Ammonium removal by native microbes and activated sludge within the Jialu River basin and the associated microbial community structures

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ABSTRACT

To explore the availability of native microbes and activated sludge for ammonium removal, the native microbes and activated sludge in Jialu River basin were investigated in terms of ammonium-removing activities and their microbial communities using spectrophotometry and high-throughput sequencing. NH$_4^+$-N and total nitrogen (TN) in the targeted river ranged from 2.45 ± 1.76 to 8.56 ± 2.54 mg/L and from 3.42 ± 2.79 to 13.49 ± 5.06 mg/L, respectively. Both the native microbes and activated sludge had strong ammonium-removing activities with the removal efficiencies of more than 94%. High-throughput sequencing results indicated that, after five batches of operation, the class Gammaproteobacteria (28.55%), Alphaproteobacteria (14.55%), Betaproteobacteria (13.89%), Acidobacteria (8.82%) and Bacilli (7.04%) were dominated in native community, and there was a predominance of Gammaproteobacteria (21.57%), Betaproteobacteria (16.33%), Acidobacteria (12.41%), Alphaproteobacteria (10.01%), Sphingobacteriia (6.92%) and Bacilli (6.66%) in activated sludge. These two microbial sources were able to remove ammonium, while activated sludge was more cost-effective.

INTRODUCTION

Serious river contamination and ecosystem degradation have taken place alongside rapid industrialization, urbanization and population growth, especially in developing countries (Qin et al. 2014; Sorensen et al. 2015). River water is easily polluted by domestic wastewater effluent, industrial wastewater, rainwater runoff, agricultural runoff, even sewage is one of the main components of municipal river water in semi-arid and arid regions. Nitrogen compounds, especially ammonium (NH$_4^+$), are the most widespread. The concentrations of NH$_4^+$-N in polluted rivers generally range from 1 to 10 mg/L (Li et al. 2015; Tang et al. 2015; Zhang et al. 2015). These ammonium concentrations are often higher than standards of surface water, like grade III standard in China (i.e. National Surface Water Quality Standard GB3838-2002, [NH$_4^+$] ≤ 1 mg/L) and grade III standard in Europe (Surface Water Quality Standard for Europe, [NH$_4^+$] ≤ 1.2 mg/L).

Although the process of anaerobic ammonium oxidation (anamox) possesses excellent removing loading and rate for ammonium (Wang et al. 2016), it is believed that the traditional two-step method (autotrophic nitrification and heterotrophic denitrification) dominates in natural river for nitrogen removal (Coban et al. 2015). To date, many applications have been employed to purify river water, like floating island, bio-filtering dam and constructed wetland. Essentially, the functional components in these systems are the microorganisms (biofilms) attached to the used carriers (Feng et al. 2012; Xu et al. 2012; Zhao et al. 2012; Gu et al. 2015; Lin et al. 2015; Ijaz et al. 2016; Zhong et al. 2016). Thereby, as a potential microbial resource, it is necessary to examine the cleaning ability of native bacteria before being applied in the treatment of polluted river water.

Generally, microbes in artificially enhanced activated sludge processes are long-term acclimated to wastewater and have strong capacity for pollutant elimination. Given that contaminants in drainage rivers mainly originate from domestic wastewater effluent (Sheng et al. 2013), activated sludge may be a good microbial resource to purify polluted...
rivers. Because of the obvious differences of nutrient concentrations between river water and sewage, this study focuses on if activated sludge can adapt to the low-nutrient condition in river, and if the microbial community evolves with the changes of feeding conditions.

In this work, the Jialu River is taken as an example to elucidate the above questions regarding native microbes and activated sludge. With respect to the Jialu River, the status of persistent organic pollutants (POPs) and heavy metals have been well estimated (Fu et al. 2017; Fu et al. 2014). However, there is little knowledge involving microbial community structure and diversity is little. The objectives of this study are: (1) to determine the feasibility of the enrichment of native microorganisms and their capacity for nitrogen removal, and to identify the related community structure; (2) to determine the adaptability of activated sludge in the regional wastewater treatment plant (WWTP) to the polluted river water, and to identify the related community structure; and (3) to finally find out the suitable microbial resource for river contamination treatment. The removing abilities and community structures of the microbial sources were estimated by spectrophotometry and high-throughput sequencing, respectively.

MATERIALS AND METHODS

Study site and inoculum source

The Jialu River, about 276 km length, is located at North China plain in Henan Province, belonging to the Huai River basin. In this area, Zhengzhou city, one of the most important industrial cities in Central China, has a long history of textile and metallurgy industries and is also a major transportation hub, which has a strong impact on the surrounding aquatic environment (Zhang et al. 2011; Ma et al. 2012a). As a result, the Jialu River, as a drainage river in this region, has been easily polluted along with a rapid economic growth and urbanization.

The sampling for native microorganisms and activated sludge took place from January to August 2014. The sampling sites in the river were numbered 1–9 in order from upstream to downstream and were chosen based on the distributions of typical pollution sources, such as discharge points of WWTPs and pile sites of solid waste. Activated sludge was harvested from Matougang WWTP with anaerobic–anoxic–oxic (A²/O) process for biological N/P removal (Zhengzhou, China). All the samples, including water samples and microbial samples, were analyzed as soon as possible, or stored in −20 °C freezer.

Operation and chemical analysis

Considering there was little biomass in the river water, native microorganisms for ammonium removal were enriched using culture medium prior to treating real river water. The medium (pH = 7.2) contained (per L of sterilized water): (NH₄)₂SO₄ 0.2 g (equal to 42 mg NH₄-N /L), NaCl 0.2 g, FeSO₄·7H₂O 0.04 g, K₂HPO₄ 0.1 g, MgSO₄·7H₂O 0.05 g, CaCO₃ 1 g. Specifically, native microorganisms were harvested from 250 mL of river water by filtering through 0.22 μm membrane, and then transferred into flasks containing 100 mL of culture medium for propagation. The enrichment experiments were operated for at least two cycles. When ammonium concentration decreased <5 mg/L, one typical cycle finished and the medium was replaced. After the enrichment, 5% by volume of the enriched liquid was inoculated into the flasks containing 100 mL of real river water to test its ammonium-removing activity. The flasks were placed in the shaker at 150 rpm.

Because of the appropriate concentration and activity of biomass in the WWTP, activated sludge was directly added into real river water for adaptability test. The real river water without any amendment acted as control and all the testing experiments for pollutant removal were operated in batch mode at least twice. The concentrations of chemical oxygen demand (COD) and nitrogen compounds were measured according to standard methods (EPA of China 2002). Samples were filtered through a 0.45 μm membrane before analysis.

Microbial community and diversity analysis based on high-throughput sequencing

The compositions of the microbial communities, including the final enriched native microorganisms, the original activated sludge and their respective evolving microorganisms after treating real river water, were determined by 16S rDNA polymerase chain reaction (PCR) and sequencing as described in detail previously (Shu et al. 2015). Briefly, DNA of the collected bio-samples was extracted using the E.Z.N.A.Soil DNA Kit for Soil (Omega, USA), following manufacturer’s instructions. The hypervariable V3–V4 regions of bacterial 16S rDNA were amplified with the following universal primers: 341F (50-adaptor B-Barcode-CCT ACG GGN GGC AGC AG-30) and 805R (50-adaptor B-Barcode-GAC TAC HVG GGT ATC TAA GCC-30).

After amplification, all PCR products were purified using AMPure Beads (Beckman Coulter, USA). The
concentrations of purified products were then measured using PicoGreen dsDNA Assay Kit (Thermo Scientific, USA) on the TBS-380 Fluorometer (Turner Biosystems, USA) and the purified products were mixed in equal mol. The equal amount of amplicons were used for pyrosequencing on an Illumina MiSeq platform according to standard protocols by a commercial service (Sangon Biological Engineering Co., China).

Following high-throughput sequencing, all raw reads were initially merged using FLASH (version 1.2.7) and then processed using QIIME standard pipeline (version 1.17). Low quality reads, adaptors, barcodes and primers were trimmed off. After the quality control, the remaining high quality sequences were clustered into operational taxonomic units (OTUs) using UCLUST (version 5.2.32) with high quality sequences were clustered into operational taxonomic units (OTUs) using UCLUST (version 5.2.32) with setting 0.03 distance limit. Taxonomic classification of the bacterial sequences was conducted using the RDP Classifier with minimum confidence of 80% (Ye et al. 2011). From the cluster file, alpha diversity statistics including Chao1 richness estimator, Shannon diversity index, Good’s coverage, and Simpson diversity index were calculated in Mothur (version 1.35.1) for each biomass sample.

RESULTS AND DISCUSSION

Characteristics of water quality in the Jialu River

Changes of microbial community structure are closely related to water quality and seasonal temperature (Boulêtreau et al. 2014; Tinta et al. 2015). Surface water samples along the Jialu River (9 sites) were obtained and the pollution status was averaged on the dry and wet seasons (Figure 1). From upstream to downstream, NH$_4^+$-N increased from 2.45 ± 1.76 to 8.56 ± 2.54 mg/L and total nitrogen (TN) from 3.42 ± 2.79 to 13.49 ± 5.06 mg/L, respectively; the concentration of COD averaged 33.79 ± 16.50 mg/L (data not shown), far higher than the grade III standard of surface water (i.e. National Surface Water Quality Standard GB3838-2002, China, NH$_3$-N < 1.0 mg/L, TN < 1.0 mg/L, COD < 20 mg/L). The pollution status in the Jialu River was similar to the case of Taihu, China (Zhao et al. 2015). Some mutagenic and carcinogenic compounds like nitrosamines, bisphenol A and heavy metals were also present in the Jialu River (Zhang et al. 2009, 2011; Fu et al. 2011; Ma et al. 2012a), and even affected groundwater in this region (Ma et al. 2012b; Yang et al. 2012). The water quality downstream was obviously more deteriorative than that upstream, implying that the self-cleaning ability of the Jialu River was weakened or was lost, causing pollutants to accumulate along the river. In the Bibío River, Chile, and Oder River, Europe, nitrogen pollutants also accumulated along the rivers due to the attenuation of their self-cleaning abilities (Karrasch et al. 2006; Absalon & Matysik 2007).

In addition, the Jialu River acted as the main drainage river in the urban area (Sheng et al. 2015), and effluent of WWTPs with low biodegradability was one of the important water sources for the Jialu River. It is therefore necessary to estimate the availability of native bacteria for nitrogen removal and the adaptability of activated sludge to low-nutrient condition (river), with the aim of finding out an appropriate microbial resource for river pollution treatment.

Treatment of polluted river water by native microorganisms

After the enrichment of native microorganisms with the culture medium, 5% by volume of the enriched liquid was inoculated into the flasks to examine the treatment efficiencies for real river water. Same volume of real river water without sterilization as control was run under the same condition. The results are shown in Figure 2. In each batch, ammonium in the river water (control) somewhat decreased, which indicated that the native microorganisms in the river water had the ability to degrade ammonium, despite less removal efficiency than the enriched groups.

In the first two cycles of experimental groups, the initial ammonium concentrations were 9.08 ± 0.41 mg/L and 5.74 ± 1.00 mg/L. After 4 or 5 days, they decreased to 0.08 ± 0.17 mg/L and 2.28 ± 0.97 mg/L, giving removal efficiencies of 99.00 ± 0.02% and 58.00 ± 2.00%, respectively. NH$_4^+$-N removal efficiency in the second cycle was much lower than the first cycle, and even less than the control. This phenomenon suggested that the inoculated
microorganisms had an adaptable period. In fact, adaptable/ lag period is ubiquitous in various microbial systems (Kroukamp et al. 2010). In the cycle III, IV, and V, ammonium decreased from around 14 mg/L to less than 1 mg/L and all the removal efficiencies reached more than 94% (94.67 ± 2.71%, 99.74 ± 0.45% and 95.33 ± 5.50%, respectively), presenting good ammonium-removing capacity. Nitrite accumulated up to about 2 mg/L in each cycle, and eventually was converted into nitrate (12–16 mg/L) by nitrite-oxidizing bacteria within the microbial community under aerobic condition, which was consistent with other studies (Zhang et al. 2016).

Enriched native bacteria as the microbial source could purify river water. After short adaptive phase (cycle II), the removal efficiencies of ammonium reached more than 94% (last three cycles), which were comparable with the previous ecological filter systems (90% to 93.1%) (Ijaz et al. 2019; Zhong et al. 2019). However, the pre-enrichment was time consuming before the application. Overall, the native bacteria, including ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB), could eliminate ammonium. However, this community only converted ammonium into nitrate without completely removing nitrogen. Considering that denitrifiers ubiquitously distribute, the possible limited factors for TN removal might be the lack of anoxic condition and biodegradable carbon source (Rivett et al. 2008).

Structure of the native microbial community

To obtain more understanding of the nitrogen removal processes by native microorganisms, the structure of the native microbial community treating river water was surveyed by high-throughput sequencing (Figure 3).

As shown in Table 1, a total number of 14,607 and 19,452 raw sequences were obtained from the native microbial community on the 0 and 19 day, respectively. After quality control, total high-quality reads of 13,855 and 17,584 were retained, respectively. After batch operation for five cycles, the number of OTUs with 97% of similarity was 3,318, which was almost two times more than that (1,867) on the 0 day. The coverages were 90.48% and 88.44%, respectively, indicating that the obtained reads were relatively complete. Meanwhile, significantly higher diversity was encountered for the sample on the 19 day compared with the sample on the 0 day based on the diversity indices such as Chao-1 (7,437 vs. 5,730), Shannon (6.12 vs. 4.54), and Simpson (0.014 vs. 0.055). The diversity observed here (Shannon index in the range of 4.54–6.12) was higher than those described elsewhere in heavily modified lakes or rivers, like Dongting Lake, China (Shannon index: 2.39–2.92) and Ohio River, USA (Shannon index: 2.29–4.23) (Schultz et al. 2016; Wu et al. 2016).

The changes of relative OTU abundance in terms of taxonomic groups by class are presented in Figure 3 (by genus in Table S1, available with the online version of this paper). On the 0 day, the native microbial community predominantly consisted of Gammaproteobacteria (26.69%), Betaproteobacteria (23.50%), Alphaproteobacteria (15.51%), Actinobacteria (10.59%) and Nitrospira (10.16%). After running for five cycles, there was a predominance of Gammaproteobacteria (28.55%), Alphaproteobacteria (14.55%), Betaproteobacteria (13.89%), Acidobacteria (8.82%) and Bacilli (7.04%).

All the results supported an evidence that a population shift happened and a higher diversity evolved after running for five cycles. A genus level, Nitrosomonas (9.64%) and
Nitrospira (10.13%) existed within the native microbial community on the 0 day and were responsible for the potential nitrogen-removing capacity in the Jialu River. Interestingly, despite the populations of Nitrosomonas and Nitrospira obviously decreasing to below 1% on the 19 day (Table S1), the native microorganisms presented stable ammonium removal in the last cycles (Figure 2), which may result from its higher diversity, such as Nitrosomonas (0.13), Nitrospira (0.87), Candidatus Nitrososphaera (0.04), Nitrosococcus (0.07) and unclassified Nitrosomonadaceae (0.56) (Cardinale 2011). Koops & Pommerening-Röser (2001) reported that Nitrosomonas had low substrate affinity constant ($K_s$) of 1.9–4.2 μM (Koops & Pommerening-Röser 2001), which gives Nitrosomonas a niche for inhabiting oligotrophic rivers. Compared with microorganisms in real rivers, the microorganisms in this experiment had longer biomass retention time and limited treated space, which facilitated pollutant elimination. Microorganism immobilization for long retention times and high biomass will therefore be a good choice for purifying river water in situ, such as bio-filter bed (Xu et al. 2012; Lin et al. 2015).

Polluted river water treatment by activated sludge

The collected activated sludge was divided into two parts: one for testing basic parameters of activated sludge and

### Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Raw reads</th>
<th>High-quality reads</th>
<th>OTUs</th>
<th>Chao-1</th>
<th>Shannon</th>
<th>Simpson</th>
<th>Coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 day</td>
<td>14,607</td>
<td>13,855</td>
<td>1,867</td>
<td>5,730</td>
<td>4.54</td>
<td>0.055</td>
<td>90.48</td>
</tr>
<tr>
<td>19 day</td>
<td>19,452</td>
<td>17,584</td>
<td>3,318</td>
<td>7,437</td>
<td>6.12</td>
<td>0.014</td>
<td>88.44</td>
</tr>
</tbody>
</table>
the other for testing its adaptability to river water. MLSS (mixed liquid suspended sludge) and SVI30 (sludge volume index for 30 min) of the sludge were around 3,000 mg/L and 100 mL/g, respectively. Five percent by volume of the sludge was directly inoculated into the flasks and the operation conditions were the same as those of native microbes.

The trends of pollutant removal by activated sludge are similar to those of native microorganisms, except for the first cycle (Figure 4). In the first cycle, ammonium in the experiment groups was immediately decreased to 0 mg/L within 1 day, while it degraded slowly from 9.13 to 5.38 mg/L in the control. In the second cycle, the removal rates of ammonium in the experiment groups were much lower than the first cycle. In the next three cycles, the activated sludge groups had a stable performance of ammonium removal. Ammonium concentrations decreased from 14.46 ± 1.27 mg/L to 0.22 ± 0.19 mg/L (Cycle III), from 14.19 ± 0.16 mg/L to 0.29 ± 0.29 mg/L (Cycle IV), and from 14.05 ± 0.00 mg/L to 0.85 ± 0.65 mg/L (Cycle V), with the removal efficiencies of 98.45 ± 1.42%, 97.98 ± 2.06% and 94.07 ± 4.64%, respectively. Supposing that $[\text{TN}] = [\text{NH}_4^+ - \text{N}] + [\text{NO}_2^- - \text{N}] + [\text{NO}_3^- - \text{N}]$, then N pollution in the form of nitrate remained in the river (Figure 4), which was consistent with the previous studies (Liang et al. 2012; Blevins et al. 2014; Zhang et al. 2014).

Activated sludge as microbial source could also purify river water. Similarly, the removal efficiencies of ammonium (more than 94%, last three cycles) were comparable with the enriched native bacteria (this manuscript) and the previous biological systems (90% to 93.1%) (Ijaz et al. 2016; Zhong et al. 2016). In comparison with the enriched native bacteria, activated sludge could be directly applied to river water purification and have the comparable effect, which would be preferable in practice. In conclusion, activated sludge can adapt to the river nutrient environment and retain the ability to oxidize ammonium. In order to completely eliminate N pollution, it is necessary to provide the anoxic microenvironment and available carbon source for denitrification.

**Microbial community structure of activated sludge**

High-throughput sequencing was conducted to acquire the evolving microbial community structure of activated sludge (Table 2 and Figure 5). In total, 35,321 and 18,761 raw sequences were obtained from the activated sludge samples on 0 day and 18 day, respectively. After quality control, in total 31,895 and 17,417 high-quality reads were retained, with the former having almost twice as many reads than the latter. The numbers of OTUs (97% of similarity) were 3,947 vs. 3,325, Chao-1 indices were 9,804 vs. 7,600 and the coverages were 92.44% and 88.40%, respectively, for the 0 and 18 day. Regarding Shannon and Simpson indices, it was concluded that a higher diversity was encountered for the sample on the 18 day. Meanwhile, there were comparable diversities between the native microbes and activated sludge. It is believed that diverse communities promote ecosystem stability, productivity and sustainability, and further improve water quality through niche partitioning (Girvan et al. 2005; Cardinale 2011). Therefore, the high ammonium removal capacity by both native microorganisms and activated sludge may result from their microbial diversity.

On the 0 day, the microbial community of activated sludge predominantly consisted of *Gammaproteobacteria* (31.87%), *Alphaproteobacteria* (15.25%), *Betaproteobacteria* (14.06%), *Acidobacteria* (11.56%), *Planctomycetacia*...
(6.36%) and Bacilli (5.68%). After running for five cycles, there was a predominance of Gammaproteobacteria (21.57%), Betaproteobacteria (16.33%), Acidobacteria (12.41%), Alphaproteobacteria (10.01%), Sphingobacteria (6.92%) and Bacilli (6.66%).

At phylum level, population shift mainly happened in Proteobacteria and Bacteroidetes. The former decreased from 63.61% to 50.44% and the latter increased from 4.98% to 13.66%, whilst the others changed little. Regardless of the acclimation for five cycles, the known ammonium oxidizing microorganisms, including *Nitrosomonas*, *Nitrospira* and *Nitrosospina*, and the nitrite-oxidizing microorganisms, including *Nitrospira* and *Nitrospina*, always ranged from 0.2% to 2% (Table S2, available with the online version of this paper). Jiao et al. (2014) reported that after augmentation, AOB+NOB increased from 0.1% to 1.3% with more than 60% of ammonium removal (Jiao et al. 2014). This indicated that such ratio of nitrogen-removing microorganisms could be acceptable for ammonium removal (>94% within 4 days, here). Certainly, other nitrogen-removing microorganisms, like *Cyanobacteria* and *Actinobacteria* also existed within the activated sludge (de-Bashan & Bashan 2010). Although the living conditions between river and WWTPs, especially food availability,
were significantly different, activated sludge could handle the relative oligotrophic environment, maintain the stable structure and clean the polluted water.

From these results, both native bacteria and activated sludge as inoculum source could efficiently remove ammonium in the contaminated river (efficiencies >94%). However, the native bacteria that was used as the inoculum source in river water treatment needed consecutive enrichment processes to harvest sufficient biomass, which were time- and labour-consuming, whereas activated sludge could be directly used after appropriate dilution. Thus, activated sludge was more suitable for purifying the river. In conclusion, ammonium in the river water was easily converted into nitrate (Figures 2 and 4) and activated sludge can be good choice as microbial resource for river pollution removal. Considering that denitrifiers ubiquitously distribute, the possible limited factors for TN removal might be the lack of anoxic conditions and biodegradable carbon sources. Measures to create anoxic microenvironments and available carbon source will be needed to completely eliminate N pollution.

CONCLUSIONS

The enriched native microbes and activated sludge could adapt to the relative oligotrophic river environment, and perform strong ammonium-removing activity. The microbial community structures shifted after acclimation and possessed relative high diversity. The native community had a predominance of Gammaproteobacteria (28.55%), Alphaproteobacteria (14.55%), Betaproteobacteria (13.89%), Acidobacteria (8.82%) and Bacilli (7.04%), whilst the composition of the activated sludge was dominated with Gammaproteobacteria (21.57%), Betaproteobacteria (16.33%), Acidobacteria (12.41%), Alphaproteobacteria (10.01%), Sphingobacteriia (6.92%) and Bacilli (6.66%). Low rate of nitrifying bacteria can accomplish ammonium removal. Activated sludge can be chosen as a cost-effective microbial source for river pollution removal.

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REFERENCES


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