The microbial community in filamentous bulking sludge with the ultra-low sludge loading and long sludge retention time in oxidation ditch

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Sludge bulking is a major problem that restricts the development of the activated sludge process. The microbial community responsible for sludge bulking varies depending on water quality and operational conditions. This study analysed the microbial community of bulking sludge in oxidation ditch with ultra-low sludge loading and long sludge retention time using high-throughput sequencing. The study found that the relative abundance of bacterial genus Saprospiraceae_norank was the highest in bulking sludge, reaching 13.39–28.83%, followed by Comamonadaceae_unclassified, Ardenticatenia_norank and Tetrasphaera, with the relative abundance of 4.59–11.08%, 0.52–16.60% and 0.17–8.92% respectively. In contrast, the relative abundance of bacteria that easily caused sludge bulking including Microthrix (0.54–2.47%), Trichococcus (0.32–1.71%), Gordonia (0.14–1.28%), and Thiothrix (0.01–0.06%) were relatively low. Saprospiraceae_norank was predominant and induced sludge bulking in oxidation ditch. The relative abundance of fungal genus Trichosporon was the highest in bulking sludge, reaching 16.95–24.98%, while other fungal genera were Saccharomycetales_unclassified (5.59–14.55%), Ascomycota_norank (1.45–13.51%), Galactomyces (5.23–11.23%), and Debaryomyces (7.69–9.42%), whereas Trichosporon was the dominant fungal genus in bulking sludge. This study reported that excessive Saprospiraceae_norank can induce sludge bulking for the first time, which provides important knowledge to control sludge bulking.

Activated sludge process is widely applied in wastewater treatment plants (WWTPs) because of low investment, high treatment efficiency and strong adaptability1. It is reported that about 50% of WWTPs used oxidation ditch process in China2. Complete biodegradation in the aeration basin and good separation of mud and water in the secondary sedimentation tank are critical in the activated sludge process. However, sedimentation performance of activated sludge is often a problem. Sludge settling performance is generally measured using sludge volume index (SVI). Generally, good activated sludge has SVI of 50–150 mL/g, filamentous sludge bulking with poor sludge settling property occurs at an SVI over 150 mL/g, while severe sludge bulking occurs at SVI of greater than 250 mL/g3. There are two kinds of sludge bulking4: (1) filamentous sludge bulking5: (1) filamentous sludge bulking induced by the mass proliferation of filamentous bacteria and (2) viscous sludge bulking caused by highly viscous substances produced by bacterial micelles5. Past research studies have reported that filamentous sludge bulking accounts for more than 90% of sludge bulking occurred in WWTPs and significantly affects the effluent quality and the operation and management of WWTPs6. Among several methods available to investigate sludge bulking, Gaussian Process Regression (GPR) is preferred since it can accurately predict and diagnose sludge bulking by monitoring the SVI values7,8.

Past studies have found that the main microbial communities responsible for sludge bulking in WWTPs are Microthrix6, Eikelboom type 021N9, Flavobacterium11, Haliscomenobacter hydrossis12, Nocardia13, Thiothrix14, Tetrasphaera15, Trichococcus Nostocoida limicola I16, Beggiatoa17, Trichosporon18, Geotrichum19, and Penicillium20 etc.

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Among them, the dominant bacterial communities that often induce sludge bulking in oxidation ditch are *Microthrix*<sup>9</sup>, *Flavobacterium*<sup>21</sup>, *Haliscomenobacter hydrosis*<sup>22</sup>, *Tetrasphaera*<sup>20</sup>, and *Trichococcus Nostocoida limicola*<sup>16</sup>. Similarly, the dominant fungal communities that induce sludge bulking in oxidation ditch are *Trichosporon*<sup>8</sup> and *Geotrichum*<sup>37</sup>

Factors such as water temperature<sup>22</sup>, dissolved oxygen (DO)<sup>9</sup>, sludge retention time (SRT)<sup>23</sup>, pH<sup>14</sup>, influent quality<sup>25</sup>, nutrient ratio<sup>26</sup> and sludge loading<sup>27</sup> are responsible for filamentous sludge bulking. The microbial community responsible for sludge bulking varies depending on the water quality and operational conditions. For example, for bacterial communities, *Microthrix* proliferated at low sludge loading and low temperature<sup>28,29</sup>, whereas *Eikelboom type 021N* induced sludge bulking at high sludge loading and high temperature<sup>10</sup>. *Flavobacterium* proliferated and caused sludge bulking at low influent carbon/nitrogen (C/N) ratio and long hydraulic retention time (HRT)<sup>11</sup>. The mass propagation of *Haliscomenobacter hydrosis* caused sludge bulking and resulted in high sludge loading and long SRT<sup>29</sup>, whereas *Nocardia* induced sludge bulking when the sludge loading was less than 0.5 kg BOD<sub>5</sub>/kg MLSS-d<sup>13</sup>. *Thiothrix* proliferated and caused sludge bulking at high chemical oxygen demand (COD) concentration, low DO and low nutrient<sup>10</sup>. Excessive proliferation of *Tetrasphaera* and *Trichococcus Nostocoida limicola* caused sludge bulking at low temperature<sup>16</sup>. Further, *Beggiaota* proliferated and resulted in sludge bulking when the sludge loading was less than 0.51 kg BOD<sub>5</sub>/kg MLSS-d and the DO lower than 1.5 mg/L<sup>17</sup>. For fungal communities, excessive propagation of *Trichosporon* caused sludge bulking at low DO<sup>16</sup>, while *Geotrichum* caused sludge bulking at low pH and high temperature<sup>19</sup>.

Although filamentous bacteria causing sludge bulking under different operational conditions have been widely investigated, sludge bulking is still a major problem hindering the operation of the activated sludge process. High-throughput sequencing is a revolutionary reform to traditional sequencing since the former does not require a pure culture and can sequence hundreds of thousands to millions of deoxyribonucleic acid (DNA) molecules rapidly and accurately<sup>32</sup>. In this study, the microbial community in the sludge collected from an oxidation ditch that has been experiencing sludge bulking constantly in recent years was analysed using high-throughput sequencing technology with the ultra-low sludge loading and long SRT. The outcomes of this study are expected to provide valuable knowledge required to control the sludge bulking.

**Results**

**Treatment efficiency of the WWTP.**  The removal efficiencies of the WWTP from January 2016 to January 2018 are presented in Table 1. The average influent biochemical oxygen demand (BOD) to COD ratio was 0.46, indicating a good biochemical property of sewage. The COD, BOD<sub>5</sub>, suspended solids (SS), total phosphorus (TP), and total nitrogen (TN) in the design influent to the WWTP were 450 mg/L, 200 mg/L, 250 mg/L, 5 mg/L, and 40 mg/L. As evident in Table 1, the actual influent COD, BOD<sub>5</sub>, SS, TP, and TN concentrations during the sampling period were 2–3 times higher than the design influent concentrations in the WWTP. The transformation of influent TN may generate substantial levels of free ammonia and free nitrous acid, which can have adverse impacts on microbial community<sup>10,13</sup>. The effluent from the WWTP met the second-level discharge standard<sup>15</sup>. Despite the sludge bulking, the sewage treatment efficiency was good.

**Activated sludge settling property.**  As shown in Fig. 1, SVI of the activated sludge samples were 162–250 mL/g indicating poor settling property and the activated sludge in the oxidation ditch was in the state of constant sludge bulking. Furthermore, filamentous sludge bulking occurred in the WWTP according to the microscopic examination.

**Bacterial community analysis based on 16S rRNA sequencing.**  The total effective readings of the seven bulking sludge samples were between 30457 and 55170. The coverage indexes of all samples were more than 0.988, indicating the detection of most microbial communities in this sequencing with high data reliability. The operational taxonomic units (OTUs), Chao, Shannon values are presented in Table 2. The Chao and Shannon indexes represent the richness and diversity of the microbial community, respectively. Higher Chao index indicates higher species richness and higher Shannon index suggests higher diversity of the communities<sup>30</sup>. In January 2016 (CJ1), the SVI value was the largest, while the Chao and Shannon values were the lowest, suggesting the lowest richness and diversity of the bacterial community. In January 2018 (CJ7), SVI value was the lowest, whereas the Chao and Shannon values were the highest, indicating the highest richness and diversity of the bacterial community. The richness and diversity of the bacterial community are lower when significant sludge bulking occurred.

A total of 35 bacterial phyla were detected in seven sludge samples. In at least one sample, there were 12 bacterial phyla with relative abundances of over 1%, accounting for 97.05–99.25% of the total bacterial effective sequences (Fig. 2). The dominant bacterial phyla were Bacteroidetes (25.86–47.56%), Proteobacteria (21.98–37.77%), Chloroflexi (4.28–24.96%), Actinobacteria (3.29–14.12%), and Firmicutes (1.20–4.65%).

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**Table 1. Treatment efficiencies of the WWTP.**

| Indicator | Influent (mg/L) | Effluent (mg/L) | Removal efficiency (%) |
|-----------|----------------|----------------|------------------------|
| COD       | 715–1233       | 66–92          | 91–94                  |
| BOD<sub>5</sub> | 265–625       | 17–27          | 91–96                  |
| SS        | 267–490        | 9–27           | 92–98                  |
| TN        | 66–94          | 4–31           | 58–93                  |
| TP        | 5.4–14.2       | 0.1–1.9        | 81–99                  |

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**Table 2. Operational taxonomic units (OTUs), Chao, Shannon values.**

| Indicator | OTUs | Chao | Shannon |
|-----------|------|------|---------|
| DJ2       | 3070 | 30457| 5.67    |
| DJ3       | 55170| 55170| 7.35    |
| CJ1       | 30457| 30457| 5.67    |
| CJ2       | 30457| 30457| 5.67    |
| CJ3       | 30457| 30457| 5.67    |
| CJ4       | 30457| 30457| 5.67    |
| CJ5       | 30457| 30457| 5.67    |

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According to the microscopic examination, the sludge samples were observed and classified into various bulking sludge samples, indicating the lowest richness and diversity of the bacterial community.
531 bacterial genera were present in seven sludge samples. In at least one sample, there were 73 bacterial genera with relative abundances of over 0.5%, accounting for 78.32–83.21% of the total bacterial effective sequences (Fig. 3). The dominant bacterial genera observed included Saprospiraceae_norank (11.87–28.83%), Comamonadaceae_unclassified (4.59–11.08%), Ardenticanetia_norank (0.52–16.60%) and Tetrasphaera (0.17–8.92%). The relative abundance of filamentous bacteria related to sludge bulking such as Microthrix (0.54–2.47%), Trichococcus (0.32–1.71%), Gordonia (0.14–1.28%) and Thiothrix (0.01–0.06%) was relatively low, among which Saprospiraceae_norank was predominant in all bacterial genera.
Fungal community analysis based on 18S rRNA sequencing. The total effective readings of the three bulking sludge samples were between 31191 and 50116. The coverage indexes of all samples achieved 1.000, suggesting that the fungal community was detected in this sequencing. The OTUs, Chao, Shannon values are summarized in Table 3. In December 2016 (CJ3), SVI value was the largest, while the Chao and Shannon values were the lowest, suggesting the lowest richness and diversity of the fungal community. In January 2018 (CJ7), the SVI value was the smallest, whereas the Chao and Shannon values were the highest, indicating that the highest richness and diversity of the fungal community. The richness and diversity of the fungal community are generally lower for significant sludge bulking to occur.

A total of 22 fungal phyla were detected in three sludge samples. In at least one sample, there were 8 fungal phyla with relative abundances of over 1%, accounting for 97.56–99.01% of the total fungal effective sequences (Fig. 4). The dominant fungal phyla were Ascomycota (58.83–69.69%) and Basidiomycota (23.71–25.68%), while other phyla included Ciliophora (1.74–7.02%), Choanoflagellida (0.55–2.52%), Cryptomycota (0.39–2.77%), and Chytridiomycota (0.06–0.43%).

89 fungal genera were present in three sludge samples. In at least one sample, there were 32 fungal genera with relative abundances of over 0.5%, accounting for 96.34–97.12% of the total fungal effective sequences. Fungi are less common than bacteria, though more evenly distributed than bacteria (Fig. 5). The top five dominant fungi in the relative abundance of bulking sludge samples were Trichosporon (16.95–24.98%), Saccharomycetales_unclassified (5.59–14.55%), Ascomycota_norank (1.45–13.51%), Galactomyces (5.23–11.23%), and Debaryomyces (7.69–9.42%), among which Trichosporon was predominant in all fungal genera.

Discussion

The dominant bacterial phyla obtained were Bacteroidetes (25.86–47.56%), Proteobacteria (21.98–37.77%), Chloroflexi (4.28–24.96%), and Actinobacteria (3.29–14.12%) and are not significantly different from the dominant bacterial phyla in the oxidation ditch process of WWTP in China37–39. However, the relative abundance of these phyla is different. Bacteroidetes, which plays an important role in wastewater treatment by degrading macromolecular organic pollutants40, were present at 25.86–47.56% in bulking sludge samples, and were the dominant bacterial phylum. Kragelund et al. found that the relatively high abundance of Bacteroidetes can cause sludge bulking problems41. Proteobacteria, which is a conventional bacterial phylum in WWTPs with the ability to degrade organic pollutants and remove nutrients such as biological nitrogen and phosphorus42, were present at 21.98–37.77% in all bulking sludge samples. Xu et al. found that Proteobacteria (33.90–50.90%) was the dominant bacterial phylum in the oxidation ditch without sludge bulking38. Chloroflexi is chiefly filamentous bacteria, which exists in flocculent sludge clump inside the body in the form of flocs skeleton. It plays a role in sludge flocculation, but rarely induces sludge bulking43. The relative abundance of Chloroflexi was between 4.28% and 24.96% in all bulking sludge samples. Furthermore, the mass proliferation of Actinobacteria can cause filamentous sludge bulking44. Wang et al. found that Actinobacteria was dominant with a relative abundance of 50% in
the WWTP, where excessive sludge bulking occurred\(^3\). In this study, the relative abundance of Actinobacteria (3.29–14.12%) was low and it was not the main bacterial phylum in bulking sludge of the WWTP.

The dominant bacterial genera obtained were Saprospiraceae\textit{unrank} (11.87–28.83%), Comamonadaceae\textit{unclassified} (4.59–11.08%), Ardenicatenia\textit{unrank} (0.52–16.60%), and Tetrasphaera (0.17–8.92%). Martins \textit{et al.} reported that Microthrix was the dominant filamentous bacterial genus that caused sludge bulking, despite the different operational conditions in different WWTPs\(^3\). Microthrix (15.11%) was the dominant filamentous bacterial genus at low temperature in WWTP in China\(^3\). Miłobędzka \textit{et al.} studied the filamentous bacteria of WWTPs in Poland and found that Microthrix (25%) was dominant with the long SRT\(^12\). Madoni \textit{et al.} reported that excessive growth of Microthrix (53.20%) resulted in sludge bulking when the sludge loading was 0.1–0.2 kgBOD\(_5/\) (kg MLVSS·d)\(^4\). Knoop \textit{et al.} reported that Microthrix has a strong reproductive advantage at low temperature.

\textbf{Figure 4.} Variation of relative abundance of fungal phylum in bulking sludge samples.

\textbf{Figure 5.} Variation of relative abundance of fungal genus in bulking sludge samples.
| Geographical names | Dominant filamentous bacteria | Main reasons | Reference |
|-------------------|------------------------------|--------------|-----------|
| China             | Microthrix, Tetrasphaera, Trichococcus | Low DO, low temperature and high influent NH$_4$+ -N concentration | 15,31 |
| Australia / Poland | Type 0914, Microthrix | Long SRT | 12,20 |
| Portugal (16 activated sludge systems), Italy | Type 0041/0675/0092, Microthrix | Low SRT and low sludge loading | 26,84 |
| Germany           | Microthrix, Gordonia, Thiothrix | Low DO, long SRT, low temperature, high COD concentration | 16,36 |

Table 4. Filamentous bacteria of WWTPs with sludge bulking in different regions, water quality and operational conditions.

(≤12–15 °C) and is the dominant bacterial genus responsible for sludge bulking in cold areas$^{28}$. Xinjiang is a dry and cold region with the winter lasting for five months. The influent temperature remains at 7–15 °C in winter and 16–24 °C in summer in the WWTP. However, in this study, Saprospiraceae_norank was the predominant bacterial genus in oxidation ditch bulking sludge and its relative abundance varied between 11.87% and 28.83%, whereas Yang et al. found that the relative abundance of Saprospiraceae_norank was between 2.10% and 3.53% in non-bulking activated sludge in WWTP$^{21}$. Muszynski et al. reported that the abundance of Saprospiraceae_norank was dependent on the season$^{45}$. Additionally, Saprospiraceae_norank, which existed in sludge flocs and is capable to produce extracellular enzymes to degrade protein and is crucial for partial nitrification, denitrification and sludge fermentation$^{46}$. Saprospiraceae_norank belongs to phylum Bacteroides, class Sphingobacteriia, order Sphingobacterales, and family Saprospiraceae. Shchepogolova et al. found that Saprospiraceae_norank was the inductor of activated sludge bulking and foaming$^{47}$. Yao et al. found that sludge bulking was inhibited due to the addition of an anaerobic step$^{48}$. In this study, Saprospiraceae_norank was the predominant bacterial genus that induced sludge bulking in oxidation ditch, while the relative abundance of Microthrix was only between 0.54% and 2.47% in bulking sludge, and was far less than the relative abundance reported when the proliferation of Microthrix caused sludge bulking in WWTPs. Microthrix was not the dominant bacterial genus that caused sludge bulking in oxidation ditch in WWTP.

Tetrasphaera belongs to Actinobacteria and plays a role in the biological phosphorus removal in WWTPs$^{49}$. According to Wang et al., Tetrasphaera (6.75%) was generally found in activated sludge systems, where filamentous sludge bulking occurred at low temperature and contributed to sludge bulking in WWTPs$^{31}$. The relative abundance of Tetrasphaera was between 0.17% and 8.92% in all bulking sludge samples. Trichococcus (3.91%) was the dominant filamentous bacterial genus that caused sludge bulking in WWTP at low temperature and low DO$^{16,31}$. The relative abundance of Trichococcus (0.32–1.71%) was low and was not the dominant filamentous bacterial genus in oxidation ditch bulking sludge. Similarly, Gordonia (5.1%) was the dominant bacterial genus in WWTPs with low DO, long SRT and low temperature$^{16}$. A long-term study that was conducted to identify the filamentous bacteria in a full-scale WWTP found that Thiothrix (51.9%) was the dominant filamentous bacterial genus with the high COD concentration, low DO and nutrient deficit$^{85}$. However, in this study, the relative abundance of Gordonia (0.14–1.28%) and Thiothrix (0.01–0.06%) were low and were not the dominant filamentous bacterial genera in oxidation ditch bulking sludge. Speirs et al. studied the bacterial community structure in oxidation ditch of a WWTP with severe sludge bulking in South Australia and found that the dominant bacterial genus was Type 0914 (35%) with the long SRT$^{50}$. Dos Santos et al. studied the dominant filamentous bacteria in WWTPs and found that Type 0041/0675 (19%) and Type 0092 (14%) induced sludge bulking with the low sludge loading and long SRT$^{35}$, while Type 0914, Type 0041/0675 and Type 0092 were not detected in all bulking sludge samples. The dominant filamentous bacteria in WWTPs with sludge bulking in different regions, water quality and operational conditions are listed in Table 4. Though Past studies did not report that excessive proliferation of Saprospiraceae_norank can induce sludge bulking, this study found that excessive Saprospiraceae_norank can induce sludge bulking for the first time.

Ascomycota (58.83–69.69%) and Basidiomycota (23.71–25.68%) were the dominant fungal phyla obtained by fungal sequencing in WWTP, which is consistent with the results obtained in other WWTPs in China. Ascomycota and Basidiomycota were conventional fungal phyla in activated sludge of WWTPs and Ascomycota (51.82%) was dominant in all fungal phyla$^{28}$. Basidiomycota mainly affects the formation of sludge flocs by reducing sludge settling properties$^{85}$ and thereby inducing sludge bulking. In this study, Basidiomycota was the dominant fungal phylum that caused sludge bulking in the oxidation ditch.

Trichosporon (16.95–24.98%) was the dominant fungal genus obtained by fungal sequencing and was responsible for inducing sludge bulking$^{35}$, whereas Trichosporon (25%) was the dominant fungal genus when sludge bulking occurred in WWTP$^{15}$. This is consistent with the dominant fungal genus obtained in this study. Further, Trichosporon induced sludge bulking in a sequencing batch reactor (SBR) in a previous laboratory study$^{14}$. The study found that Trichosporon was the filamentous fungal genus causing sludge bulking under different operational conditions. Saccharomyces_unclassified (5.59–14.55%) and Debaryomyces (7.69–9.42%) are common yeasts found in the oxidation ditch of WWTPs in China$^{34}$. Saccharomyces_unclassified and Debaryomyces can produce hydrolytic enzymes and collectively degrade some pollutants in sewage$^{35}$. In this study, Saccharomyces_unclassified and Debaryomyces existed in all bulking sludge in the oxidation ditch of the WWTP. However, they are not filamentous fungi and do not generally induce sludge bulking. Galactomyces is a common filamentous fungal genus in activated sludge of WWTPs and can induce sludge bulking$^{26}$. Comparatively high relative abundance of Galactomyces (5.23–11.23%) in oxidation ditch bulking sludge could contribute to the sludge bulking in WWTP, while the relative abundance of Geotrichum$^{19}$ related to sludge bulking was low,
only 2.17–3.02%. In other words, *Trichosporon* was the dominant filamentous fungal genus when sludge bulking occurred in WWTP, which was consistent with past study results. In this study, sludge bulking in WWTP occurred due to high influent pollutant concentrations, ultra-low sludge loading and long SRT. They are discussed in detail below.

1. **High influent pollutant concentrations.** The influent quality (COD, BOD₅, SS, TN, and TP) of the WWTP was generally higher than that of other WWTPs in China, where the influent pollutant concentration is generally high. China has 656 domestic sewage treatment plants in 70 cities including Shenyang, Changchun, Dalian, Harbin, Tianjin, Shanghai, Beijing, Wuhan, Zhengzhou, Shijiazhuang, etc. The average influent COD, BOD₅, SS, TN, and TP concentrations in these WWTPs were 395 mg/L, 220 mg/L, 250 mg/L, 55 mg/L, and 8.7 mg/L, respectively. In this study, the average influent COD, BOD₅, SS, TN, and TP concentrations in WWTP from January 2016 to January 2018 were 938 mg/L, 441 mg/L, 377 mg/L, 77 mg/L, and 10.6 mg/L, respectively. High influent pollutant concentration may be one of the reasons for sludge bulking in oxidation ditch.

2. **Ultra-low sludge loading operation.** From January 2016 to January 2018, the SVI values and sludge loading change trend of WWTP are shown in Fig. 6. In January and September 2016, the sludge loading were the lowest, only 0.007 kg COD/(kg MLSS·d), while the SVI values were the highest, reaching 250 mL/g. In January 2018, the maximum sludge loading was 0.016 kg COD/(kg MLSS·d), with the minimum SVI value of 177 mL/g. The SVI value decreased with the increase in sludge loading. High, medium and low sludge loadings were 0.12 ± 0.016 kg COD/(kg MLSS·d), 0.07 ± 0.015 kg COD/(kg MLSS·d), and 0.04 ± 0.004 kg COD/(kg MLSS·d) respectively. However, in this study, the sludge loading was less than 0.02 kg COD/(kg MLSS·d) due to ultra-low sludge loading operation. Sludge bulking occurs when the activated sludge system operates under low sludge loading condition for a long time.

3. **Long SRT.** The design SRT of oxidation ditch in WWTP was 25 d, while the actual sludge retention time was 40 d. The particle size distribution of sludge floc and sludge settling property are directly affected by increasing SRT. Settling test conducted on activated sludge with sludge retention time ranging from 0.25 to 12 days showed that increased SRTs resulted in an exponential decrease in percent dispersion (non-floculent or pinpoint floc), and an increase in the particle size of the floc, leading to a good sludge settling property. However, SRTs greater than 12 d resulted in a reduce in the diameter of the floc and the decline in sludge settling property. Significantly long SRT can lead to poor sedimentation performance of activated sludge, resulting in further sludge bulking.

**Materials and Methods**

**Description of the WWTP and sample collection.** The WWTP located in the Changji city of Xinjiang, northern China, with the treatment design capacity of 10 × 10⁴ m³/d uses the carousel oxidation ditch process and has been operating since 2000. The influent of Changji WWTP is mainly domestic wastewater. Activated sludge samples, CJ1, CJ2, CJ3, CJ4, CJ5, CJ6, and CJ7, were collected from the end of the aerobic stage of the oxidation ditch. Sampling date, sludge index volume and operating parameters of WWTP are presented in Table 5. The SVI values of the samples were more than 150 mL/g, confirming that all samples were bulking sludge.

**Analysis methods.** COD, BOD₅, TN, TP, SS and mixed liquor suspended solids (MLSS) were assayed according to the standard method. The temperature was measured using a thermometer. SVI values were determined by reading the percentage of sludge volume in the mixture of water and sludge after 30 min settling in a 100 mL measuring cylinder and counted from the dry weight in MLSS. Microscopic examination was conducted using a photonic microscope. The morphology of activated sludge filaments and flocs was characterized daily.
DNA extraction and PCR amplification. The E.Z.N.A. soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA) was used for microbial DNA extraction from eight samples according to the manufacturer’s instructions. The NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA) was used for measuring the final DNA concentration and purification, while the quality of the DNA was checked by 1% agarose gel electrophoresis. The V4-V5 hypervariable regions of 16S rRNA gene of all samples were amplified with primers 515F(5′-GTGCCAGCMGCCGCGG-3′) and 907R(5′-CCGTCAATTGCGCTACHT-3′), while the fungal 18S rRNA gene of three sludge samples (CJ3, CJ6 and CJ7) were amplified with primers SSU 0817F(5′-TTAGCATGGAATAATRRAATAGGA-3′) and 1196B(5′-TCCTCGAGCGGTGGA GTTTCC-3′) by thermocycler PCR system (GeneAmp 9700, ABI, USA). The PCR reactions were executed using 20μL reaction mixtures, containing 4μL of 5× FastPfu Buffer, 2μL of 2.5mM dNTPs, 0.8μL of each primer (5μM), 0.4μL of FastPfu Polymerase and 10 ng of template DNA. The triplicate amplicons were pooled together for each sample. The AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) was used together with the quantification using QuantifiFluor-ST (Promega, USA) for the extraction of PCR products from a 2% agarose gel and for further purification according to the manufacturer’s introductions.

High-throughput sequencing and Data analysis. Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard introductions of Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The raw readings were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: SRP197666). The i-Sanger platform (http://www.i-sanger.com/) was provided by Majorbio Bio-Pharm Technology Co. Ltd (Shanghai, China) for conducting data analysis. The Chao estimator and the Shannon diversity index were used to calculate the microbial phylotype richness levels. The Mothur program version v.1.30.1 (http://www.mothur.org/wiki/Schloss_SOP#Alpha_diversity) was used to calculate the Chao estimator, the Shannon diversity index, and the coverage percentage. These analyses were performed using the R Programming Language software.

Table 5. Sampling date, sludge settling property, sludge concentration and water temperature of the operation.

| Sample | Sampling date | MLSS (mg/L) | SVI (mL/g) | SV(%) | Water temperature (°C) |
|--------|---------------|-------------|------------|-------|------------------------|
| CJ1    | 2016.01.25    | 3355        | 262        | 88    | 13.7                   |
| CJ2    | 2016.03.02    | 4091        | 220        | 90    | 13.5                   |
| CJ3    | 2016.12.23    | 4265        | 211        | 90    | 15.2                   |
| CJ4    | 2017.03.31    | 5107        | 192        | 98    | 13.5                   |
| CJ5    | 2017.07.26    | 5132        | 187        | 96    | 24.5                   |
| CJ6    | 2017.11.24    | 5092        | 189        | 96    | 16.3                   |
| CJ7    | 2018.01.23    | 5279        | 188        | 99    | 11.7                   |

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Author Contributions
J.Y. and Y.C. designed the study. M.Z., X.W. and Y.H. sampled the activated sludge and analysed the data. M.Z. and J.Y. edited the manuscript. All authors reviewed the manuscript and approved the manuscript for publication.

Additional Information
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