Unravelling riverine microbial communities under wastewater treatment plant effluent discharge in large urban areas

Yang Huo^{1,2} · Yaohui Bai¹ · Jiuhui Qu¹

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Abstract In many highly urbanized areas, effluent from wastewater treatment plants (WWTPs) represents a significant proportion of the water source for receiving rivers. Microbial communities are major components of riverine ecosystems and mediate the processes of nutrients and organic matter produced by treated and untreated WWTP effluent. To date, the impacts of WWTP effluent discharge on riverine microbial communities remain poorly understood. Based on 16S rRNA gene sequencing and water quality analysis, we investigated the microbial community compositions and predicted functions in the effluents of five municipal WWTPs and their receiving rivers. The results showed that the microbial compositions in the five WWTP effluents with different treatment processes were similar. Significant differences in the microbial community were not noted between the effluent, upstream, and downstream sites for both sampling months. However, dissimilarity of microbial composition between two sampling periods was observed. The temperature, pH, dissolved oxygen, and ammonium were major environmental factors associated with microbial community changes. Functional annotations of microbial communities based on 16S amplicons identified xenobiotic degradation and

metabolism functions in effluent and river samples. Quantitative polymerase chain reaction revealed the dominance of ammonia-oxidizing bacteria (AOB) over ammonia-oxidizing archaea (AOA) in the WWTP effluents and rivers, and significant positive correlation between AOB abundance and nitrate concentration was observed. These findings will help increase our understanding of the impact of effluent discharge on urban river ecosystems.

Keywords WWTP effluent · Receiving river · Microbial community · High-throughput sequencing

Introduction

Wastewater treatment plants (WWTPs) carry large loads of domestic and industrial wastewater and frequently discharge effluent into open lotic ecosystems. Despite a series of treatment processes, WWTP effluent still contains contaminants such as nutrients and emerging micropollutants (Schwarzenbach et al. 2006). Previous studies have revealed the ecosystem impacts of WWTP effluent, including increased nutrient loading (Waiser et al. 2011), eutrophication (Gucker et al. 2006; Pernet-Coudrier et al. 2012), and community structure disruption of algae, invertebrates, and fish (Bunzel et al. 2013; Dyer et al. 2003; Spanhoff et al. 2007). These detrimental effects have increased public environmental concern and pose threats to both riverine and human health. Evaluating and predicting aquatic responses to WWTP effluent, notably in urbanized watersheds in arid and semi-arid regions where river flows are perennially dominated and dependent on WWTP effluent (Brooks et al. 2006), is a continuing challenge.

As the economic and political center of China, Beijing is a good representative to assess the impacts of WWTP effluent on receiving river ecosystems. Beijing is one of the fastest growing

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✉ Jiuhui Qu
jhqu@rcees.ac.cn

Yang Huo
huo0814@outlook.com

¹ Key Laboratory of Drinking Water Science and Technology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, People's Republic of China

² University of Chinese Academy of Sciences, Beijing 100049, China

municipalities and suffers intense pressure from water scarcity and wastewater discharge. The city's average annual precipitation is only 590 mm, compared with 1090 mm in New York at the same latitude (Probe International Beijing Group 2010). In 2014, Beijing discharged 1.61 billion cubic meters of wastewater, 86.1% of which was treated by 15 central municipal WWTPs (Beijing Water Authority 2014). Five of Beijing's largest WWTPs (Qinghe, Beixiaohe, Jiuxianqiao, Gaobeidian, and Xiaohongmen) treat approximately 2.28 million cubic meters of wastewater daily and account for 90% of Beijing's treated wastewater discharge into the tributaries of the Haihe River System (HRS) (Qi et al. 2015). The HRS connects two megacities in the North China Basin (Beijing and Tianjin), and 95% of the river water from the HRS is diverted for agricultural irrigation.

Ubiquitous and highly abundant microorganisms in streams and rivers can mediate carbon and nutrient cycling (McClain et al. 2003) and organic matter decomposition (Logue et al. 2016; Tank et al. 2010). Although the relationship between WWTP discharge and river ecosystem health has been widely investigated, previous findings have been primarily based on macroinvertebrate bioindicators (Prat et al. 2013; Wright et al. 1995). Research on riverine microbial communities was largely restricted due to conventional cultivation-dependent techniques. In recent years, high-throughput 16S rRNA gene sequencing has become a widely employed tool for assessing composition (Sergeant et al. 2012) and functional profiles (Arndt et al. 2012; Asshauer et al. 2015; Langille et al. 2013) of microbial communities. Several studies have explored the effect of WWTP effluent on the composition and function of microbial communities in the receiving river (Atashgahi et al. 2015; Drury et al. 2013; Marti and Balcazar 2014; Wakelin et al. 2008). However, more research are still needed especially for riverine ecosystem in large urban area, which is much influenced by WWTP effluent treated by various techniques. In this study, we attempted to address several issues, including (1) whether microbial community composition in WWTP effluent varies by different treatment techniques; (2) whether WWTP effluent microbial communities are significantly different from river communities; and (3) which environmental factor(s) is/are strong driving force(s) for changes in WWTP and riverine microbial communities. We applied 16S rRNA gene-based sequencing for the identification of microbial communities in effluent from the five largest WWTPs of Beijing and their receiving rivers. The structural and functional profiles of the microbial communities in these sites were analyzed using a series of bioinformatics tools.

Materials and methods

Sampling of the WWTP effluents and receiving rivers

The waterways of the receiving rivers and sampling sites in this study are shown in Fig. S1. All these rivers discharge into

the Wenyu River, upper reach of the HRS. The characteristics of the five WWTPs and the receiving rivers are described in Table S1. The geopositions of the sampling sites are shown in Table S2. Sampling campaigns were performed in the end of April and November 2015 without recent rainfall. For each WWTP and its receiving river, we selected five sampling sites: the effluent, two sites upstream of the effluent outfall, and two sites located downstream of the discharging point (E, U1, U2, D1, and D2). The distance of each sampling site was at least 150 m for adequate mixing of effluent and river water. For each site at each sampling time, a 10-L surface water sample was collected using sterile containers. Temperature and pH were measured in the field using an ULTRAMETER II™ 6PFC^E (MYRON L Company, Carlsbad, CA, USA). Dissolved oxygen (DO) was measured by a HQ30d portable meter with an LDO101 probe (HACH, Loveland, CO, USA). The collected samples were immediately placed at 4 °C in the dark for further processing. Selected physicochemical parameters, including total phosphorus and nitrogen (TP, TN), soluble phosphate (PO₄), ammonium and nitrate (NH₃-N, NO₃-N), total organic carbon (TOC), and chemical oxygen demand (COD) were analyzed using methods provided by the State Environmental Protection Administration of China (See Table S3 for details).

DNA extraction and 16S rRNA gene sequencing

Prior to DNA extraction, each water sample was filtered using a 0.45-μm pore size cellulose nitrate membrane on a vacuum pump. Total environmental DNA was extracted from the membranes in triplicate using a PowerWater DNA isolation kit (Mo-Bio Laboratories, Carlsbad, CA, USA). Successful extraction was confirmed by agarose gel electrophoresis. Prepared DNA was stored at −80 °C until further analysis. The extracted DNA samples were used for 16S rRNA gene sequencing. The V4 region of the 16S rRNA gene was amplified using primers 515F (5'-GTGCCAGCMGCCGCGGT AA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') with dual index and Illumina adapters for polymerase chain reaction (PCR). Targeted PCR amplicons were sequenced on a MiSeq platform (Illumina, Carlsbad, CA, USA), which produced 2 × 250 bp paired-end reads. The raw sequence data can be accessed from the NCBI Sequence Read Archive (SRA) with accession number [SRA:SRP068719].

Bioinformatics analysis for community composition

The Illumina paired-end sequences were processed using the UPARSE + QIIME pipeline (Pyro et al. 2014). In brief, forward and reverse Illumina reads were merged with QIIME v1.9.0 (Caporaso et al. 2010). USEARCH was used for dereplication and filtering of noisy sequences and chimeras (Edgar 2010). The operational taxonomic unit (OTU)

clustering was performed by the UPARSE method (Edgar 2013) and then assigned to taxonomy using *uclust* with a 97% sequence identity threshold. Representative sequences were aligned against the Greengenes database May 2013 release (DeSantis et al. 2006). Alpha diversity was calculated using the Chao 1 (Chao 1987) and Shannon diversity indices. Unweighted-Unifrac beta diversity was determined using QIIME and analyzed by non-metric multidimensional scaling (NMDS). The relationship between environmental variables and log-transformed OTU abundances was analyzed through redundancy analysis (RDA), and their pairwise Pearson's *r* correlations were calculated using QIIME.

Taxonomy-based functional prediction of microbial communities

To determine the putative functional profiles of the microbial communities based on 16S rRNA gene data, we performed taxonomic-to-phenotypic mapping by *METAGENassist*, a web-based tool for comparative metagenomic analysis (Arndt et al. 2012). To further investigate the organic pollutant degradation and metabolism profiles in the five zones, we applied *PICRUSt* (Langille et al. 2013), another 16S rRNA gene functional prediction tool. Functional predictions were aligned in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and the functional parameters were assigned to Tier 3. The predicted results were then analyzed with *STAMP* software v2.1.3 (Parks et al. 2014) and plotted using *heatmap* package in R (version 3.2.3). Functional analyses were performed following the corresponding online tutorials (*METAGENassist*: <http://www.metagenassist.ca/METAGENassist/faces/>

Docs/Tutorial.jsp; *PICRUSt*: http://picrust.github.io/picrust/tutorials/metagenome_prediction.html#metagenome-prediction-tutorial).

Quantification of ammonia-oxidizing microbes by real-time PCR

The quantitative distributions of bacteria, archaea, ammonia-oxidizing bacteria (AOB), and ammonia-oxidizing archaea (AOA) in the river, and WWTP effluents were determined by real-time PCR. Bacterial and archaeal 16S rRNA or *amoA* genes were quantified by the LightCycler96 real-time PCR system (Roche, Switzerland) using the SYBR Green I method (Noble and Fuhrman 1998). Information on the primer pairs and thermal programs are provided in Table S5. Standard DNA plasmids for absolute quantification were prepared as previously established (Bai et al. 2012). The real-time PCR reaction mixture (20 μ L) contained 1 μ L of template DNA, 0.4 μ L of each primer, 10 μ L of 2 $\times$ FastStart SYBR Green Master (Roche, Switzerland), and 8.2 μ L of dH₂O.

Each sample was performed in triplicate. The qPCR efficiencies ranged from 93 to 108%, and the linear regression of amplification efficiency (R^2) was all over 0.99. The relationship between log-transformed *amoA* gene abundance and concentrations of NH₃-N and NO₃-N was analyzed. The Glejser test was performed to detect data heteroscedasticity (Glejser 1969). A Spearman's correlation was also calculated to identify the linear relationship between AOB abundance and concentration of NO₃-N.

Results

Physicochemical properties of WWTP effluent and river samples

The physicochemical properties of the water samples are summarized in Table S3. The water temperature ranged from 18.3 to 24.0 °C in April and from 3.7 to 14.5 °C in November. The pH varied between 7.16 and 8.54. The concentrations of NH₃-N, TP, and TN revealed high nutrient levels at most sampling sites compared with those of the Chinese Environmental Quality Standards for Surface Water (GB 3838-2002). To investigate the impact of effluent and sampling time on river water quality, a two-way analysis of variance (ANOVA) was performed. The results showed that the effluents did not significantly alter the nutrient level of the receiving rivers although concentrations of NO₃-N ($P < 0.01$), TN ($P < 0.01$), and TP ($P < 0.01$) in November samples significantly increased and concentrations of TOC in November samples significantly decreased ($P < 0.01$). These findings suggest that WWTP effluent did not induce significant changes between upstream and downstream, which might be due to the upstream sites being dominated by previously discharged WWTP effluent.

Alpha and beta diversity of the microbial communities

Using Illumina paired-end 16S rRNA gene sequencing, a total of 1,814,055 raw sequences were produced, including 1,703,308 high-quality reads with an average merged length of 253 bp after processing. The sequences were clustered into a total of 8383 OTUs at the 97% similarity level, with a Good's library coverage from 97.7–99.3%. Rarefaction curves revealed that bacterial diversity almost reached a saturated sequencing depth (Fig. S2). Sequence libraries for the sites in April ranged between 446 and 1477 OTUs, and in November ranged between 557 and 1929 OTUs. The rarefaction curve, Chao 1 value, and Shannon index were calculated as metrics for species richness and diversity (Fig. S2). The lowest and highest Chao 1 values were found downstream of BXH and upstream of JXQ, respectively, indicating distinct diversity between these sites in regard to microbial

community richness. Beta diversity was visualized via NMDS (Fig. 1). The NMDS graph showed clear and significant differences in bacterial community composition between the two sampling periods for most of the riverine samples (ANOSIM, $P < 0.01$). However, no consistent significant differences between the upstream and downstream sites in both seasons were observed at most sampling sites, revealing that effluent did not significantly change in microbial compositions. Only the bacterial community composition at downstream of Xiaohongmen was more similar to its effluent in both seasons, suggesting that bacterial community composition of Xiaohongmen was influenced by its effluent. Additionally, most effluent samples were closely clustered in both seasons at Gaobeidian, Jiuxianqiao, and Qinghe, indicating little composition variability in the microbial community among these effluent sites (Fig. 1).

Microbial community composition and functional characterization

From the OTU assignment, a total of 57 phyla, 155 classes, and 924 genera were identified across all samples. The dominant taxa based on classes with more than 1% relative abundance are summarized in Fig. 2. *Proteobacteria* (41–82%) and *Bacteroidetes* (7–39%) were the most abundant phyla in almost all samples. Within *Proteobacteria*, *Betaproteobacteria* dominated in most April and most November samples (20–55%). *Epsilonproteobacteria* and *Gammaproteobacteria* were more abundant in November samples (Fig. 2). In

Bacteroidetes, *Flavobacteria*, and *Bacteroidia* dominated at most sampling sites. These results indicate that effluent discharge did not alter the microbial community composition between upstream and downstream. In addition, 21 OTUs were identified as core microbial taxa present across all samples (Table S4). *Comamonadaceae* was the largest family found in the *Betaproteobacteria* class and contributed more than a quarter of the total core community. The genera *Arcobacter* and *Flavobacterium* were also identified as abundant taxa in the core community.

Putative changes in microbial community function based on composition were predicted by *METAGENassist* (Fig. 3). Two functions that are assigned as a characteristic of the greatest number of 16S rRNA sequences, on average, were annotated as ammonia oxidizers (12,598 sequences) and sulfur oxidizers (9653 sequences), indicating possible involvement of ammonia and sulfur oxidizer microbes in the samples. For pollutant metabolism and degradation profiles, a high number of 16S rRNA sequences were assigned to degradation of aromatic hydrocarbons and xylan (2787 and 3406 sequences on average, respectively). Atrazine degradation, chlorophenol degradation, and naphthalene degradation were also identified in river and effluent samples. Functional properties with xenobiotic degradation and metabolism (XDM) were further investigated by *PICRUSt* assigned up to level 3 in the KEGG database (Fig. S3). We identified 8 out of 20 XDM features with statistical significance, including bisphenol degradation ($P < 0.01$) and polycyclic aromatic hydrocarbon degradation ($P = 0.02$). A high percentage of

Fig. 1 Non-metric multidimensional scaling (NMDS) biplot showing the beta diversity among five WWTP effluents and their receiving rivers. Samples collected in April and November are in light blue and orange, respectively. Sampling sites are connected with lines of different dash types according to their locations and displayed in different shapes (upper triangle: upstream sites; lower triangle: downstream sites; circle: effluent sites)

Fig. 2 Relative abundances of microbial communities (classes) among the five sampled effluents and rivers in April and November. Taxa that accounted for less than 0.1% of the relative abundance or present in less

than three samples were omitted from the chart. Text colors in the legend represent different phyla

sequences were assigned to caprolactam degradation and chloroalkane and chloroalkene degradation. These findings reveal that microbes in the effluents and rivers were possibly involved in the degradation and metabolism of a variety of organic pollutants. However, neither seasonal nor regional associations were found among these functional profiles.

Relationship between environmental variables and microbial communities

RDA was performed to gain an overview on the associations between physicochemical water characteristics and microbial community composition (Fig. 4). Forward selection tests indicated that temperature ($P < 0.01$), pH ($P < 0.01$), DO ($P < 0.01$), and $\text{NH}_3\text{-N}$ ($P < 0.01$) were the four main

environmental variables (total percentage of contribution = 71.7%) that accounted for changes in the bacterial communities with statistical significance. Ten bacterial classes that best fit the RDA were analyzed. *Alphaproteobacteria* and *Betaproteobacteria* were positively associated with pH and temperature, whereas *Gammaproteobacteria*, *Epsilonproteobacteria*, and *Fusobacteriia* were affected by variability of nutrients. Relationships between environmental factors and other bacterial classes were investigated by calculating a Pearson's r correlation (Fig. S4). *Acidimicrobiia*, *Anaerolineae*, and *Pedosphaerae* were positively associated with pH ($r = 0.37$, $P < 0.01$; $r = 0.39$, $P = 0.01$; $r = 0.34$, $P = 0.02$, respectively). *Alphaproteobacteria* and *Chloroplast* were negatively correlated with $\text{NO}_3\text{-N}$ ($r = -0.28$, $P = 0.04$ and $r = -0.27$, $P = 0.05$, respectively). Negative correlations

sum, which adjusted for varying sequencing coverage among samples by normalizing to the same total abundance for each sample. Data were then analyzed for “metabolism by phenotype” and Euclidean distance measures and average clustering algorithms were used. Changes are visualized on a relative scale, with enrichment in *red* and depletion in *blue*

Fig. 4 Redundancy analysis (RDA) biplot of bacterial communities at the class level in association with environmental variables. The ten classes that best fit the analysis are displayed

were also found between *Bacilli* and TOC ($r = -0.29$, $P = 0.04$) and between *Cytophagia* and DO ($r = -0.25$, $P = 0.08$), but with lower statistical significance.

Quantitative analysis of 16S rRNA and *amoA* genes

The abundances of archaeal 16S rRNA, bacterial 16S rRNA, and *amoA* genes in different water samples are shown in Fig. S5. The copy number of the bacterial 16S rRNA gene

was higher than that of the archaeal 16S rRNA gene in almost all samples. The *amoA* results revealed the dominance of AOB rather than AOA in all environmental samples, with the AOB/AOA ratios ranging from 8.07 to 184.44. The *amoA*/16S rRNA ratio in the five WWTP effluents and rivers ranged from 10^{-1} to 10^{-4} . A non-linear relationship between ammonium concentration and AOB abundance was found, whereas there seemed a possibly downward trend of AOB with increases of ammonium (Fig. 5a). A significant positive

Fig. 5 Scatter plots illustrating the relationship between copy number of ammonia-oxidizing microbes and concentration of ammonium/nitrate, including AOB and ammonium (a), AOB and nitrate (b), AOB/AOA and ammonium (c), and AOB/AOA and nitrate (d)

correlation between AOB and nitrate concentration was identified (Fig. 5b). The abundance ratio of AOB to AOA showed almost no relationship with ammonium and nitrate concentrations (Fig. 5c, d). These results demonstrated that AOB were the dominant ammonia-oxidizing microbes in all effluents and river samples. The abundance of AOB was associated with levels of nitrate in the effluents and rivers although the dominance over AOA did not change with the concentrations of both ammonium and nitrate.

Discussion

The objective of this study was to understand the impact of WWTP discharge on the composition and function of urban riverine microbial communities. Previous studies have reported both an increase (Goni-Urriza et al. 1999; Wakelin et al. 2008) and decrease (Drury et al. 2013) in downstream riverine microbial diversity with the impact of WWTP effluent. In our study, however, we did not observe significant differences in the microbial community from almost all upstream to downstream sites of these receiving rivers impacted by WWTPs in both sampling seasons. We considered that this similarity is mainly because of the receiving rivers being dominant by discharged WWTP effluent. First, as Beijing has encountered long-term water scarcity notably during the recent couple of decades, effluent from WWTPs rather than rainfall has been the major water source of these rivers in the urban area; second, the average velocity of effluent discharge is over 7.5 times higher than the velocity of the receiving river, which could bring effluent flow into these upstream sites. Furthermore, potential sources listed in Fig. S1 may contribute pollutants such as nutrients to the river and could cause similar water quality along the sampling sites. Through the NMDS analysis, we also found that the microbial community composition in the majority of the effluents falls in a similar range, indicating that the microbial community shifts along the upstream and the downstream is likely not caused by treatment technology (Fig. 1). Member profiling of the microbial communities revealed dominant abundance of *Proteobacteria*, except for the *Comamonadaceae* family that belonged to the *Proteobacteria* that has been frequently reported in river ecosystems (Cai et al. 2016; Crump et al. 2009). The genus *Arcobacter*, another member of the *Proteobacteria*, has been reported as potential human pathogens (Fisher et al. 2014). *Bacteroidetes* in freshwater ecosystems are known to degrade organic compounds with high molecular weight (Thomas et al. 2011). Therefore, the higher abundance of *Bacteroidetes* indicates the result of complex organic compounds with higher concentration in the sampling sites (Drury et al. 2013). For the upstream of Xiaohongmen in April, *Chloroplast* was richer because of the large nutrient load at that time. *Chloroplast* belonged to the *Cyanobacteria*

phylum and *Cyanobacteria* proliferation is mainly associated with water body eutrophication (Riedinger-Whitmore et al. 2005). These results revealed that the effluent-dominated rivers with heavy pollution negatively affect the safety of river in a microbial ecological context.

Through the workflow of *METAGENassist* and *PICRUSt*, we annotated the microbial functional features for the WWTP effluent and river samples. Notably, several xenobiotic degradation functions were identified in the effluent and river samples, as also reported in WWTP effluent and river samples using shotgun metagenome sequencing (Bai et al. 2014). In this study, it is still unclear to what extent the abundance of 16S sequences that likely encoding these functional genes shifted between effluents and samples with different concentrations of toxicants, or whether these related genes in the effluent were still expressed once they migrated to the rivers. One recent study found that functional genes associated with xenobiotic, carbohydrate, and amino acid metabolism were overrepresented in upstream and deep downstream river sediments. By contrast, wastewater discharge in shallow downstream sediments resulted in an increase in genes associated with nitrogen, sulfur, purine, and pyrimidine metabolism (Li et al. 2016). Another recent study showed that toxic chemicals increased the microbial metabolic enzymes and pathways, and polycyclic aromatic hydrocarbons and nitrobenzene were negatively correlated with bacterial community variation (Lu et al. 2016). However, further investigations are needed to understand the flow and impact of these functional genes on the relationship between wastewater discharge and receiving rivers.

In this study, bacterial and archaeal *amoA* genes were detected using real-time PCR. Ammonia oxidation by functional AOA and AOB is the key rate-limiting step of the nitrogen cycle and therefore plays an important role in riverine ecosystem function. Previous research has demonstrated that the biotransformation kinetics of pollutants during WWTP treatment are associated with the activity of the *amoA* gene, rather than WWTP process parameters (Helbling et al. 2012). The larger copy number of AOB *amoA* genes in both WWTP effluent and river water samples demonstrated the dominance of AOB in ammonia oxidation, in accordance with recent research (Lee et al. 2016). Earlier studies also revealed higher abundances of AOA in river ecosystems (Zhang et al. 2015), and the dominance of AOB was found in WWTP effluent (Gao et al. 2014; Sonthiphand et al. 2013) and rivers (Mosier and Francis 2008; Santoro et al. 2008; Wankel et al. 2011). Here, correlation analysis demonstrated significant associations between AOB and nitrate concentration. The greater number of AOB *amoA* gene copies over that of AOA suggests that AOB are a major contributor to nitrification (Magalhães et al. 2009).

This study provided structural and functional insights into the microbial communities of urban river ecosystems experiencing heavy anthropogenic stresses. With the rapid

development of high-throughput sequencing technologies and increasing advances in the Earth microbiome project, further exploration of microbial communities will continue to improve our understanding of microbial responses and processes to a wide range of pollutants in water bodies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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