

# Advanced nitrogen removal from municipal wastewater treatment plant secondary effluent using a deep bed denitrification filter

Xiaowei Zheng, Shenyao Zhang, Jibiao Zhang, Deying Huang and Zheng Zheng

## ABSTRACT

With the improvement of wastewater discharge standards, wastewater treatment plants (WWTPs) are continually undergoing technological improvements to meet the evolving standards. In this study, a quartz sand deep bed denitrification filter (DBDF) was used to purify WWTP secondary effluent, utilizing high nitrate nitrogen concentrations and a low C/N ratio. Results show that more than 90% of nitrate nitrogen ( $\text{NO}_3\text{-N}$ ) and 75% of chemical oxygen demand (COD) could be removed by the 20th day of filtration. When the filter layer depth was set to 1,600 mm and the additional carbon source  $\text{CH}_3\text{OH}$  was maintained at  $30 \text{ mg L}^{-1}$  COD ( $20 \text{ mg L}^{-1}$  methanol), the total nitrogen (TN) and COD concentrations of DBDF effluent were stabilized below 5 and  $30 \text{ mg L}^{-1}$ , respectively. Analysis of fluorescence revealed that DBDF had a stronger effect on the removal of dissolved organic matter (DOM), especially of aromatic protein-like substances. High throughput sequencing and qPCR results indicate a distinctly stratified microbial distribution for the main functional species in DBDF, with quartz sand providing a good environment for microbes. The phyla *Proteobacteria*, *Bacteroidetes*, and *Chloroflexi* were found to be the dominant species in DBDF.

**Key words** | 3D-EEM, deep bed denitrification filter, methanol as additional carbon source, microorganism

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## INTRODUCTION

Municipal wastewater treatment plants (WWTPs) are a crucial part of modern water pollution control, however many developed regions have recognized the limitations of WWTPs in the removal of various pollutants, resulting in effluents being a potential source of aquatic pollution (Lowenberg *et al.* 2016). Even in the most stringent Chinese municipal WWTP pollutant discharge standard (GB18918–2002 1A), the concentration limits for nitrogen and phosphorus are higher than those of Class III – IV in the Environmental Quality Standard for Surface Water (GB3838-2002) (MOEP 2002) (Table 1). Nitrogen removal forms an important aspect of WWTP management, with nitrogen from secondary effluents negatively affecting both the aquatic environment and human health (Cao *et al.* 2016). Restrictions on effluent nitrogen concentrations are critical for the prevention of eutrophication in water bodies and cyanobacterial blooms (Abell *et al.* 2010). Due to

growing concern regarding aquatic environmental health, many cities have established more stringent drainage requirements, resulting in the need for further upgrading of WWTPs to meet standards. However, upgrades are a difficult task and require additional manpower, land, money, and time (Jokela *et al.* 2002); therefore, establishing cost-effective WWTP development schemes is of significant research interest.

In recent years, numerous intensive studies have been conducted on advanced nitrogen removal for WWTP secondary effluent, which can be classified into three types: (1) physical and chemical technologies, such as coagulation (Tang *et al.* 2013); activated carbon adsorption (Chen *et al.* 2017) and membrane filtration (Lee *et al.* 2012); (2) biological technologies, such as bamboo charcoal filters (BCF) (Cao *et al.* 2016), deep bed denitrification filters (DBDF) (Joseph A 2012), and partial-denitrification (denitrification to nitrite)

**Table 1** | Comparisons of Chinese surface water environmental quality standards and the wastewater treatment plant discharge standards

	COD (mg/L)	NH <sub>4</sub> -N (mg/L)	TN (mg/L)	TP (mg/L)
Municipal WWTP pollutant discharge standard (GB18918-2002 1A)	50	5	15	0.5
Environmental Quality Standard for Surface Water (GB3838-2002 V)	40	2	2	0.2
Environmental Quality Standard for Surface Water (GB3838-2002 IV)	30	1.5	1.5	0.1
Environmental Quality Standard for Surface Water (GB3838-2002 III)	20	1.0	1.0	0.05

sequencing batch reactors (PDSBR) (Cao *et al.* 2017); (3) ecological technologies, such as constructed wetlands (Xiong *et al.* 2011), oxidation ponds (Zhao *et al.* 2013), and ecological floating-bed processes (Wang 2012). Among these, physical and chemical technologies have various reported disadvantages such as relatively high operating costs and possible secondary pollution, to name a few. Ecological technologies generally require a large area of land, complex management, and contribute secondary pollution to the environment. In contrast, biological technologies have the advantages of low running costs and high efficiency, with DBDF being particularly well suited to advanced nitrogen removal of the secondary effluent.

Domestic wastewater contains both persistent organics and easily-biodegradable organic matter (OM), which affect denitrification processes (Cao *et al.* 2017). Although the OM of wastewater plant influents fluctuates significantly, OM concentrations in the secondary effluent remain relatively constant, with especially low bioavailable. Consequently, there is insufficient denitrification of carbon sources during secondary processing (Guo *et al.* 2010). To provide OM for further denitrification, additional carbon sources are required and commonly, two broad categories of carbon sources have been utilized: traditional and emerging. Traditional carbon sources include methanol, ethanol, acetate, glucose, and other low molecular organic compounds and carbohydrates. Emerging carbon sources include wheat straw, pruned plant matter, corncob, food waste leachate, etc. However, methanol has been the most commonly used additional carbon source due to its low cost.

The aim of the present research was to find a efficient improvement scheme to effectively treat WWTP secondary

effluent. Methanol was utilized as the carbon source, with a quartz sand deep bed filter used as the denitrification reactor to investigate microbial dynamics with varying DBDF packing layer depth. The microorganism community in the DBDF denitrification processes was analyzed via high-throughput sequencing, and the obtained results provide a comprehensive basis for the management of WWTP upgrades. In addition, the influence on the denitrification process of additional carbon sources and varying packing depths were studied.

## METHODS

### Experimental set-up and influent quality

The experimental site was located in a WWTP (north latitude: 31°45'54.60", east longitude: 117°20'51.80") in Hefei City (Anhui Province, China), which applied a micro-aeration oxidation ditch treatment process for water treatment. The experiment was conducted for a six-month period, from April 1 2017, to October 1 2017. Methanol was applied as an additional carbon source. The influent supplied to experimental devices was the effluent produced by the WWTP secondary sedimentation tank, with effluent quality characteristics outlined in Figure 1.

### Design of deep bed denitrification filters (DBDF)

The experimental setup is displayed in Figure 2; the experimental DBDF was constructed of polymethyl methacrylate. WWTP secondary effluent was pumped from the sedimentation tank effluent to the setup influent tank. From there it was pumped to the DBDF using a controlled volume pump. The flow in the DBDF was from top to bottom. The DBDF had an inner diameter of 0.20 m, and a height of 5.05 m. The main layers were a supporting layer, a quartz sand layer and a water layer. The supporting layer consisted of pebbles with a diameter of 3–38 mm, to a total height of 0.45 m; the filling layer consisted of quartz sand with a diameter of 2–3 mm, to a total height of 2.00 m. The hydraulic retention time (HRT) was 2 h. The backwashing system adopted was air and water combined recycle, with a 48 h operation cycle. The first 5 min formed the water backwash stage, followed by 15 min of air and water combined backwash, and finally 5 min of air backwashing. The water backwashing strength was 14.7 m<sup>3</sup> (m<sup>2</sup>·h)<sup>-1</sup> and the air backwashing strength was

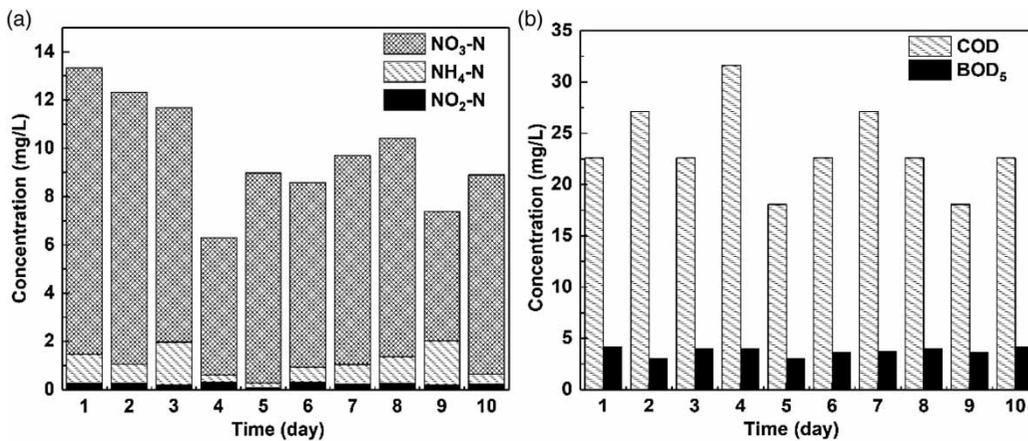


Figure 1 | Concentrations of NO<sub>3</sub>-N, NH<sub>4</sub>-N, NO<sub>2</sub>-N, COD and BOD<sub>5</sub> in effluent from secondary sedimentation tank.

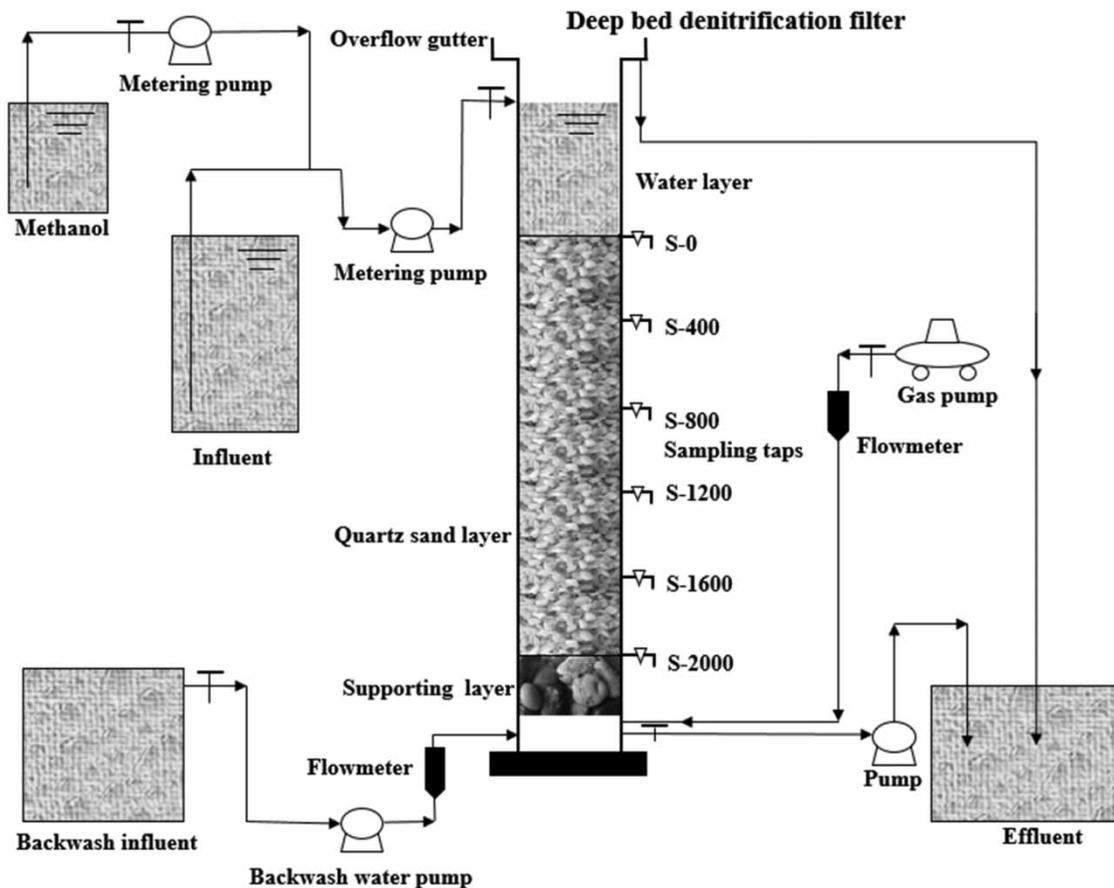


Figure 2 | Scheme of the pilot-scale set-up of the utilized deep bed denitrification filter.

91.4 m<sup>3</sup> (m<sup>2</sup>·h)<sup>-1</sup>. The carbon source was added through a tank containing methanol and dosed using a metering pump. There were six sampling openings, spaced

400 mm apart, from the top to the bottom of the filter layer, numbered as S-0, S-400, S-800, S-1200, S-1600, and S-2000.

## Experimental analysis

### Wastewater quality analysis

Influent and effluent water samples were analyzed for concentrations of ammonium (NH<sub>4</sub>-N), nitrate nitrogen (NO<sub>3</sub>-N), nitrite nitrogen (NO<sub>2</sub>-N), total nitrogen (TN), chemical oxygen demand (COD), and biochemical oxygen demand at 5 days (BOD<sub>5</sub>). Parameters were measured according to *Standard Methods for the Examination of Water and Wastewater* (APHA 2012). Dissolved oxygen (DO) concentration and water temperature were measured immediately, using a DO meter (YSI-550A).

### 3D-EEM Characterization

Chemical features of DOM were characterized via 3D-EEM spectra, established using a Hitachi F-4600 fluorescence spectrometer (Hitachi, Japan) equipped with a 150-W continuous output xenon arc lamp. The monitored excitation wavelengths (Ex) were in the range of 220–400 nm, the emission wavelengths (Em) ranged from 280 to 500 nm, and sampling intervals were 5 nm for both. The response time was 0.004 s and the scan speed was 2,400 nm min<sup>-1</sup>. To prevent any internal filter effects, samples were diluted using purified water to provide a UV absorbance at 260 nm of 0.1. Samples were filtered through a 0.45 μm pore size polycarbonate filter before measurements were taken using a fluorescence spectrometer.

### DNA extraction and PCR

When the DBDF achieved stable operation, six samples were taken from the sampling port of the filter. Samples contained quartz sand and water. Therefore, they were vibrated strongly to strip microbes from the surface of the quartz sand to the liquid prior to DNA extraction. Extraction of each sample was then performed using an E.Z.N.A.<sup>TM</sup> Mag-Bind Soil DNA Kit for soil extraction (Omega Bio-tek, Inc., USA), according to the manufacturer's protocol. Concentration and purity of the extracted DNA were determined using a spectrophotometer (NanoDrop-2000, Thermo Scientific, USA) and confirmed via agarose gel (1%) electrophoresis. 16S rRNA gene segments (16S rDNA) were polymerase chain reaction (PCR)-amplified using the forward primer 341F (CCTACGGGNGG CWGCAG) and the reverse primer 805R (GACTACHVGGGTATCTAATCC), targeting the V3-V4 hypervariable region. The first PCR amplification was performed in a reaction system (30 μL) containing

10–20 ng of genomic DNA, 15 μL 2×Taq master mix, 1 μL bar-PCR primer F, 1 μL primer R, and H<sub>2</sub>O. PCR was performed under the following conditions: 94 °C for 3 min; 5 cycles of 94 °C for 30 s, 45 °C for 30 s and 65 °C for 30 s; 20 cycles of 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s; and a final extension at 72 °C for 10 min. The second PCR amplification was also performed in a reaction system (30 μL), introducing Illumina bridge type PCR compatible primers. This system contained 20 ng genomic DNA, 15 μL 2×Taq master mix, 1 μL primer F, 1 μL primer R, and H<sub>2</sub>O. The second amplification was performed under the following conditions: 95 °C for 3 min; 5 cycles of 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s; and a final extension at 72 °C for 5 min. PCR products were checked via agarose gel (1%) electrophoresis and the DNA library was constructed and run on the Miseq 2×300 bp by Sangon Biotech Co., Ltd (Shanghai, China).

### High-throughput sequencing of 16S rRNA genes

The Qubit3.0 DNA detection kit was used to quantify the recovered DNA, allowing convenient sequencing at a 1:1 ratio when the DNA volume of each sample was 10 ng, providing a final sequencing concentration of 20 pmol. Following this, the DNA library was constructed and run on the Miseq 2×300 bp by Sangon Biotech Co., Ltd (Shanghai, China). After sequencing, data were processed and the sample sequence was distinguished via barcode, with the sequence of samples then going through QC to remove non-specific amplification sequences and chimeras.

## RESULTS AND DISCUSSION

### Quality of secondary effluent

The characteristics of the WWTP secondary sedimentation tank effluent are presented in Figure 2. The concentration of NO<sub>2</sub>-N was lowest at 0.23 ± 0.06 mg L<sup>-1</sup>, while NO<sub>3</sub>-N concentrations accounted for more than 85% of the TN content. The COD levels ranged between 18–32 mg L<sup>-1</sup> and BOD<sub>5</sub> levels remained below 5 mg L<sup>-1</sup>. Calculation of the BOD<sub>5</sub>/COD ratio was 0.16 ± 0.03, significantly below 0.3, which forms the lower limit of biodegradability in wastewater (Ying *et al.* 2012). This finding shows that the bioavailability of OM in the secondary sedimentation tank effluent is very low and therefore, further denitrification required additional OM. Previous studies have also reported low concentrations of biodegradable organic carbon in

WWTP secondary effluent, resulting in the need for external organic carbon sources for further denitrification (Park *et al.* 2009).

### Bioreactor performance

Biofilms are an essential component of quartz sand denitrification filters, with attachment of sufficient microbes to the bed filter being key to an effective DBDF operation. This study utilized continuous flow water to prepare the biofilm, with microbial growth facilitated via addition of a methanol concentration of 30 mg L<sup>-1</sup> (COD). At the beginning of the experiment, the COD removal rate was about 50%, while the NO<sub>3</sub>-N removal rate was only 8%. This may be because the bed filter had a good filtration effect on OM, intercepting and removing part of the OM component. The results also show the removal of NO<sub>3</sub>-N during the DBDF start-up period. By the 20th day, results show that the removal rate of NO<sub>3</sub>-N was stable at above 90%, while the COD removal rate also increased to more than 70%, indicating successful biofilm formation.

To investigate the influence of different concentrations of COD on quartz sand DBDF performance, the removal rates of NO<sub>3</sub>-N, NH<sub>4</sub>-N, TN, and COD were studied, with the addition of varying concentrations of methanol resulting in additional COD levels of 20 mg L<sup>-1</sup>, 30 mg L<sup>-1</sup>, and 40 mg L<sup>-1</sup>. HRT was set at 2 h and the operational cycle time was set at 48 h. With the increase in COD dosage from 20 mg L<sup>-1</sup> to 30 mg L<sup>-1</sup>, the average removal rates of TN and NO<sub>3</sub>-N significantly increased from 81.2 ± 6.8% to 87.2 ± 5.7% and 88.1 ± 4.5% to 92.8 ± 5.3%, respectively. Previously reported findings (Hidaka *et al.* 2003) showed that the degree of denitrification increased with higher C/N ratios and COD concentrations; however, in the present study, the average removal rates of TN and NO<sub>3</sub>-N decreased to 85.8 ± 5.9% and 91.1 ± 4.6% with further increased COD dosage of 40 mg L<sup>-1</sup>. This phenomenon may be due to the high concentration of methanol limiting microbial activity, as previously demonstrated by Fernandez-Nava *et al.* (2010), when effluent COD concentrations exceeded 30 mg L<sup>-1</sup>. Additionally, the average removal rate of NH<sub>4</sub>-N showed a similar trend (Figure 3). For the WWTP used in the present study, large fluctuations in influent COD levels are common; therefore, it is important to find the appropriate COD dosage required to stabilize the effluent and meet emission requirements. When COD was supplemented at a maximum of 30 mg L<sup>-1</sup>, optimal rates of removal of TN and NO<sub>3</sub>-N were observed, with effluent concentrations of NH<sub>4</sub>-N, and TN stabilized lower than

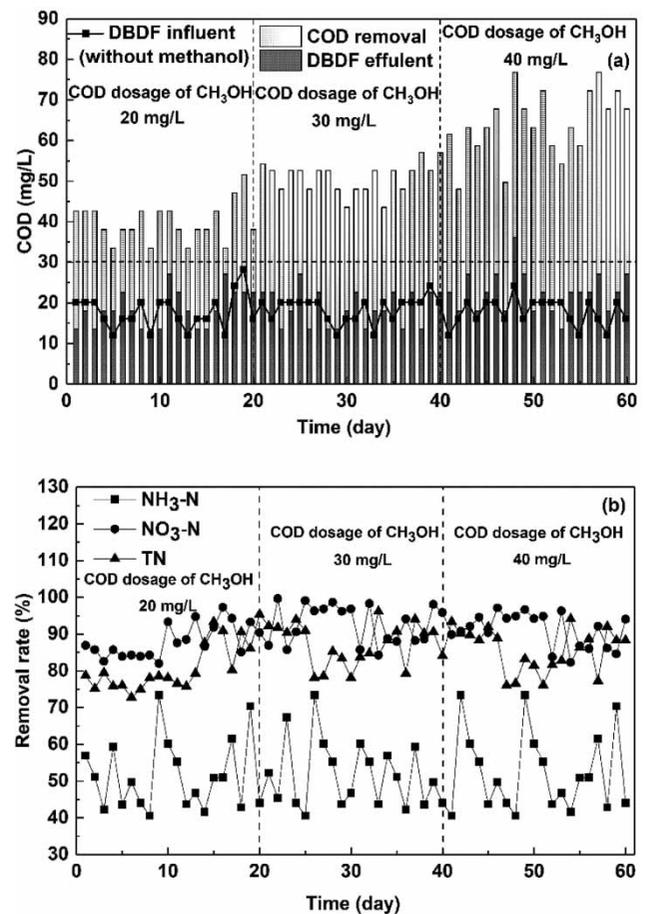
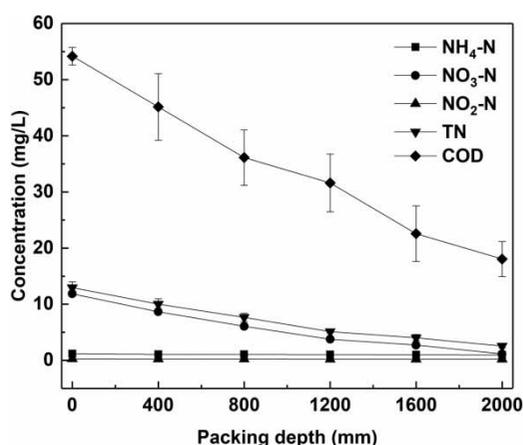


Figure 3 | Changes of nitrogen and COD removals at different CH<sub>3</sub>OH dosages.

1.5 mg L<sup>-1</sup> and 5 mg L<sup>-1</sup>, respectively. Moreover, the ratio of consumed COD to removed NO<sub>3</sub>-N was 3.65–4.99 mg (mg)<sup>-1</sup>, which is consistent with previously reported findings (Shi *et al.* 2015). As a result, the optimal COD concentration was established as 30 mg L<sup>-1</sup>.

Six samples were obtained from different sampling points along the direction of water flow in the quartz sand DBDF. Under the conditions of 30 mg L<sup>-1</sup> COD, the concentrations of COD, TN, NO<sub>3</sub>-N, NH<sub>4</sub>-N, and NO<sub>2</sub>-N varied according to different packing layer depths (Figure 4). NO<sub>2</sub>-N is an intermediate product formed in the process of denitrification, accumulating when the carbon source is insufficient (Karanasios *et al.* 2016). Several previous studies have reported this situation in denitrification experiments (e.g. Cao *et al.* 2016); however, no NO<sub>2</sub>-N accumulation was observed in the present study. Therefore, the 30 mg L<sup>-1</sup> COD dosage provided sufficient levels of carbon source for the denitrification process. No significant change was observed in the concentration of NH<sub>4</sub>-N with varying depths of the filter layer, possibly due to the low



**Figure 4** | Changes of nitrogen and COD concentrations at different DBDF depths (September 10–15).

concentration of NH<sub>4</sub>-N in the influent and the main NH<sub>4</sub>-N removal method being absorption by microbes.

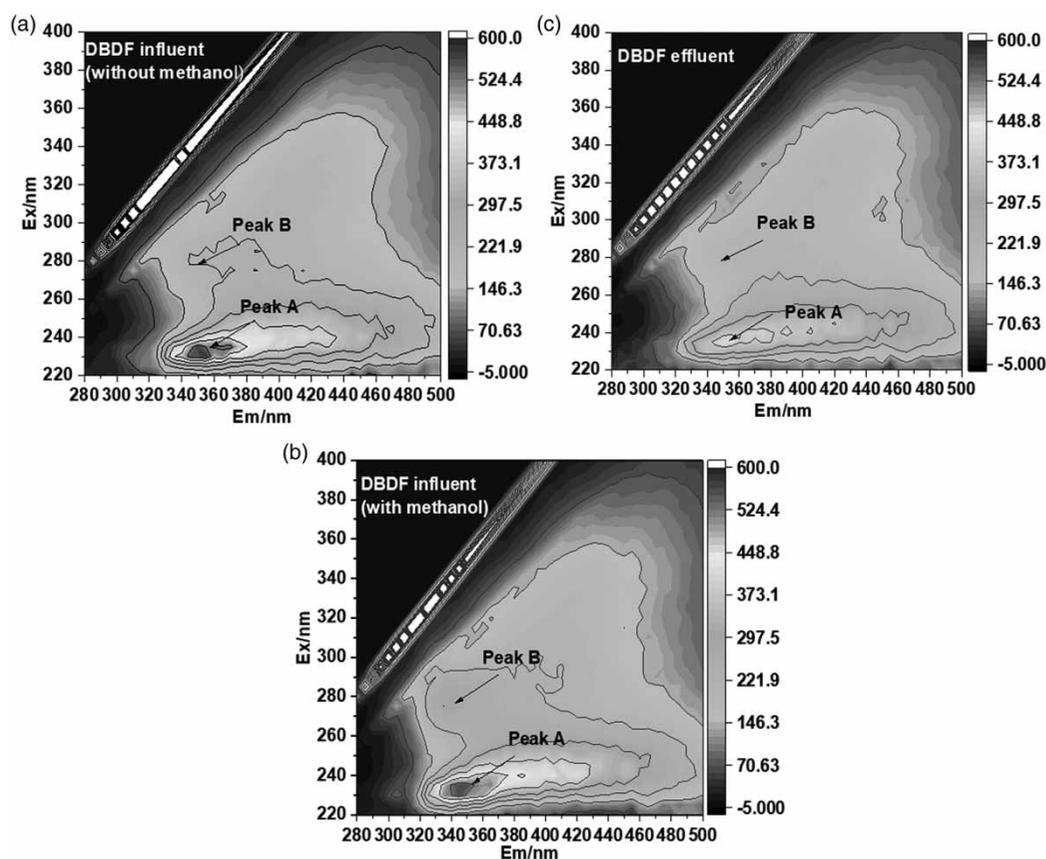
In contrast, the concentrations of COD, TN, and NO<sub>3</sub>-N significantly changed with varying layer depth, with upper packing layer concentrations of COD, TN, and NO<sub>3</sub>-N being 54.18, 12.97, and 11.87 mg L<sup>-1</sup>, respectively. At a

packing layer depth of 1,600 mm, concentrations decreased to 22.58, 4.04, and 2.73 mg L<sup>-1</sup>, respectively. The resulting effluent readily met all requirements of the GB18918-2002 1A discharge standard (MOEP 2002) and when taking the economic cost into account, this is optimal when the depth of filter layer is set to 1,600 mm.

### Change in DOM levels

OM is an important component in aquatic ecosystems in which DOM is the most easily released and active substance; therefore, DOM plays a more important role in the ecosystem overall (Liu *et al.* 2018). Consequently, after treatment with additional carbon sources by DBDF, it is necessary to study the composition and removal of DOM to minimize the effects of DOM on the water environment.

Fluorescence EEM analysis provides a qualitative estimate of the DOM removal performance of the quartz sand DBDF, as shown in Figure 5. Fluorophores in DOM were classified as: Peak A (Ex/Em = 225–237/340–381 nm); Peak B (Ex/Em = 275–285/340–345 nm); Peak C



**Figure 5** | Fluorescence EEMs (RU) of water samples.

(Ex/Em = 315–350/415–440 nm); Peak D (270–280/415–430 nm); and Peak E (220/310 nm). Based on previously reported literature, peaks A, B, C, D, and E were related to aromatic protein-like, tryptophan protein-like, humic acid-like, fulvic acid-like, and tyrosine protein-like substances, respectively (Liu et al. 2018).

As shown in Figure 5(a), two main peaks (peak A and B) were observed in the EEM fluorescence spectra of DOM extracted from the secondary sedimentation tank effluent. This implies that oxidation ditches may not be ideal for the removal of aromatic protein-like substances. Figure 5(b) shows the EEM fluorescence spectrum after the addition of methanol (30 mg L<sup>-1</sup> based on COD) to the secondary sedimentation tank effluent. In comparison to Figure 5(a), the fluorescence intensity of peaks A and B were enhanced, particularly in peak B.

A strong decolorizing effect can be seen in Figure 5(c) and, compared to Figure 5(a), the fluorescence spectrum of peak A was significantly weaker. Furthermore, peak B was also attenuated, showing that the DBDF with quartz

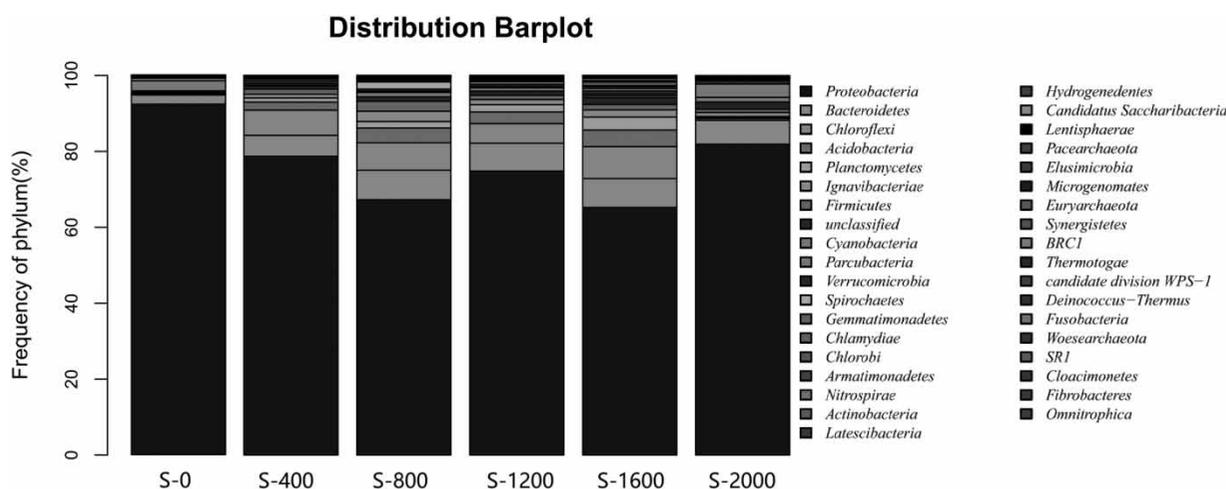
sand has a better removal rate for DOM, especially aromatic protein-like DOM, than the WWTP's oxidation ditch.

## Microbial analysis

Microorganisms are a key factor for effective wastewater treatment by biological means. Microbial biochemistry processes occurring in the DBDF are functional in wastewater treatment, with each microbial species present determining which pollutants will be effectively removed. Therefore, microbial analysis can provide a comprehensive understanding of the active treatment mechanisms and aid in characterization of mechanisms and pathways (Hua et al., 2018). To investigate microbial community dynamics during the experimental process, six samples were taken from various filter depths. 16S rRNA gene based high-throughput sequencing technology was utilized to analyze the microbial communities present in these samples. After removing low quality sequences and

**Table 2** | Richness and diversity of the water samples at various filter depths

Sample	Mean length	OTU counts	Shannon index	ACE index	Chao1 index	Coverage	Simpson
S-0	426.98	1,845	2.31	2,831.69	2,538.01	0.988	0.42
S-400	424.85	2,299	3.81	3,359.32	3,166.01	0.987	0.16
S-800	422.12	2,255	5.05	3,150.47	3,064.44	0.985	0.038
S-1200	423.92	2,540	4.49	3,620.08	3,447.36	0.983	0.091
S-1600	422.23	2,665	5.09	3,395.45	3,287.88	0.989	0.043
S-2000	424.72	2,110	3.04	2,848.46	2,668.92	0.990	0.33

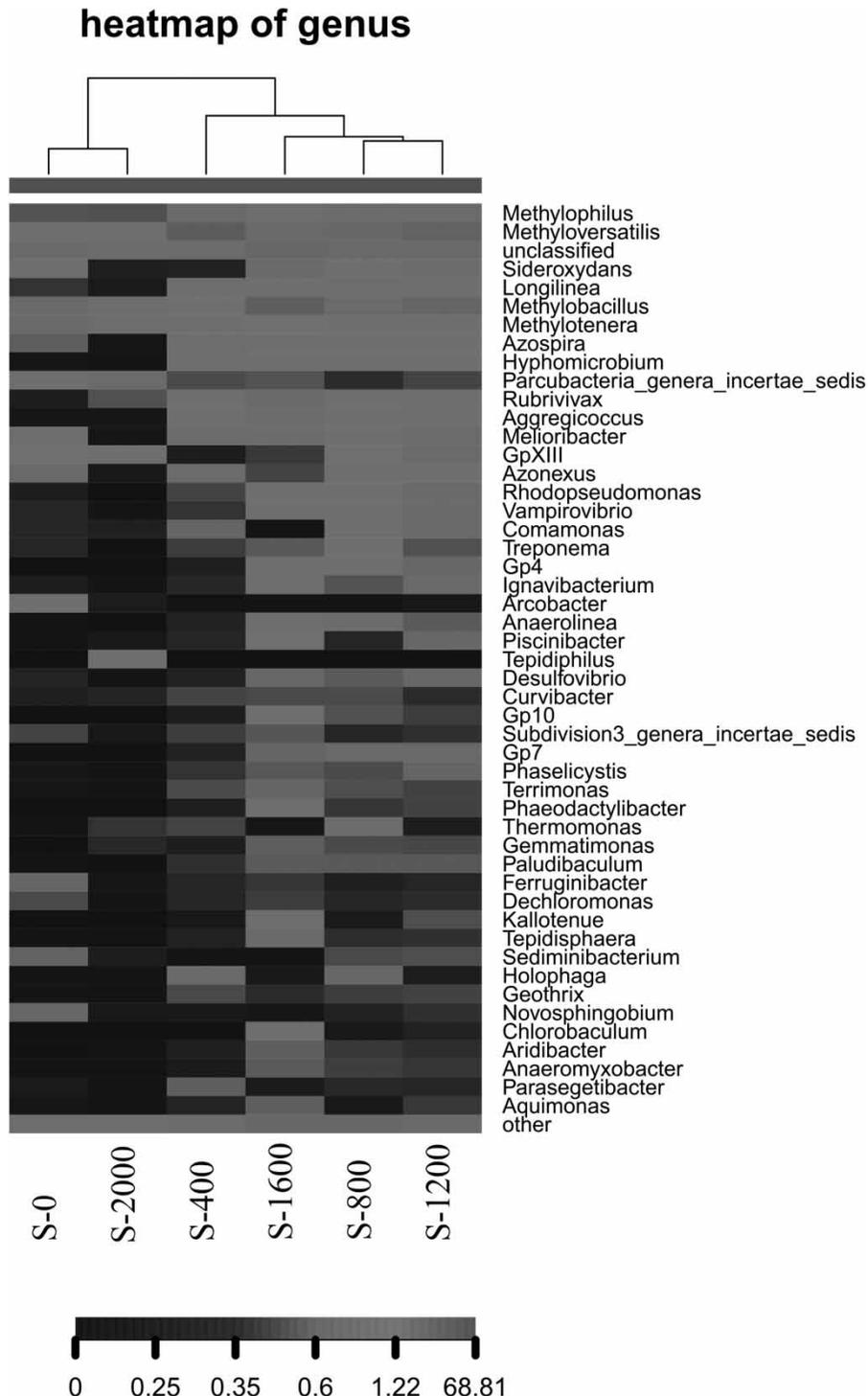


**Figure 6** | Bacterial community distribution in water samples at phylum level (September 25).

chimeras, approximately 51,419–68,134 effective sequences were obtained, with an average length ranging from 422 bp to 427 bp (Table 2). These effective sequences were further classified into operational taxonomic units

(OTUs) at a 3% distance, to assess the diversity and richness of the bacterial community (Cao *et al.* 2017).

From S-0 to S-1600, the Shannon and Simpson diversity indices showed that the bacterial diversity increased



**Figure 7** | Heatmaps of bacterial genera relative abundance of water samples at different DBDF depths (25 September).

significantly (Table 2), clearly indicating that quartz sand can provide an effective living environment for bacteria. The OTU counts from S-0 to S-2000 showed that the species richness and diversity in S-1600 were noticeably higher than S-2000, which was located between quartz sand and pebbles. Furthermore, the DO level of the S-2000 layer ( $1.8 \pm 0.08 \text{ mg L}^{-1}$ ) was higher than that of the S-1600 layer ( $1.2 \pm 0.11 \text{ mg L}^{-1}$ ), which may also be a contributing factor.

The most significantly abundant phylum in the influent S-0 samples was *Proteobacteria*, accounting for 92.34% of the sequences, while in other samples *Proteobacteria* were also dominant with an average proportion of 76.71% (Figure 6). A high abundance of *Proteobacteria* has been previously observed in some wastewater treatment bioreactors (Ma et al. 2015). *Proteobacteria* dominance decreased, from 92.34% in S-0 samples to 65.23% in S-1600 samples, suggesting that DO had an adverse effect on these microbes. Previous research (Cao et al. 2017) demonstrated that the concentration of OM has a noticeable impact on *Proteobacteria*. In contrast, in the present study, the phyla *Bacteroidetes* and *Chloroflexi* showed a significant increase, from 2.41% and 0.17% in S-0 samples to 7.61% and 8.42% in S-1600 samples, respectively.

To gain a deeper insight into the variations in bacterial genera, a heatmap of hierarchical clustering was prepared for the 50 most abundant genera, as shown in Figure 7. Enrichment of *Methyloversatilis*, *Sideroxydans*, *Longilinea*, *Azospira*, and *Hyphomicrobium* was identified due to their increased abundance in the sand layer, in comparison to influent concentrations. *Methylophilus* and *Methyloversatilis* were two of the most significantly changing genera. Lu et al. (2012) previously reported that *Methyloversatilis* induced incomplete denitrification, reducing nitrates or nitrites to  $\text{N}_2\text{O}$  instead of  $\text{N}_2$  (Lu et al. 2012). *Methylophilus* is a genus of aerobic Gram-negative bacteria, which can use methanol as carbon and energy source (Jenkins et al. 1987).

## CONCLUSIONS

The quartz sand DBDF in this experiment showed very good nitrogen and COD removal performance (TN removal >85%,  $\text{NO}_3\text{-N}$  removal >90%, and COD removal >75%). The DBDF start-up period was short, with steady running achieved within 20 days. When the influent TN concentration was below  $15 \text{ mg L}^{-1}$  and COD levels above  $50 \text{ mg L}^{-1}$ , the effluent TN and COD could be stabilized to less than 5 and  $30 \text{ mg L}^{-1}$ , respectively. 3D-EEM results

show that the DBDF had a better removal capacity for DOM, especially aromatic protein-like substances, than the WWTP oxidation ditch at which it was operated. High throughput sequencing and qPCR results showed a distinctly stratified distribution of the main functional microbial species in DBDF. These findings demonstrate that quartz sand can provide an effective living environment for microbes.

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