factors, we adopted GeoChip, a high-throughput microarray-based tool, to examine microbial genes in four large-scale, in-operation MBRs located in Beijing, China. The results revealed substantial microbial gene heterogeneity (43.7–85.1% overlaps) among different MBRs. Mantel tests indicated that microbial nutrient cycling genes were significantly ($P < 0.05$) correlated to influent COD, $\text{NH}_4^+ - \text{N}$, TP or sulfate, which signified the importance of microbial mediation of wastewater constituent removal. In addition, functional genes shared by all four MBRs contained a large number of genes involved in antibiotics resistance, metal resistance and organic remediation, suggesting that they were required for degradation or resistance to toxic compounds in wastewater. The linkages between microbial functional structures and environmental variables were also unveiled by the finding of hydraulic retention time, influent COD, $\text{NH}_4^+ - \text{N}$, mixed liquid temperature and humic substances as major factors shaping microbial communities. Together, the results presented demonstrate the utility of GeoChip-based microarray approach in examining microbial communities of wastewater treatment plants and provide insights into the forces driving important processes of element cycling.

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1. Introduction

Membrane bioreactor (MBR) has been widely applied to treat municipal wastewater (Huang et al., 2010). With a combination of biological treatment process and membrane technology, MBR has remarkable advantages in producing high-quality reclaimed water over conventional activated sludge process. The high biomass retained in the systems by membrane rejection and the long sludge retention time (SRT) were thought to be important for the stable performance of MBRs (Drews et al., 2005). However, it remains unclear whether and how microbial community in activated sludge is linked to the MBR performance. Most of the current studies focused on the understanding of the influence of specific influent components, single operational conditions or the reactor configuration on microbial communities. Furthermore, most of them were conducted in lab-scale or pilot-scale plants (Xia et al., 2012), which did not reflect actual conditions in large-scale municipal MBRs due to substantial differences in design, scale, operational time and parameters, as well as influent components and the fluctuations (Table S1) (Shen et al., 2012). In addition to differences in influent alkalinity, biochemical oxygen demand (BOD), chemical oxygen demand (COD), BOD/COD and C/N/P ratios, real wastewater often contains antibiotics, heavy metals and other organic pollutants from domestic and industrial discharges, which seldom appears in

the synthetic wastewater treated by lab-scale MBRs. To date, few studies were done with large-scale, operational MBR plants except for two recent studies (Wan et al., 2011; Hu et al., 2012). These two studies targeted bacterial phylogenetic or ammonia-oxidizing community composition. Thus, microbial functional structure and metabolic potentials remained elusive.

High-throughput functional gene array (e.g., GeoChip) technology has been proven to be powerful in examining microbial functional potentials, since it targets a wide range of functional genes involved in C, N, P, S cycling, metal resistance and organic contaminant degradation (such as aromatics, herbicides and pesticides related compounds) and so on. To date, it has been used to profile microbial communities in various habitats, including soil, marine sediments, contaminated groundwater and lab-scale bioreactors (Van Nostrand et al., 2009; Yang et al., 2013; Zhong et al., 2012). However, its utility in examining microbial communities of wastewater treatment plants is yet to be demonstrated.

In this study, we applied GeoChip to examine four large-scale, in-operation MBRs located in Beijing, China. These plants, each with a capacity over 10,000 m³/d, were combined with a common nutrient-removal anaerobic-anoxic-oxic process (Table 1). Considering that different tanks might have similar microbial profiles due to activated sludge circulated between each tanks by return sludge pumping system, and on the other hand membrane tank has unique features enduring

Table 1 – Process and environmental variables of MBRs.

Factors		B1	B2	B3	B4
Processes and membrane information	Process ^a	A ₁ -A ₂ -O-MBR			
	Capacity (m ³ /d)	60,000	35,000 ^b	40,000 ^b	30,000 ^b
	Commissioned date	2007.11	2007.11	2010.05	2009.11
	Membrane type	PVDF, 0.04 μm	PVDF, 0.1 μm	PVDF, 0.4 μm	PVDF, 0.4 μm
	Wastewater type	Domestic	80% domestic + 20% industrial	80% domestic + 20% industrial	Domestic
Operation conditions	HRT (h)	17.3	40.0	30.0	16.5
	SRT (d)	20.0	27.0	28.3	26.0
	MLSS (g/L)	10.1	2.2	5.9	4.0
	F/M ^c	54.6	239.3	11.6	69.8
Influent characters	COD (mg/L)	550	520	68	276
	NH ₄ ⁺ -N (mg/L)	26.1	20.1	21.5	39.6
	TN (mg/L)	52.6	43.5	42.1	45.0
	TP (mg/L)	4.8	10.7	2.0	4.5
Mixed liquid properties	Temperature (°C)	16.7 ± 1.4	15.3 ± 0.6	8.5 ± 0.2	13.7 ± 0.2
	pH	7.9 ± 0.2	7.7 ± 0.1	7.2 ± 0.1	7.4 ± 0.1
	DO (mg/L)	6.4 ± 0.5	7.6 ± 0.9	10.9 ± 0.5	7.9 ± 1.4
	Viscosity (mPa·s)	5.2 ± 0.5	1.3 ± 0.0	2.3 ± 0.1	2.1 ± 0.2
	Conductivity (μS/cm)	941.0 ± 10.2	1307.0 ± 4.3	1193.3 ± 5.5	1025.0 ± 21.9
	SVI	9.4 ± 0.5	9.9 ± 0.6	16.0 ± 0.7	20.8 ± 2.2
	MLVSS/MLSS	0.6 ± 0.0	0.6 ± 0.0	0.4 ± 0.1	0.7 ± 0.1
	TOC (mg/L)	9.2 ± 0.4	13.3 ± 0.5	22.3 ± 1.7	11.5 ± 1.8
	Polysaccharides (mg/L)	7.7 ± 0.3	7.3 ± 0.1	17.0 ± 0.5	7.8 ± 0.8
	Proteins (mg/L)	1.6 ± 0.3	2.2 ± 0.5	2.8 ± 1.1	3.3 ± 0.6
	Humics (mg/L)	4.8 ± 0.6	5.6 ± 0.4	8.4 ± 0.3	3.6 ± 0.6

PVDF: polyvinylidene fluoride.

^a A₁: anaerobic; A₂: anoxic; O: oxic; MBR: membrane bioreactor.

^b These plants' actual capacity is around 40–80% of the design. B1 plant reached full-capacity operation.

^c Sludge load.

“harsh” environment such as intense aeration and high shear force performed for fouling control, higher sludge concentration and soluble microbial products (SMPs) retained by membrane interception, activated sludge in membrane tank was focused in this study. We hypothesize that (1) microbial community functional structures in the sludge were different among different MBRs, considering that these MBRs differ in process, operation and wastewater constituents; (2) key functional gene categories (C, N, P and S cycling) are related to influent constituents; and (3) microbial genes present in all four MBRs were consistent with wastewater constituents. To test these hypotheses, activated sludge samples in membrane tanks of MBRs were collected in triplicates and analyzed by GeoChip 4.0. To our knowledge, this is the first study to examine microbial community functional structures in large-scale, in-operation MBRs.

2. Experimental procedures

2.1. MBRs and sample analysis

The activated sludge samples were obtained from four large-scale MBRs (B1, B2, B3 and B4) located in Beijing, China. The four plants share a common nutrient-removal anaerobic-anoxic-oxic process enhanced with membrane bioreactor ($A_1/A_2/O$ -MBR). Three replicate sludge samples were collected in the membrane tanks from each plant during October to December, 2010. All precipitated sludge samples were sealed into sterile sampling tubes, stored in a portable dry ice container, directly shipped to the lab and stored at $-80\text{ }^\circ\text{C}$ before DNA extraction. The untreated samples were kept at $4\text{ }^\circ\text{C}$ for mixed liquor property analyses, which were performed immediately after sampling. The mixed liquor properties, the operation conditions and influent compositions (average data at the inlet of wastewater treatment plants in half a month collected before sampling) were measured and summarized. The physicochemical properties of mixed liquid were classified into apparent parameters (temperature, pH, dissolved oxygen (DO), conductivity), rheological behavior (viscosity), settle ability (sludge volume index, SVI), sludge compositions (mixed liquor suspended solid (MLSS), mixed liquor volatile suspended solid (MLVSS), and extracellular polymeric substances (EPS)), and supernatant fractions (organics and nutrients).

Temperature, pH, DO and conductivity were determined using portable meter (Orion 4-StarTM, Thermo Scientific; SevenGoTM, Mettler Toledo). Viscosity was measured using a rotational viscosity meter (Model DV-II+Pro, Brookfield, USA). COD, NH_4^+-N , total nitrogen (TN) and total phosphorous (TP), MLSS, MLVSS, SVI were measured according to standard methods (APHA, 1998). The polysaccharide, protein and humic substance were measured according to the procedures described in the literature (Frolund et al., 1995; Dubois et al., 1956). Before protein determination, divalent cations were removed using gel-type cation exchange resins (Amberlite IR-120 Na, Acros Organics, Belgium) in order to get rid of the interference of calcium and magnesium (Shen et al., 2013). The detailed information is shown in Table 1.

2.2. Genomic DNA extraction, GeoChip 4.0 assay and data pre-processing

Microbial genomic DNA was extracted from 2 g sludge sample according to the method (Zhou et al., 1996); the crude DNA was further purified using commercial kit (Wizard[®] SV Gel and PCR Clean-up System, Promega Corporation, USA). The purified DNA quality, integrity and concentration were assessed by 260/280 nm and 260/230 nm ratios (Nanodrop 2000, Thermo Scientific), gel electrophoresis and Quant-It[®] TMPicoGreen[®] kit (Invitrogen, Carlsbad, CA, USA), respectively. The purified DNA (2 μg) was first labeled with Cy3 using random primers and the Klenow fragment of DNA polymerase I, then purified and dried in a SpeedVac at $45\text{ }^\circ\text{C}$ for 45 min (ThermoSavant) before hybridization. The hybridization was performed at $42\text{ }^\circ\text{C}$ with 40% formamide for 16 h on a MAUI hybridization station (BioMiro, UT, USA) using GeoChip 4.0 (NimbleGen, WI, USA), which contains 83,992 50-mer oligonucleotide probes targeting 152,414 genes in 410 categories regarding different microbial functional processes. Common oligo reference standard (CORS) probes were placed randomly throughout the chips and used for signal normalization. The GeoChips were scanned (MS 200 Microarray Scanner, NimbleGen) at a laser power of 100% after hybridization. The signal intensities were measured based on the scanned images. The spots with a signal to noise ratio ($\text{SNR} < 2.0$) were removed as poor-quality spots (He et al., 2010). Genes that were detected in only one sample out of the three replicas were considered ineffective and removed. Subsequently, the GeoChip data were normalized by relative abundance and then transformed into the natural logarithmic form.

2.3. Statistical analysis

Principal component analysis (PCA) and the dissimilarity test using Adonis algorithm were used as previously described (He et al., 2010). The relationship among the operational conditions, influent characters, mixed liquor properties, and functional microbial community was explained by Mantel test (r and P values indicated the strength of correlation and the significance of the relationship, respectively) and canonical correspondence analysis (CCA); the contribution of operational conditions (O), influent characters (I) and mixed liquor properties (M) to the variation of functional microbial community was further quantified via variation partitioning analysis (VPA) using the vegan package (v. 1.17–9) in R (v.2.12.2). To compare gene abundance difference among the four MBRs, gene abundance values were normalized by the abundance in B1.

3. Results

3.1. Process and environmental variables of MBRs

Process and environmental variables of MBRs are detailed in Table 1. The process of four plants in Beijing was similar, which was an A^2O process with a membrane bioreactor, but they varied in commission time and wastewater treatment capacities. Substantial differences in operation and influent

condition were notable, HRT, SRT, influent NH_4^+-N , COD, TN and TP. B1 and B2 had higher influent COD than other two plants. The influent NH_4^+-N was the highest in B4, and the TP concentration was highest in B2. Accordingly, the mixed liquor properties were different among four plants. B1 and B2 had higher mixed liquid temperature than B3 and B4. The soluble microbial products (e.g., polysaccharides, proteins and humic substances) showed a relatively high level in B3. Other parameters such as conductivity and SVI also varied among four MBRs.

3.2. Microbial heterogeneity of MBRs

The most microbial genes were detected in B1 (34,897 genes), while fewest genes were detected in B4 (16,427 genes) (Table 2). A total of 15,421 genes were present in common in all four MBRs, accounting for 38.6% of total detected genes. B1 and B2 shared the most overlapping genes (32,809; 85.1%), and B1 and B4 had the fewest (16,633; 43.7%). Therefore, microbial gene heterogeneity among MBRs was apparent.

The within-MBR gene heterogeneity was smaller than the between-MBR gene heterogeneity, as shown by the nonparametric statistics test of adonis algorithm (Table S2). Consistently, PCA analysis showed that microbial community functional structures were clustered by MBRs (Fig. 1). B1 and B2 were close to each other, indicating that their microbial communities were more similar than other MBR plants.

3.3. Key genes related to MBR performance

3.3.1. Functional genes involved in carbon degradation

Carbon compound is a major constituent of municipal wastewater, accounting for approximately 40% of organic contents (Ellis, 2004). Therefore, carbon compound removal is a key performance index of wastewater treatment. Our results showed that microbial genes involved in the degradation of starch, hemicelluloses, cellulose and chitin were detected in all of four MBRs (Fig. 2). Among them, gene abundances between B1 and B2 were similar except for endochitinase and cellobiose dehydrogenase (*cdh*, an extracellular flavocytochrome involved in cellulose degradation). In contrast, abundances of acetylglucosaminidase, xylanase and *pula* were lower in B3 than those in B1 and B2, while the abundance of endochitinase was higher in B3 than B2. Most of gene abundances were significantly different in B4. For example,

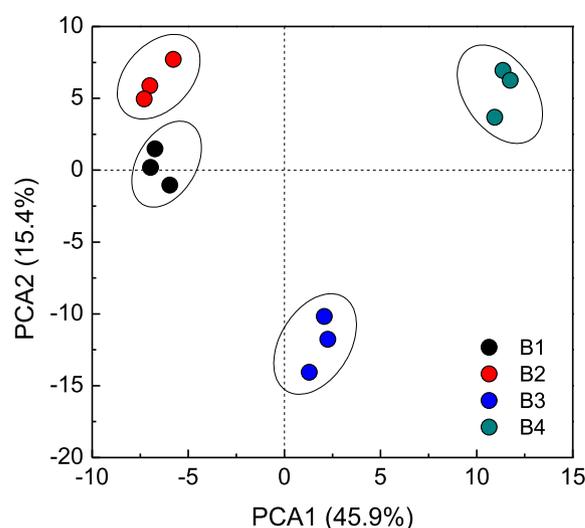


Fig. 1 – Principal component analysis (PCA) of functional genes detected from the four MBRs. Black circles represent B1, Red circles represent B2, Blue circles represent B3 and Green circles represent B4. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

abundances of acetylglucosaminidase, cellobiase and glucoamylase were lowest in B4, but its abundances of endoglucanase, *cdh* and *ara* were the highest. Notably, *amyA*, endochitinase and arabinofuranosidase, which were mainly derived from *Streptomyces*, *Vibrio* and *Bifidobacterium*, were abundant in all of the samples.

Microbial communities might be influenced by nutrient contents in the influent. In order to determine whether and how the functional genes involved in C cycling were related to the influent COD, Mantel test was performed. Mantel test result revealed a significant correlation ($r = 0.5647$, $P = 0.003$) between all of carbon compound degradation genes and influent COD (Table 3). At the individual gene level, *npIT* showed the strongest correlation with influent COD ($r = 0.7224$, $P = 0.001$); exochitinase showed the weakest, albeit still significant, correlation with influent COD ($r = 0.3334$, $P = 0.021$).

3.3.2. Functional genes involved in nitrogen cycling

Removal of nitrogen, mostly in the form of urea, protein and ammonium, from wastewater influent is important for preventing eutrophication (Gallert and Winter, 2005). Our Geochip results indicated that a number of functional genes related to ammonification, nitrification and nitrogen fixation, denitrification, dissimilatory nitrogen reduction and assimilatory nitrate/nitrite reduction were present in MBRs (Fig. 3). For example, the results indicated that *ureC* was the most abundant but *gdh* was the least abundant in B4. Since *ureC* and *gdh* functioned to convert urea into ammonia and vice versa, respectively, the combined effect of *gdh* and *ureC* appeared to shift the balance of urea metabolism toward urea ammonification and hence increased the nitrogen mineralization potential. The most abundant *ureC* genes in B4 were derived

Table 2 – Functional gene uniqueness, overlap and total gene numbers of four MBRs.

	B1	B2	B3	B4
B1	2447 (6.6)	32,809 (85.1)	26,520 (69.6)	16,633 (43.7)
B2		1224 (3.54)	25,480 (69.2)	16,289 (45.1)
B3			374 (1.35)	16,746 (58.0)
B4				384 (2.2)
Gene number	34,897	32,609	25,920	16,427

Notes: Values in parentheses are percentages. Italicized values indicate the number of overlapping genes between samples; bold-face values indicate the number of unique genes in each sample; gene number indicates the detected total numbers in each MBR.

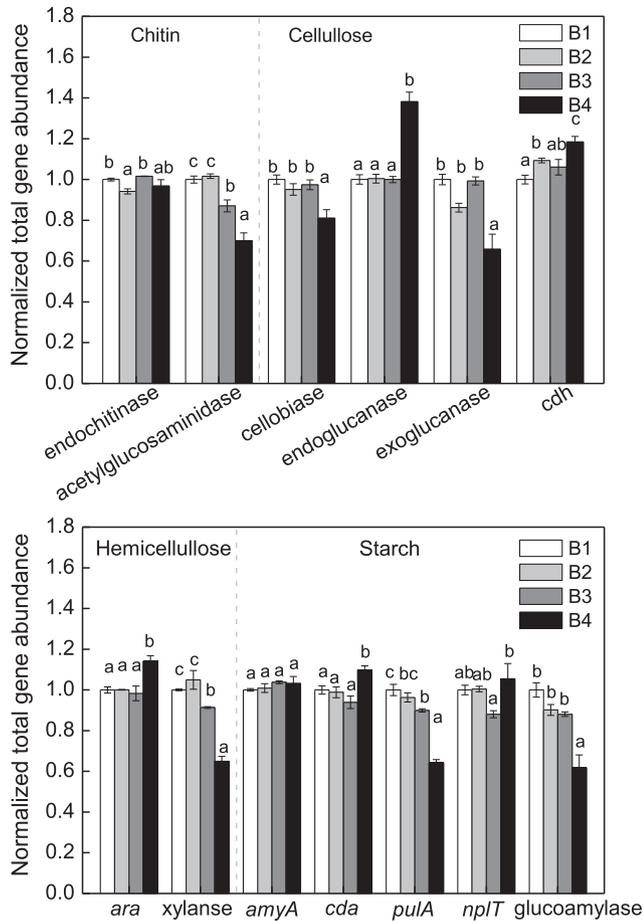


Fig. 2 – The total abundance of genes involved in carbon compound. Functional genes related to chitin, cellulose, hemicelluloses and starch degradation. *cdh*: cellobiose dehydrogenase, *amyA*: amylase, *ara*: arabinofuranosidase, *cda*: cyclomalto-dextrin dextrin-hydrolase, *pulA*: pullulanase, *nplT*: neopullulanase. The data are presented as mean ± s.e. One way anova was performed to assess the significance among four MBR plants, with a, b, and c indicating significant differences at the $P < 0.05$ level.

from *Streptomyces*, *Mycobacterium*, *Methylobacterium* and *Pseudomonas*.

amoA encodes ammonia monooxygenase, which is responsible for a key step of nitrification to oxidize ammonia into nitrite (Wang et al., 2011). The abundance of *amoA* was the highest in B4 but the lowest in B1. However, the ratios of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) were similar among four MBRs (data not shown).

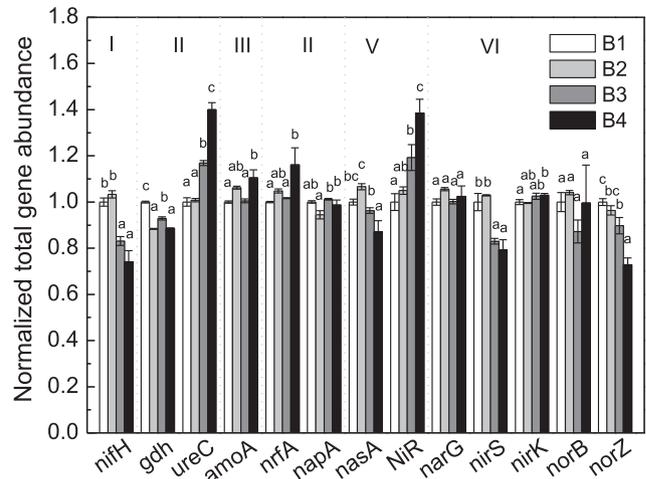


Fig. 3 – The total abundance of genes involved in the nitrogen cycle. These genes are classified into five groups: I: nitrogen fixation, *nifH* encoding nitrogen asereductase; II: ammonification, *gdh* encoding glutamate dehydrogenase, *ureC* encoding urease; III: nitrification, including genes *amoA* encoding ammonia monooxygenase; IV: dissimilatory N reduction (reduction of nitrate to ammonia in two enzymatic steps), including *nfrA* and *napA* encoding nitrite reductase; V: assimilatory nitrate/nitrite reduction, including *nasA* encoding nitrate reductase and *NiR* encoding nitrite reductase; VI: denitrification, including *narG*, *nirS* and *nirK* for nitrite reductase, *norB* for nitric oxide reductase, *nosZ* for nitrous oxide reductase. The data are presented as mean ± s.e. One way anova was performed to assess the significance among four MBR plants, with a, b, and c indicating significant differences at the $P < 0.05$ level.

For denitrification genes, the abundance of *narG* encoding nitrate reductase and *norB* encoding nitric oxide reductase were similar in all of the MBRs, whereas abundances of *nirS* converting nitrite into NO and *nosZ* converting N_2O into N_2 were low in B3 and B4. These results unveiled low functional potentials of denitrification in these two MBRs. The majority of the denitrification genes in MBRs were from uncultured bacteria, signifying the importance to further analyze the denitrification process of MBR systems.

A very strong and significant correlation ($r = 0.7186$, $P = 0.001$) was observed for nitrogen cycling genes and influent ammonium (Table 3). At the individual gene level, *ureC* showed the strongest correlation with influent ammonium ($r = 0.7672$, $P = 0.003$). *hao* showed the weakest correlation with influent ammonium ($r = 0.1513$, $P = 0.108$).

Table 3 – Significant relationships ($P < 0.05$) of selected functional categories of GeoChip data to influent characters through Mantel test.

Gene category	Functional parameters	Statistic (r)	Sig. (P)
Carbon compound degradation	Influent COD	0.5647	0.003
Nitrogen cycling	Influent ammonia	0.7186	0.001
Phosphorus cycling	Influent TP	0.2627	0.042
Sulfur cycling	Influent sulfate	0.3231	0.015

3.3.3. Functional genes involved in phosphorus cycling

Phosphorus is another pollutant contributing to eutrophication. During the municipal wastewater treatment, phosphorous removal is implemented by the uptake and removal of phosphate-accumulating organisms (PAOs) under the anaerobic/oxic alternative conditions. In this study, exopolyphosphatase (*ppx*), polyphosphate kinase (*ppk*) and phytase were detected by GeoChip (Fig. 4). In the four MBRs, abundances of *ppk* and phytase in B3 were significantly ($P < 0.05$) different from the others.

Significant correlation ($r = 0.2627$, $P = 0.042$) was observed for phosphorus cycling genes and influent phosphorus (Table 2). At the individual gene level, *ppx* showed the strongest correlation with influent phosphorus ($r = 0.2967$, $P = 0.015$), but phytase was not significantly correlated with influent phosphorus ($r = 0.0243$, $P = 0.274$).

3.3.4. Functional genes involved in sulfur cycling

Abundances of sulfite reduction genes *dsrA/B* were low in B4 but that of sulfur oxidation gene *sox* was high (Fig. S1), suggesting a shift of sulfur metabolism toward oxidized sulfur in B4 compared to the others. Sulfur oxidation gene *sox* was derived from *Rhodobacteraceae*, *Bradyrhizobiaceae*, *Chlorobiaceae* and *Methylobacteriaceae*. Consistently, abundances of *APS_aprA* and *aprA*, which oxidized sulfite into adenylylsulfate, were also high in B4.

Significant correlation ($r = 0.3232$, $P = 0.015$) was observed for sulfur cycling genes and influent sulfate (Table 3). At the individual gene level, *sox* showed the strongest correlation with influent sulfate ($r = 0.3382$, $P = 0.017$). *APS_aprB* showed the weakest correlation with influent sulfate ($r = 0.2617$, $P = 0.029$).

3.4. Genes present in all of the MBRs

We examined the 15,421 genes present in all of the four MBRs, accounting for 38.6% of total detected genes, since they might represent functional potentials critical for MBR performance. The results indicated that most of them were derived from *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes* of bacteria; *Ascomycota* of eukaryote; and *Crenarchaeota* of Archaea.

The average signal intensity of genes present in all of MBRs was roughly 3.5 times of unique genes (Fig. 5). Notably, the genes present in all of MBRs contained a large number of genes involved in antibiotic resistance, metal resistance and organic remediation (Fig. 6a). These gene categories also had higher percentages in detected genes than the percentages of their probes designed on GeoChip 4.0. These categories included antibiotic resistance (4.1% vs. 2.8%), metal resistance (12.5% vs. 7.8%) and organic remediation (27.0% vs. 14.4%). It was noted that these functional categories were mainly derived from bacteria, such as *Actinobacteria* and *Proteobacteria* (Fig. 6b).

3.5. The linkages between microbial community functional structures and environmental variables

The observations of significant correlation between nutrient cycling genes and influent nutrients provoked us to examine possible linkages between microbial community functional structures and environmental variables. The correlation

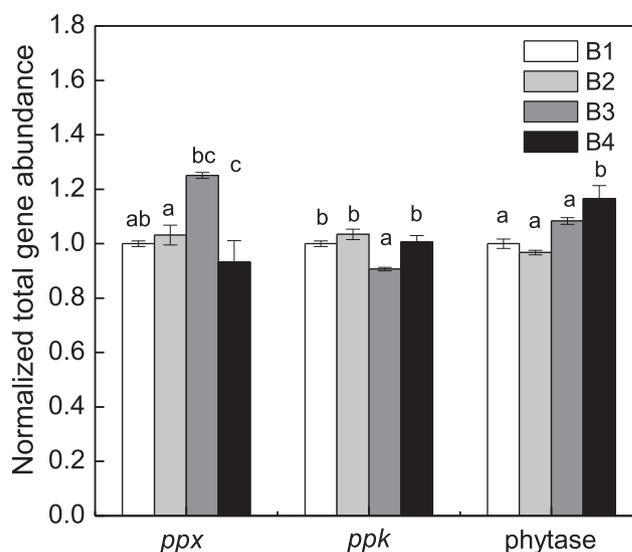


Fig. 4 – The total abundance of genes involved in the phosphorus cycle. The data are presented as mean \pm s.e. One way anova was performed to assess the significance among four MBR plants, with a, b, and c indicating significant differences at the $P < 0.05$ level.

analysis indicated that microbial community was significantly ($P < 0.01$) correlated with operational conditions, influent characters and mixed liquid properties (Fig. 7). To discern the major environmental variables correlated with microbial community, Canonical correspondence analysis (CCA) was carried out. Five variables (HRT, influent COD, influent $\text{NH}_4^+\text{-N}$, temperature and supernatant humic substances) were selected on the basis of variance inflation factor (VIF), which formed a significant ($P = 0.005$) CCA model. Both axes combined explained 76.4% of microbial community

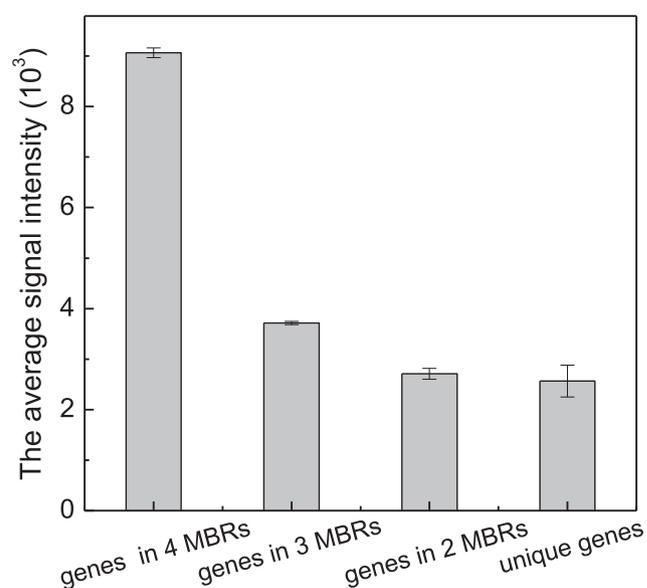


Fig. 5 – The difference in average abundance of genes present in the four MBRs and unique genes. The data are presented as mean \pm s.e.

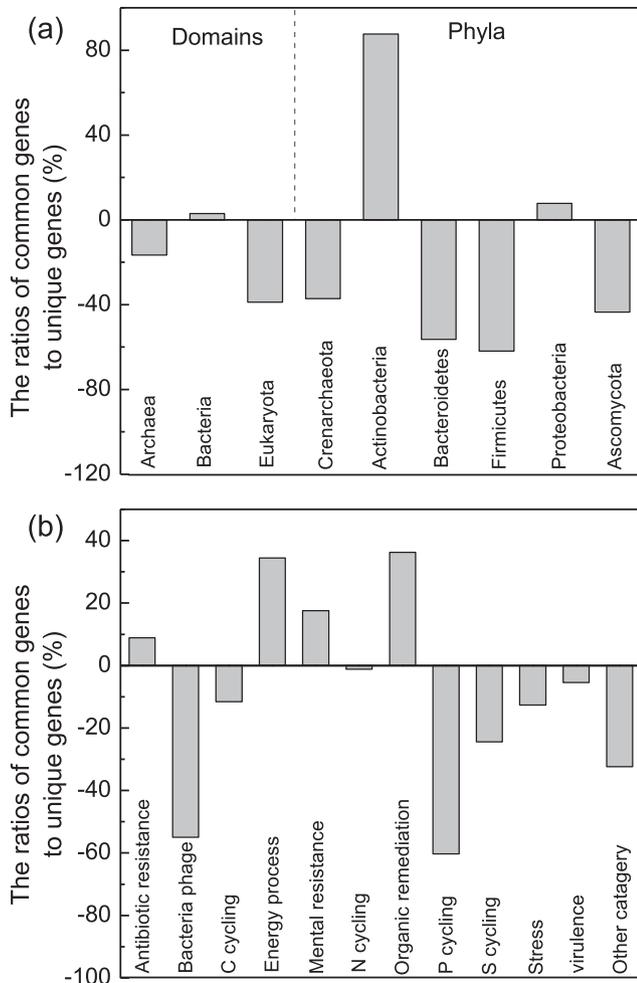


Fig. 6 – The ratios of genes present in the four MBRs to unique genes, (a) indicating the ratios of gene categories, (b) indicating the ratios of microbial phyla.

variations (Fig. 8a), indicating that these five variables were major factors shaping microbial communities. This finding was further verified by Mantel tests as HRT, influent COD and NH_4^+-N showed correlations ($P < 0.05$) with microbial functional structures (Table 4). Similarly, temperature, humic substances, proteins and SVI also demonstrated significant correlations with functional genes (Table 4).

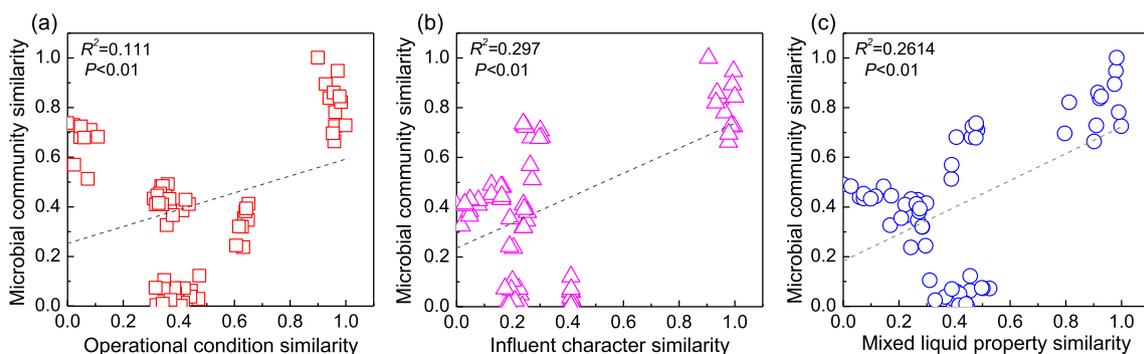


Fig. 7 – The correlation between (a) microbial community and operational conditions, (b) microbial community and influent characters, and (c) microbial community and mixed liquid properties.

Variation partitioning analysis (VPA) was performed to assess the contributions of individual variables. The results indicated 81.5% of the variations in functional microbial community composition and structure could be explained by operational condition HRT (12.3%); influent variables (COD and NH_4^+-N , 16.5%) and mixed liquid properties (temperature and humic substances, 6.2%) (Fig. 8b). The interactive contribution between mixed liquid properties and influent variables were both substantial and significant ($P = 0.005$), indicating that they were major environmental factors shaping microbial communities.

4. Discussion

4.1. Microbial heterogeneity in MBRs

The microbial functional structure and metabolic potentials of large-scale, in-operation MBR plants have been previously examined. In this study, we showed that microbial functional structures were distinct in different MBR plants, and changes of N, C, P, S-cycling, antibiotics resistance, metal resistance, organic remediation genes were in consistency with changes of influent COD, NH_4^+-N , TP, sulfate and toxic compounds expected in wastewater, which generally supports our hypotheses.

The distinct microbial functional structures can partially be attributed to the process variable, as HRT alone contributed to 13.4% of microbial community variations (Fig. 8b). Consistently, operational condition HRT was also found to contribute to the overall differences between the communities in a pilot-scale submerged MBR (Molina-Munoz et al., 2009) and conventional activated sludge bioreactors (Valentin-Vargas et al., 2012). In addition, our results indicated that the influent COD and mixed liquid temperature were also important for shaping microbial communities (Fig. 8 and Table 4), which was supported by a previous finding that pyrosequencing analysis of bacterial diversity in wastewater treatment indicated that influent COD was a major factor to shape microbial compositions (Wang et al., 2012). As shown by PCA (Fig. 1) and higher gene overlap (Table 2), microbial community structures of B1 and B2 were similar, which could be attributed to influent COD and mixed liquid temperature, since both were higher in B1 and B2 than that of B3 and B4 (Table 1). Since influent COD and mixed liquid temperature under MBR

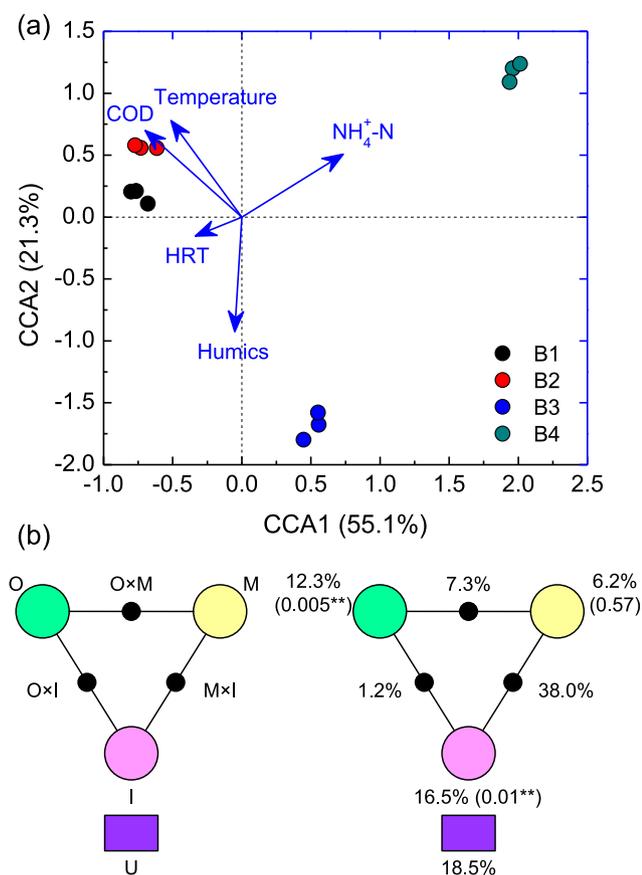


Fig. 8 – (a) Canonical correspondence analysis (CCA) of functional genes and operational conditions and mixed liquid properties. (b) Variation partitioning analysis (VPA) of functional microbial community explained by environmental parameters. The conceptual model and results are shown in the left and right, respectively. Each node represents the indicated variable. The black nodes represent interaction effects between each pair of variables. M (mixed liquor properties): temperature and humics; O (operational conditions): HRT; I (influent characters): influent $\text{NH}_4^+ - \text{N}$ and COD; U: unknown factors. The values in parentheses are *P* values.

environments were favorable to microbial growth, it could result in higher microbial biomass and diversity. Our GeoChip results complemented those studies and suggested that microbial functional profiles, in addition to phylogenetic profiles, were strongly correlated with influent characteristics.

4.2. Linkage between functional genes and wastewater constituents

Previous studies indicated that the availability of C and N in the influent had important impacts on microbial diversity and abundance (Xia et al., 2008). Removals of carbon compound (e.g., COD) and nitrogen (e.g., $\text{NH}_4^+ - \text{N}$) in municipal wastewater are major goals of wastewater treatment. Since carbon and nitrogen are indispensable for microbial growth, microbial communities are influenced by these wastewater characteristics, which are verified by our findings in this study.

Removals of N, C, P and S from wastewater are mediated by microbial communities. The functional gene *amoA* was shown to vary substantially with influent such as municipal, industrial and synthetic wastewater (Pal et al., 2012), which established a linkage between functional genes and these wastewater constituents. Taking advantages of the high-throughput metagenomics tool, here we provide substantially more comprehensive analysis of microbial metabolic genes for their linkages with wastewater treatment. For example, the exceptionally high level of $\text{NH}_4^+ - \text{N}$ in the B4 influent (Table 1) was related with the response of nitrogen-related functional genes. Notably, the abundance of *amoA* was high in B4.

In this study, it was exciting to note that DNA abundances were linked to constituent contents. It is generally accepted that microbial community DNA measures the metabolic potential, but it is less reliable than mRNA in representing functional activity. However, detection of mRNA imposes a number challenges including interference from rRNA and tRNA, strict storage requirement, rapid turnover and severe instability, rendering the profiling and quantification very difficult, if not intractable in many environmental samples. In contrast, DNA profiling and quantification are substantially reliable compared to mRNA. The linkages discovered in this study suggested that the metabolic potential of microbial communities, represented by microbial DNA abundance, can be directly linked to the targeted chemicals of wastewater treatment. However, it is yet unclear whether it is caused by the fact that microbial communities in MBRs has been domesticated and adapted for removal of targeted chemicals, or it is a common phenomenon detectable in other engineered systems or even natural ecosystems.

4.3. Abundance of functional genes and microbes in MBRs

Municipal wastewater is generally enriched with antibiotics, metal and organic pollutants. The residual antibiotics and their metabolites are released from feces or urine of the organism finally into wastewater (Uyaguari et al., 2011). Previous studies have reported that antibiotics in wastewater must play important roles in stimulating antibiotic resistance genes (Uyaguari et al., 2011), rendering wastewater treatment plants as potential reservoirs for antibiotic resistance genes. The MBRs had longer SRT and it might be more profitable to the enrichment of antibiotic resistance genes (Xia et al., 2012). Meanwhile, there were a number of metal resistance genes present in all four MBRs, which might be caused by the presence of metal contaminants in wastewater. Previous studies have found that heavy metals such as iron, chromium and arsenic have impacts on altering microbial communities in different habitats (Wang et al., 2012). In addition to antibiotics and metal, organic pollutants also impose adverse effect on microbial growth (Kelly et al., 2004; Le-Minh et al., 2010). However, organic pollutants can enrich specialized microbial communities for organic degradation. It has been reported that microbial communities were enriched in the deep-sea oil plume during the deep water horizon oil spill in the Gulf of Mexico in 2010 (Lu et al., 2012). Therefore, microbial communities are highly sensitive to antibiotics, metal and organic pollutants, which are verified by our findings in this study.

Table 4 – Relation of whole GeoChip data with operational conditions, influent characters and mixed liquid properties revealed by Mantel test.

Operational condition	Statistic (r)	Sig. (P)	Mixed liquid property	Statistic (r)	Sig. (P)
SRT	0.1745	0.096	Temperature	0.2507	0.049*
HRT	0.2360	0.042*	pH	0.3880	0.014*
MLSS	0.1918	0.077	DO	0.1380	0.122
F/M	0.1481	0.121	Viscosity	0.1305	0.153
Influent characters	Statistic (r)	Sig. (P)	Conductivity	0.2026	0.070
COD	0.5840	0.005**	SVI	0.9018	0.001**
NH ₄ ⁺ –N	0.7467	0.002**	MLVSS/MLSS	0.1027	0.146
TN	0.1748	0.090	TOC	0.0858	0.192
TP	0.2122	0.078	Polysaccharide	0.1247	0.124
SO ₄ ²⁻	0.3087	0.032*	Protein	0.3173	0.016*
			Humics	0.3332	0.012*

Note: Boldface values indicate significant correlation (*P < 0.05; **P < 0.01).

Our GeoChip results indicated that *Proteobacteria* was the most abundant phylogenetic group in MBR systems. Consistently, it was reported that *Proteobacteria* were the most dominant phylum, followed by *Actinobacteria* and *Firmicutes* in the lab-scale MBR (Lim et al., 2012). Another study showed that the abundance of *Sphingobacteria*, *Flavobacteria* and *Proteobacteria* were high in a lab-scale MBR (Falk et al., 2009). Also, *Proteobacteria* and *Bacteroidetes* were abundant in full-scale MBR plants (Hu et al., 2012). Furthermore, *Proteobacteria* are the predominant microbes involved in the removal of carbon, nitrogen and phosphorus compounds (Wagner and Loy, 2002). Accordingly, a number of microbial N, C, P and S-cycling genes derived from *Proteobacteria* were detected by GeoChip. Meanwhile, our study unveiled a number of microbial genes from eukaryotes and archaea in MBRs. Although they were not as diverse as that of bacteria, they might play vital roles during wastewater treatment, which has not been previously explored.

It was intriguing to note that the average signal intensity of genes present in all of MBRs was roughly 3.5 times of unique genes (Fig. 5). While it suggests that common genes presented in all of MBRs tend to be abundant, the low abundance of unique genes raises the possibility that they are part of a “rare biosphere”. To date, rare biosphere is largely unexplored due to the mask by dominant population and limits of current technologies. Although we cannot exclude the possibility that some of its members might act as “keystone” species in ecosystems, most of its members might be dormant, inactive species with the potential to become numerically important when environmental conditions change (Epstein, 2009). The rare biosphere has drawn much attention for its role as a genomic reservoir that contributes to microbial genome novelty and a functional reservoir that helps microbial community adapt for or recover from adverse conditions (Huber et al., 2007).

4.4. The major factors shaping microbial functional structure in MBRs

In the CCA biplot, temperature appeared to be a major factor affecting the microbial functional structure in B1 and B2 (Fig. 8a). Consistently, the importance of temperature for microbial community composition in wastewater treatment systems was also reported in previous studies (Siggins et al., 2011; Wang et al., 2012). In addition, supernatant humic substances were vital for microbial community functional

structure of B3. The high level of humic substances in B3, which was soluble microbial products (SMPs), was indicative of more complicated operational conditions. B3 runs at very low influent load level (Table 1), supplying less nutrients to activated sludge microbes. Also, B3 runs at low temperature due to the outdoor location of membrane tank, which affected SMPs generation as well as microbial functional structures.

5. Conclusions

To the best of our knowledge, this is the first study to profile functional gene compositions and explore major environmental parameters that shape microbial communities in large-scale, operational MBRs. The results showed that functional gene heterogeneity was present in MBRs. Notably, the abundance of genes present in all of the MBRs was much higher than unique genes and functional gene categories involved in antibiotic resistance, metal resistance and organic remediation were abundant. Key functional gene categories were related to influent constituents, signifying their roles in the nutrient removal and MBR performance. Influent COD, NH₄⁺–N, mixed liquid temperature and humic substances were the major factors shaping microbial communities. In summary, the findings of remarkable linkages between microbial metabolic potentials and wastewater constituents were influential, which demonstrated that microbial functional structures can be linked to community metabolism, making it possible to monitor microbe-mediated ecosystem functioning by profiling microbial genes. Future studies will be helpful to further investigate this structure-function relationship and dynamic correlations between microbial functional genes profiles and influent characteristics over time. However, these findings need to be validated by further studies with integrated technologies, since GeoChip provides only a snapshot of the microbial functional profiles.

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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.2014.03.003>

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