Mixed carbon source improves deep denitrification performance in up-flow anaerobic sludge bed reactor

Hong Xiao, Jiaojiao Wu, Hong Peng and Zhongyao Jiang

ABSTRACT

To investigate the advantages of mixed carbon source over a single one in deep denitrification, sodium acetate, glucose and their mixture were used as carbon sources in present study. Denitrification performance, effluent pH, microbial community and carbon source cost were taken into account. With the same influent NO_3^--N concentration of 50 mg/L and the same C/N ratio of 1.5, the NO_3^--N removal rate with the mixed carbon source (96.53%) was slightly lower than that with sodium acetate (98.15%), but significantly higher than that with glucose (74.69%). The specific denitrification rates of the sodium acetate, glucose and sodium acetate/glucose reactor were 47.7, 29.7 and 45.4 mg N/g VSS d, respectively. The effluent pH with sodium acetate varied in the range of 9.13–9.60, exceeding the discharge standard limit of 9.0, whereas the sodium acetate/glucose reactor could keep pH in the range of 7.80–8.23. The 16S rRNA gene-based high-throughput sequencing revealed that carbon sources determined the microbial community structure and the sludge Shannon index with the mixed carbon source was the highest. Furthermore, cost estimation indicated that the mixed carbon source was the cheapest. This study is significant as it tests reasonable selection of carbon sources for deep denitrification in practice.

Key words | C/N, deep denitrification, microbial community, mixed carbon source, pH

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INTRODUCTION

The anoxic/oxic (A/O) process is widely used to treat the wastewater containing nitrogenous components. Although fairly good removal efficiency of ammonia nitrogen and total nitrogen can be achieved for A/O process, nitrate concentration in its effluent usually maintains at a high level when the raw wastewater contains high concentrations of nitrogen. In this case, the increasingly stringent discharge standards cannot be met. To remove residual nitrate, deep denitrification is commonly applied. Denitrification is the reduction of nitrate to N_2 carried out by denitrifying bacteria (Xu *et al.* 2018). Generally, most denitrifying bacteria are heterotrophic and therefore require organic carbon sources which have an important effect on the denitrification process for cell growth and nitrate reduction (Zhang *et al.* 2018). However, the organic carbon in the raw wastewater has been mostly consumed during the A/O process, resulting in C/N ratio of the secondary effluent insufficient for further denitrification. Hence, the addition of an external carbon source is needed.

Traditionally, liquid organic substances such as methanol, ethanol, glucose and acetate serve as external carbon sources for nitrate removal (Calderer *et al.* 2010; Ribera-Guardia *et al.* 2014). Methanol was once the most commonly employed external carbon source due to being easily assimilated by denitrifying bacteria and its low cost (Rabah & Dahab 2004). Ethanol was found to be considerably more readily available as a carbon source for denitrification than was methanol (Christensson *et al.* 1994). However, both methanol and ethanol are flammable carbon sources, presenting security hazards for transportation, storage and operation (Li *et al.* 2016). Thus, glucose and acetate received more attention in recent years.

Glucose and acetate proved to be beneficial for denitrification (Calderer *et al.* 2010; Chen *et al.* 2015; Xu *et al.* 2018). It was reported that high denitrification rate could be obtained with acetate as carbon source instead of glucose (Yang *et al.* 2012). However, in the acetate system, pH often increased significantly to exceed the limit of 9.0 (Yang *et al.* 2012; Ma *et al.* 2015). As regards glucose used as carbon

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source, relatively low denitrification rate and undesirable accumulation of NO_2^--N and NH_4^+-N hindered its application (Srinandan *et al.* 2012). Although utilizing sodium acetate or glucose as the single carbon source has its own limitations, employing their mixture as carbon source may be a reasonable alternative. Unfortunately, the denitrification performance of using binary mixture of sodium acetate and glucose as carbon source remained unclear.

In this study, sodium acetate, glucose and their mixture were adopted as external carbon source to promote biological denitrification, respectively. Denitrification performance with different carbon sources was explored. Special attention has been paid to effluent pH. Meanwhile, microbial community was investigated to shed light on the microbial utilization mechanism of different carbon sources. In addition, cost estimation with different carbon sources was carried out.

MATERIALS AND METHODS

Experimental materials

The experiment set-up was shown in Figure 1. Three identical up-flow anaerobic sludge bed (UASB) reactors with an internal diameter of 70 mm and a height of 280 mm were designed. The working volume of each reactor was 1 L. The synthetic influent for the three reactors was mainly composed of $NaNO_3$, different carbon source, KH_2PO_4 and trace element solution. Sodium acetate, glucose and their mixture were used as carbon sources in the influent, respectively. To simulate the condition of low C/N ratio, the influent NO_3^--N concentration was kept at 50 mg/L and the C/N ratio was set as 1.5 during the whole experiment. The detailed characteristics of the influent were shown in Table 1.

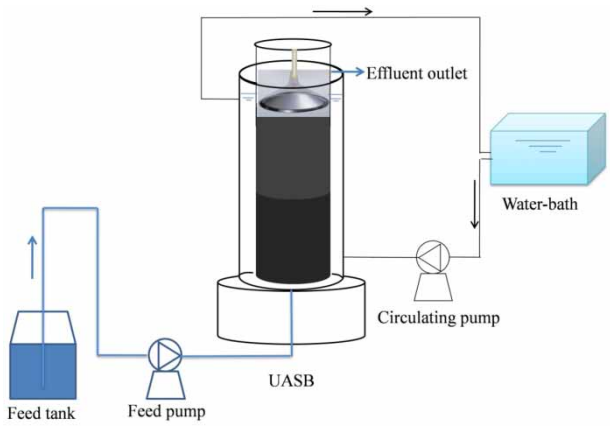

Figure 1 | Schematic diagram of the experimental set-up.

The inoculated sludge was obtained from an anoxic tank of an industrial wastewater treatment plant in Chengdu, China. The ratio of volatile suspended sludge (MLVSS) to total suspended sludge (TSS) was 0.61 with an initial MLVSS of 5,730 mg/L.

Experimental procedures

All reactors were operated at 30 ± 2 °C. The synthetic wastewater was pumped into the bottom of the reactor with a peristaltic pump. Three reactors (i.e. the glucose, sodium acetate and sodium acetate/glucose reactor) were operated under a continuous mode with a hydraulic retention time (HRT) of 4 h. The steady-operation phase lasted for 48 days.

Analytical methods

Prior to analysis, samples were filtered through a 0.45 μm polyethersulfone membrane. NO_3^--N was analyzed by the phenol disulfonic acid method. NO_2^--N was measured by the N-(1-naphthalene)-diaminoethane photometry method. pH was measured with a pH electrode (Thermo, 3-star 310p-02, USA). MLSS and MLVSS were measured according to the standard methods (APHA *et al.* 2005). All tests were performed in triplicate.

The specific denitrification rate (SDR, mg N/g VSS d) was defined as follows:

$$SDR = (C_{in} - C_{ef}) \times Q/(V \cdot X_v) \quad (1)$$

where C_{in} is the initial NO_3-$N + NO_2$-N concentration (mg/L); C_{ef} is the effluent NO_3-$N + NO_2$-N concentration (mg/L); Q is the flow rate (L/d); V is the reactor volume (L); and X_V is the MLVSS concentration (g/L).

Table 1 | Characteristics of synthetic influent

Influent characteristics	Sodium acetate reactor	Sodium acetate/glucose reactor	Glucose reactor
$NaNO_3$ (mg/L)	303.57	303.57	303.57
Sodium acetate (mg/L)	256.25	64.06	/
$C_6H_{12}O_6$ (mg/L)	/	140.75	187.67
KH_2PO_4 (mg/L)	5	5	5
pH	7.3 ± 0.2	7.3 ± 0.2	7.3 ± 0.2

Microbial community analysis

DNA extraction and PCR

Four sludge samples (R0, R1, R2 and R3) were collected. R0 represented the inoculated sludge and R1, R2 and R3 represented the sludge sample collected from the sodium acetate, sodium acetate/glucose and glucose reactor on the end of experiments, respectively. All samples were freeze-dried by a lyophilizer (LABCONCO Co., Free Zone, USA). DNA was extracted from dried sludge using the E.Z.N.ATM Mag-Bind Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) following the manufacturer's protocol. DNA concentration and purity of the extracted DNA were evaluated by using a spectrophotometer (UV2700, Shimadzu, Japan) and checked by agarose gel (1%) electrophoresis.

The bacterial 16S rRNA genes were PCR-amplified with forward primer 341F (CCTACGGGNGGCWGCAG) and reverse primer 805R (GACTACHVGGGTATC TAATCC) for the V3 and V4 region. The PCR reaction mixture contained 15 μL 2 × *Taq* master mix, 1 μL forward primer (10 μM), 1 μL reverse primer (5 μmol/L), 10 ng genomic DNA and added H_2O to 30 μL. The PCR thermal programs consisted of an initial denaturation at 94 °C for 3 min, then followed by the following conditions: five cycles of denaturing at 94 °C for 30 s, 45 °C annealing at for 20 s, and extension 65 °C for 30 s; 20 cycles of denaturing at 94 °C for 20 s, 55 °C annealing at for 20 s, and extension72 °C for 30 s, and a final extension at 72 °C for 5 min.

High-throughput sequencing and data analysis

Sequencing of amplicons from all samples was carried out on Illumina MiSeq PE300 platform. The efficient readings were selected based on the barcode and primers at the beginning and end. After removing low quality sequences and chimeras, the effective sequences of R0, R1, R2 and R3 were 63,578, 69,886, 76,645 and 81,301, the mean lengths were 419.60, 422.61, 417.83 and 420.07 bp, respectively.

Operational taxonomic units (OTUs) used 97% identity thresholds (i.e. 3% dissimilarity levels) in sequences by Usearch (Usearch 5.2.236). The species richness, rarefaction and Shannon diversity index were generated by Mothur (Mothur 1.30.1) and the taxonomy was assigned via the RDP classifier with the Silva databases.

Statistical methods

Data analyses were performed for all samples and the mean values with standard deviation are presented. One-way-analysis of variance (ANOVA) was conducted using the statistical software package SAS 9.0. For values of $p < 0.05$, the statistical analysis was considered significant.

RESULTS AND DISCUSSION

Denitrification performance

As is shown in Figure 2, the effluent NO_3^--N concentrations of the sodium acetate, glucose and sodium acetate/glucose reactor were 0.93, 12.66 and 1.74 mg/L, respectively. The corresponding NO_3^--N removal rates were 98.15%, 74.69% and 96.53%, respectively.

Only taking nitrate removal into account, sodium acetate was evidently the best and glucose the worst carbon source. Zhang *et al.* (2016a) reported a similar conclusion that when the initial NO_3^--N concentration was 40 mg/L and the HRT was 6 h, the effluent NO_3^--N concentration was 22.2 mg/L with glucose as carbon source and less than 1 mg/L with sodium acetate as carbon source. This can be explained as follows. Firstly, compared to sodium acetate, less NO_3^--N could be reduced by glucose under the same C/N ratio. In the case of sodium acetate as carbon source, the C/N ratio required for the complete reduction of NO_3^--N to N_2 by heterotrophic denitrification is 1.5, as

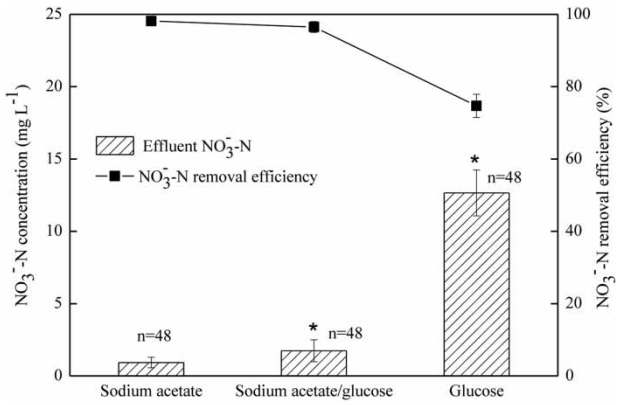

Figure 2 | NO_3^--N removal performance with different carbon sources. Data are the mean values of different measurements. Error bars represent standard deviations of statistical analysis. *n* means measurement times. The asterisk (*) indicates statistical differences ($p < 0.05$) from the sodium acetate reactor.

calculated in Equation (2) (Huang *et al.* 2013):

$$0.125CH_3COO^- + 0.1438NO_3^- + 0.1438H^+ \rightarrow 0.0122C_5H_7O_2N + 0.0658N_2 + 0.125HCO_3^- + 0.0639CO_2 + 0.1542H_2O \quad (2)$$

However, the C/N ratio increased to 1.56 if glucose was used as a carbon source, as calculated in Equation (3) (Henze 1991):

$$C_6H_{12}O_6 + 2.8NO_3^- + 0.5NH_4^+ + 2.3H^+ \rightarrow 0.5C_5H_7O_2N + 3.5CO_2 + 6.4H_2O + 1.4N_2 \quad (3)$$

Secondly, acetic acid, as the hydrolysate of acetate, can be directly inserted into the metabolic process without modification (Elefsiniotis *et al.* 2004), whereas glucose requires some enzymatic conversion before it can enter the metabolism of most denitrifying bacteria. As is known, propionic acid fermentation and butyric acid fermentation are two main fermentation types. Whichever fermentation type it is, the end product of fermentation has other ingredients besides acetic acid. In addition, glucose could not be fully fermented in time due to the relatively short HRT (4 h) used in this study.

The NO_2^--N and NH_4^+-N concentration profiles of different carbon sources were depicted in Figure 3.

The effluent NO_2^--N concentrations of the sodium acetate, glucose and sodium acetate/glucose reactor were 3.50, 9.00 and 4.93 mg/L, respectively. Obviously, NO_2^--N accumulated in all reactors. It could be ascribed to insufficient carbon source in the influent with C/N ratio as low as 1.5. The NO_2^--N accumulation with glucose was the most significant. High nitrate reduction rate than nitrite reduction of the selected species in the glucose-fed reactor could be the possible reason for nitrite accumulation (Chakravarthy *et al.* 2011). Similar observations had been obtained by Ge *et al.* (2012) and Yang *et al.* (2012). However, when mixed carbon source (sodium acetate/glucose) was added, the NO_2^--N accumulation was greatly alleviated. For NH_4^+-N, although not contained in the influent, its concentration in the effluent of the sodium acetate, glucose and sodium acetate/glucose reactor reached 0.13, 1.56 and 0.95 mg/L, respectively. The dissimilatory nitrate reduction to ammonium (DNRA) was assumed to be responsible for the NH_4^+-N production (Hardison *et al.* 2015). In general, denitrification process is the main competitor of DNRA process, and DNRA phenomenon often appears at higher C/N ratio. However, present experimental results showed that DNRA could happen when C/N ratio was as low as 1.5. Akunna *et al.* (1993) pointed out that using glucose as carbon source may foster the reduction of NO_3^--N to NH_4^+-N whereas using acetic acid as the carbon source was beneficial to the conversion of NO_3^--N to N_2. A similar result was reported by Srinandan *et al.* (2012). These are in agreement with the results in the present study that effluent NH_4^+-N concentration of glucose reactor was higher than that of sodium acetate reactor. Herein, it is worth noting that using binary mixtures of sodium acetate and glucose produced less NO_2^--N and NH_4^+-N, compared with using glucose alone.

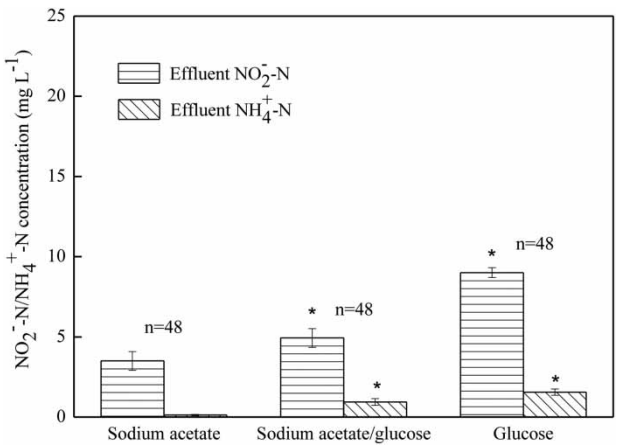

Figure 3 | NO_2^--N and NH_4^+-N concentration profiles with different carbon sources. Data are the mean values of different measurements. Error bars represent standard deviations of statistical analysis. *n* means measurement times. The asterisk (*) indicates statistical differences ($p < 0.05$) from the sodium acetate reactor.

Comparison of denitrification performance

Denitrification performance observed in this study was compared with other published studies using acetate or glucose for denitrification (Table 2).

By a simple comparison, the SDR values obtained in present study were lower than those in the references listed. This can be largely ascribed to lower nitrate concentrations or lower C/N ratios in this study compared to other studies. In fact, SDR values are correlated with many experimental factors, such as carbon source, influent nitrate concentration, C/N ratio, HRT, temperature, inoculum, reactor's operational mode and so on; thereby the reported SDR data in different literature are usually scattered so that an in-depth comparison becomes difficult.

Among various factors influencing the denitrification performance, C/N ratio and HRT are of great concern.

Table 2 | Comparison of SDRs using acetate/glucose from other literature and this study

Carbon source	Operational mode	Initial nitrate concentration (mg/L)	SDR (mg N/g VSS d)	C/N	HRT (h)	T (°C)	Source of inocula	Reference
Acetate	Batch	1,000	220[a]	2.0	15.8	37	An up-flow denitrification sludge blanket reactor	Sánchez *et al.* (2000)
Acetate	Batch	1,000	350[a]	1.3	15.8	37		
Acetate	Batch	750	238[a]	1.94	/	23	A wastewater treatment plant	Bilanovic *et al.* (1999)
Glucose	Batch	200	64.8[b]	5.4	96	30	A pilot anaerobic digester	Akunna *et al.* (1993)
Acetate	Continuous	50	47.7[b]	1.5	4	30	An anoxic tank of an industrial wastewater treatment plant	The present study
Glucose	Continuous	50	29.7[b]	1.5	4	30		
Acetate + glucose	Continuous	50	45.4[b]	1.5	4	30		

[a]Maximum value.
[b]Average value.

Thereupon, the following discussion focused on these two factors.

In terms of C/N ratio, it has been referred as a key factor to influence denitrification process. Akunna *et al.* (1992) reported that in a continuous flow anaerobic reactor fed with nitrate and glucose, methane production without denitrification took place and DNRA was the main pathway for nitrate reduction at C/N > 19.9, denitrification and methane production occurred simultaneously at $3.3 \leq C/N \leq 19.9$ and denitrification without methane production happened at C/N < 3.3. Similarly, Ruiz *et al.* (2006) found that C/N ratio showed a strong influence on the biomass activity, and therefore on the metabolic pathways of nitrate and organic matter utilization in UASB reactors fed with nitrate and sodium acetate. At C/N = 0.375, nitrate removal was poor and serious nitrite accumulation occurred; whereas at C/N > 1.875, nitrate was practically 100% eliminated. At $C/N \leq 3.75$, denitrification represented by far the main rout of organic matter consumption. At C/N = 37.5, over 97% of the organic carbon source was used to produce methane. Chiu & Chung (2003) investigated the optimal C/N ratio for complete denitrifications in a semi-continuous flow reactor with sodium acetate as carbon source. They concluded that the lower the initial nitrate concentration, the higher the corresponding optimal C/N ratio. Specifically, the optimal C/N ratios for initial nitrate concentrations of 25, 50, 100, and 200 mg/L were 5.5, 4.5, 4.0 and 2.6, respectively. In addition, one of our previous works demonstrated that 99.5% nitrate removal could be achieved at a C/N ratio as low as 1.5 in a continuous-operation biofilm-electrode reactor with sodium acetate as the carbon source (Peng *et al.* 2015). In that case, the heterotrophic denitrification accounted for 92% of nitrate removal. Therefore, the C/N ratio was set as 1.5 in the present study and pretty good nitrate removal efficiency (96.53%) was obtained. In theory, the minimum C/N ratio required for complete heterotrophic denitrification is 1.5 and 1.56, corresponding to utilizing acetate and glucose as carbon source, respectively, hence a C/N ratio lower than 1.5 is unreasonable. It is very likely that denitrification will be impeded due to insufficient carbon source, which was verified in our previous work (Peng *et al.* 2015). A sharp drop of nitrate removal rate from 99.5% to 64.1% was observed as the C/N ratio decreased from 1.5 to 0.8. On the other hand, from the perspective of further optimization, a somewhat larger C/N ratio over 1.5 would probably be better. In practice, more carbon will be demanded as it is not only used for respiration, but also for cell growth and maintenance (Constantin & Fick 1997).

As regards HRT, its effects on denitrification were investigated in our earlier work (Wu *et al.* 2018). In that work, the influent nitrate concentration of 50 mg/L and the C/N ratio of 1.5 were kept unchanged in a UASB reactor and different HRTs (8, 6, 4, 2 h) were tested in order. To attain the highest removal rates of NO_3^--N and total nitrogen, the optimal HRT was found to be 6 h with glucose as the carbon source while

it became 4 h as the carbon source was converted to sodium acetate. A conclusion was drawn that HRT, combined with carbon source types, had a significant effect on denitrification and HRT affected the balance among denitrifying bacteria, bacteria responsible for DNRA and other heterotrophic bacteria. In the present study, HRT was set as 4 h considering the mixed carbon source employed was composed of sodium acetate and glucose. What can be expected is further reduction of HRT is inadvisable. If HRT gets too short, a portion of glucose in the form of intermediate metabolites will be washed out of the reactor at the stage of acidification fermentation, as a result, they cannot provide electrons for denitrification in time. On the other hand, appropriate extension of HRT in the range of 4–6 h may be desirable and worth further research.

Overall, it must be noted that the optimal C/N ratio and HRT for a biological denitrification system treating a specific wastewater under special conditions should be determined experimentally.

Effluent pH

pH is a very important index related to denitrification performance (Ge *et al.* 2012). The effluent pH values of the sodium acetate, glucose and sodium acetate/glucose reactor varied in the range of 9.13–9.60, 7.32–7.60 and 7.80–8.23, respectively. This observation is in agreement with previous studies (Yang *et al.* 2012; Ma *et al.* 2015). In theory, regardless of what type of carbon source used, the total amount of OH^- produced by denitrification to remove the same NO_3^--N is equal. However, the difference between the effluent pH values of the sodium acetate and glucose reactor was significant. With the same pH (7.3 ± 0.2) in the influent, the effluent pH of the sodium acetate reactor increased to exceed the discharge standard limit of 9.0, whereas that of the glucose reactor changed little. This difference could be explained from two aspects. Firstly, sodium acetate, as a strong base weak acid salt, once dissolved in water, produced OH^-. Secondly, CO_2 was produced in the fermentation of glucose and the resulting CO_3^{2-} or HCO_3^- could buffer the system's pH. The experimental results demonstrated that keeping pH in a reasonable range was one of the advantages of mixed carbon sources.

Microbial community

Diversity of microbial communities

To explore the microbial utilization mechanisms of different carbon sources, the 16S rRNA gene based high-throughput sequencing was used to analyze sludge samples. The Shannon indexes of R0, R1, R2, and R3 were 6.41, 5.53, 5.74 and 5.34, respectively. The larger the Shannon value, the higher the community diversity. Obviously, the Shannon index of the mixed carbon sources was increased compared to that of a single carbon source. This result hinted that the mixed carbon source was beneficial to the improvement of microbial community diversity, which was linked to the stability of the microbial system (Hagman *et al.* 2008; Zhang *et al.* 2016b).

In order to deeply explore the effect of carbon sources on microbial community, sludge samples were analyzed at phylum level (Figure 4).

Compared with R0, *Proteobacteria* and *Firmicutes* were enriched in R1, R2 and R3. The relative abundance of *Proteobacteria* and *Firmicutes* in R0 were 41.8% and 1.54%, respectively; whereas the relative abundance of *Proteobacteria* and *Firmicutes* were 61.13% and 4.85% in R1, 60.39% and 2.53% in R2, and 68.69% and 2.24% in R3, respectively. The microbial community of denitrification was mainly distributed in *Proteobacteria* (Zhang *et al.* 2012; Ma *et al.* 2015), and *Firmicutes* was found to be responsible for denitrification process under anaerobic conditions and could survive under the conditions lacking organic substrates (Wang *et al.* 2009). By contrast, the relative abundance of *Bacteroidetes*, *Planctomycetes* and *Acidobacteria* decreased from 19.54%, 8.16% and 8.27% in R0 to 10%, 3.36% and 3.52% in R1, 14.22%, 3.01% and 2.67% in R2, and 13.39%, 2.32% and 2.14% in R3, respectively. *Bacteroidetes*, *Planctomycetes* and *Acidobacteria* were found in granular sludge bed reactor via autotrophic nitrogen removal process (Wang *et al.* 2012). However, the difference of above-mentioned phyla among R1, R2 and R3 was not significant.

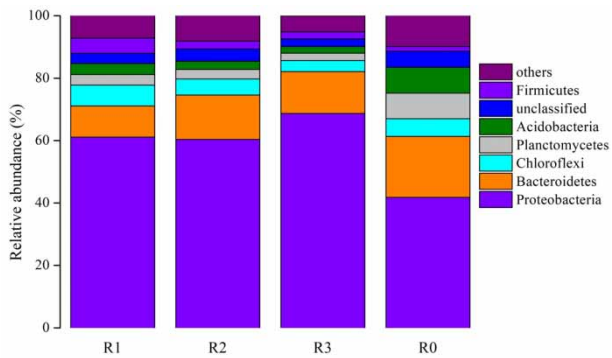

Figure 4 | Distribution of bacterial community with different carbon sources at phylum level.

The heatmap of genera with different carbon sources is shown in Figure 5. For the sodium acetate reactor (R1), *Thauera* (15.03%) and *Comamonas* (12.30%) were the two dominant genera. For the glucose reactor (R3), *Rhizobium* (12.23%), *Comamonas* (5.95%), *Aeromonas* (4.71%), *Acidovorax* (4.39%) and *Elstera* (3.53%) were the dominant

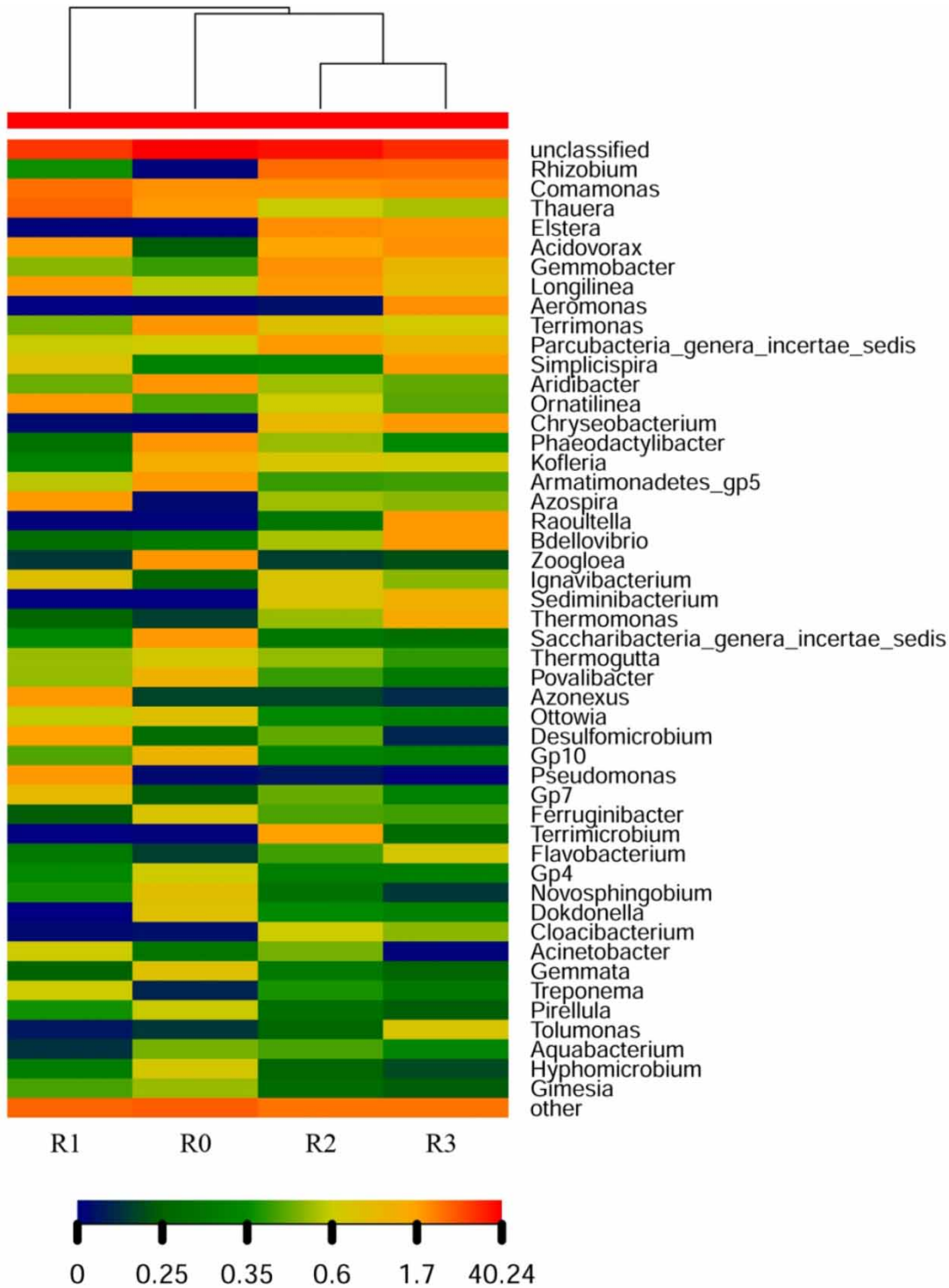

Figure 5 | Heatmap of genera with different carbon sources.

genera. As regards the sodium acetate/glucose reactor (R2), *Rhizobium* (13.15%), *Elstera* (5.05%), *Gemmobacter* (3.87%) and *Comamonas* (3.83%) were the dominant genera. Among the abovementioned genera, only *Thauera* (2.22%) and *Comamonas* (3.84%) were the dominant genera for R0. *Thauera* was reported to be the dominant denitrifying bacteria in acetate supported denitrification system (Ginige *et al.* 2005; Osaka *et al.* 2006). Su *et al.* (2020) discovered that *Comamonas* sp. exhibited excellent nitrogen removal ability under low C/N conditions (C/N = 2.5), with no accumulation of nitrite. The important role of *Comamonas* in the nitrogen removal was also confirmed in present study. Under a C/N ratio as low as 1.5, the relative abundance of *Comamonas* in R1 was the highest (12.30%), meanwhile the NO_3^--N removal rate of R1 was the highest (98.15%) with the lowest effluent nitrite concentration. Compared with glucose, sodium acetate proved to be more favorable for enriching *Comamonas.* Considering the relative abundance of *Rhizobium* in R2 (13.15%) and R3 (12.23%), which were markedly higher than that in R1 (0.37%) and R0 (0.01%), it is inferred that the enrichment of *Rhizobium* was closely correlated with glucose. According to Gonçalves Pessoa *et al.* (2019), the genus *Aeromonas* comprises D-glucose fermentative facultative anaerobic bacteria that are D-glucose fermenters with or without gas production. In addition, they are catalase and oxidase positive, reduce nitrate to nitrite, produce several enzymes and are capable of using other carbohydrates besides glucose. For *Acidovorax,* it belongs to the class *Betaproteobacteria*, and was reported to be the most dominant genus at a low COD to nitrate ratio of 1:1 (Rungkitwatananukul *et al.* 2016). In the present study, more *Acidovorax* was enriched with glucose than with sodium acetate. With regard to *Elstera,* it belongs to the family *Rhodospirillaceae* and class *α-proteobacteria*, comprising just two species so far, i.e. *Elstera cyanobacteriorum* and *Elstera litoralis. E. cyanobacteriorum* was found to be positive for nitrate reduction and denitrification (Cai *et al.* 2018). Besides, *Gemmobacter* exhibited a relative abundance of 3.87% in R2 and 1.35% in R3. *Gemmobacter* belonged to the class *α-Proteobacteria* (Liu *et al.* 2014), and had been identified as denitrifying bacteria (Wei *et al.* 2017). These results mentioned above indicated that different carbon sources led to a different microbial community structure at the genus level.

Cost estimation

Actually, the choice of carbon sources in sewage treatment plants is often affected by economic factors. Based on the denitrification performance of sodium acetate, sodium acetate/glucose and glucose system, the economic analysis of different carbon sources was conducted. It is assumed that the treatment scale of denitrification system is 1,000 m^3/d and the characteristics of the influent are the same as presented in this study, aiming to obtain an effluent nitrate concentration of 5 mg/L. Sodium acetate, sodium acetate/glucose and glucose are respectively used as the external carbon sources for denitrification. The estimated cost with different carbon sources was presented in Table 3.

Clearly, the cost of sodium acetate/glucose was the lowest, which was about 0.64 RMB/m^3. Compared with sodium acetate and glucose, sodium acetate/glucose could reduce the carbon source cost by 0.36 and 0.03 RMB/m^3, respectively. In addition, compared with sodium acetate,

Table 3 | Carbon source cost with different carbon sources

Item	Value	Unit	Remark
Operation parameter			
Wastewater treatment capacity	1,000	m^3/d	
Initial concentration	50	mg/L	
Effluent concentration	5	mg/L	
Price of carbon source			
Sodium acetate	2,500	RMB/ton	Purity (58%)
Glucose	2,400	RMB/ton	Purity (99%)
Cost of carbon source			
Sodium acetate	1,005	RMB/d	Sodium acetate (402 kg/d)
Sodium acetate/glucose	642.7	RMB/d	Sodium acetate (115 kg/d)/glucose (148 kg/d)
Glucose	669.6	RMB/d	Glucose (279 kg/d)

sodium acetate/glucose could save the chemical cost for pH adjustment.

CONCLUSIONS

Glucose and acetate are traditional external carbon sources for nitrate removal. However, they both have inherent flaws, making a preferable alternative to be expected. This study aimed to investigate the comprehensive performance of the binary mixture of sodium acetate and glucose for deep denitrification. The experimental results revealed that the mixed carbon source was advantageous over each single component. Compared with glucose, the mixture exhibited higher denitrification rate and produced less undesirable NO_2^--N and NH_4^+-N. Compared with sodium acetate, the mixture afforded an effluent pH in compliance with emission standards. In addition, the biodiversity with the mixed carbon source was the highest, whereas its cost was the lowest. These findings contribute to a reasonable selection of carbon sources for deep denitrification in practice. However, the optimizations of C/N ratio and HRT were not included in the present study. These issues deserve to be addressed in further research.

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REFERENCES

Akunna, J. C., Bizeau, C. & Moletta, R. 1992 Denitrification in anaerobic digesters: possibilities and influence of wastewater COD/N-NOx ratio. *Environmental Technology* **13** (9), 825–836.

Akunna, J. C., Bizeau, C. & Moletta, R. 1993 Nitrate and nitrite reductions with anaerobic sludge using various carbon sources: glucose, glycerol, acetic acid, lactic acid and methanol. *Water Research* **27** (8), 1303–1312.

APHA, AWWA & WEF 2005 *Standard Methods for the Examination of Water and Wastewater*, 21st edn. American Public Health Association/American Water Works Association/Water Environment Federation, Washington, DC, USA.

Bilanovic, D., Battistoni, P., Cecchi, F., Pavan, P. & Mata-Alvarez, J. 1999 Denitrification under high nitrate concentration and alternating anoxic conditions. *Water Research* **33** (15), 3311–3320.

Cai, H. Y., Cui, H. L., Zeng, Y. H., An, M. L. & Jiang, H. L. 2018 *Sandarakinorhabdus cyanobacteriorum* sp. nov., a novel bacterium isolated from cyanobacterial aggregates in a eutrophic lake. *International Journal of Systematic and Evolutionary Microbiology* **68** (3), 730–735.

Calderer, M., Gibert, O., Marti, V., Rovira, M., de Pablo, J., Jordana, S., Duro, L., Guimera, J. & Bruno, J. 2010 Denitrification in presence of acetate and glucose for bioremediation of nitrate-contaminated groundwater. *Environmental Technology* **31** (7), 799–814.

Chakravarthy, S. S., Pande, S., Kapoor, A. & Nerurkar, A. S. 2011 Comparison of denitrification between *Paracoccus* sp. and *Diaphorobacter* sp. *Applied Biochemistry and Biotechnology* **165** (1), 260–269.

Chen, H. B., Wang, D. B., Li, X. M., Yang, Q. & Zeng, G. M. 2015 Enhancement of post-anoxic denitrification for biological nutrient removal: effect of different carbon sources. *Environmental Science and Pollution Research* **22** (8), 5887–5894.

Chiu, Y.-C. & Chung, M.-S. 2003 Determination of optimal COD/nitrate ratio for biological denitrification. *International Biodeterioration & Biodegradation* **51** (1), 43–49.

Christensson, M., Lie, E. & Welander, T. 1994 A comparison between ethanol and methanol as carbon sources for denitrification. *Water Science and Technology* **30** (6), 83–90.

Constantin, H. & Fick, M. 1997 Influence of C-sources on the denitrification rate of a high-nitrate concentrated industrial wastewater. *Water Research* **31** (3), 583–589.

Elefsiniotis, P., Wareham, D. G. & Smith, M. O. 2004 Use of volatile fatty acids from an acid-phase digester for denitrification. *Journal of Biotechnology* **114** (3), 289–297.

Ge, S., Peng, Y., Wang, S., Lu, C., Cao, X. & Zhu, Y. 2012 Nitrite accumulation under constant temperature in anoxic denitrification process: the effects of carbon sources and COD/NO_3-N. *Bioresource Technology* **114**, 137–143.

Ginige, M. P., Keller, J. & Blackall, L. L. 2005 Investigation of an acetate-fed denitrifying microbial community by stable isotope probing, full-cycle rRNA analysis, and fluorescent in situ hybridization-microautoradiography. *Applied and Environmental Microbiology* **71** (12), 8683–8691.

Gonçalves Pessoa, R. B., de Oliveira, W. F., Marques, D. S. C., dos Santos Correia, M. T., de Carvalho, E. V. M. M. & Coelho, L. C. B. B. 2019 The genus *Aeromonas*: a general approach. *Microbial Pathogenesis* **130**, 81–94.

Hagman, M., Nielsen, J. L., Nielsen, P. H. & Jansen, J. L. 2008 Mixed carbon sources for nitrate reduction in activated sludge-identification of bacteria and process activity studies. *Water Research* **42** (6–7), 1539–1546.

Hardison, A. K., Algar, C. K., Giblin, A. E. & Rich, J. J. 2015 Influence of organic carbon and nitrate loading on partitioning between dissimilatory nitrate reduction to ammonium (DNRA) and N_2 production. *Geochimica et Cosmochimica Acta* **164**, 146–160.

Henze, M. 1991 Capabilities of biological nitrogen removal process from wastewater. *Water Science and Technology* **23** (4), 669–679.

Huang, B. C., Feng, H. J., Wang, M. Z., Li, N., Cong, Y. Q. & Shen, D. S. 2013 The effect of C/N ratio on nitrogen removal in a bioelectrochemical system. *Bioresource Technology* **132**, 91–98.

Li, P., Zuo, J., Wang, Y., Zhao, J., Tang, L. & Li, Z. 2016 Tertiary nitrogen removal for municipal wastewater using a solid-phase denitrifying biofilter with polycaprolactone as the carbon source and filtration medium. *Water Research* **93**, 74–83.

Liu, J., Zhang, X., Chi, F., Pan, J., Sun, C. & Wu, M. 2014 *Gemmobacter megaterium* sp. nov., isolated from coastal planktonic seaweeds. *International Journal of Systematic and Evolutionary Microbiology* **64**, 66–71.

Ma, Q., Qu, Y., Shen, W., Zhang, Z., Wang, J., Liu, Z., Li, D., Li, H. & Zhou, J. 2015 Bacterial community compositions of coking wastewater treatment plants in steel industry revealed by Illumina high-throughput sequencing. *Bioresource Technology* **179**, 436–443.

Osaka, T., Yoshie, S., Tsuneda, S., Hirata, A., Iwami, N. & Inamori, Y. 2006 Identification of acetate- or methanol-assimilating bacteria under nitrate-reducing conditions by stable-isotope probing. *Microbial Ecology* **52** (2), 253–266.

Peng, H., Liu, W. Y., Liu, S. X., Chen, Y. H. & Xiao, H. 2015 Effect of low C/N ratios on denitrification in a biofilm-electrode reactor. *Chinese Journal of Environmental Engineering* **9** (7), 3263–3268 (in Chinese).

Rabah, F. K. J. & Dahab, M. F. 2004 Nitrate removal characteristics of high performance fluidized-bed biofilm reactors. *Water Research* **38** (17), 3719–3728.

Ribera-Guardia, A., Kassotaki, E., Gutierrez, O. & Pijuan, M. 2014 Effect of carbon source and competition for electrons on nitrous oxide reduction in a mixed denitrifying microbial community. *Process Biochemistry* **49** (12), 2228–2234.

Ruiz, G., Jeison, D. & Chamy, R. 2006 Development of denitrifying and methanogenic activities in USB reactors for the treatment of wastewater: effect of COD/N ratio. *Process Biochemistry* **41** (6), 1338–1342.

Rungkitwatananukul, P., Nomai, S., Hirakata, Y., Pungrasmi, W., Puprasert, C., Hatamoto, M. & Yamaguchi, T. 2016 Microbial community analysis using MiSeq sequencing in a novel configuration fluidized bed reactor for effective denitrification. *Bioresource Technology* **221**, 677–681.

Sánchez, M., Mosquera-Corral, A., Méndez, R. & Lema, J. M. 2000 Simple methods for the determination of the denitrifying activity of sludges. *Bioresource Technology* **75** (1), 1–6.

Srinandan, C. S., D'Souza, G., Srivastava, N., Nayak, B. B. & Nerurkar, A. S. 2012 Carbon sources influence the nitrate removal activity, community structure and biofilm architecture. *Bioresource Technology* **117**, 292–299.

Su, J. F., Yang, S., Huang, T. L., Li, M., Liu, J. R. & Yao, Y. X. 2020 Enhancement of the denitrification in low C/N condition and its mechanism by a novel isolated *Comamonas* sp. YSF15. *Environmental Pollution* **256**, 113294.

Wang, L., Zheng, P., Chen, T. T., Chen, J. W., Xing, Y. J., Ji, Q. X., Zhang, M. & Zhang, J. Q. 2012 Performance of autotrophic nitrogen removal in the granular sludge bed reactor. *Bioresource Technology* **123**, 78–85.

Wang, W., Wang, Y., Zhang, Q., Qi, Y. & Guo, D. 2009 Global characterization of *Artemisia annua* glandular trichome transcriptome using 454 pyrosequencing. *BMC Genomics* **10** (1), 465–465.

Wei, H., Wang, J., Hassan, M., Han, L. & Xie, B. 2017 Anaerobic ammonium oxidation-denitrification synergistic interaction of mature landfill leachate in aged refuse bioreactor: variations and effects of microbial community structures. *Bioresource Technology* **243**, 1149–1158.

Wu, J. J., Li, Y. M., Tan, D. M., Peng, H. & Xiao, H. 2018 Effects of HRT on anaerobic denitrification in UASB. *Chinese Journal of Environmental Engineering* **12** (5), 1510–1516 (in Chinese).

Xu, Z. S., Dai, X. H. & Chai, X. L. 2018 Effect of different carbon sources on denitrification performance, microbial community structure and denitrification genes. *Science of the Total Environment* **634**, 195–204.

Yang, X. P., Wang, S. M. & Zhou, L. X. 2012 Effect of carbon source, C/N ratio, nitrate and dissolved oxygen concentration on nitrite and ammonium production from denitrification process by *Pseudomonas stutzeri* D6. *Bioresource Technology* **104**, 65–72.

Zhang, T., Shao, M. F. & Ye, L. 2012 454 Pyrosequencing reveals bacterial diversity of activated sludge from 14 sewage treatment plants. *ISME Journal* **6** (6), 1137–1147.

Zhang, Y. M., Wang, X. C. C., Cheng, Z., Li, Y. Y. & Tang, J. L. 2016a Effect of fermentation liquid from food waste as a carbon source for enhancing denitrification in wastewater treatment. *Chemosphere* **144**, 689–696.

Zhang, Y. M., Wang, X. C. C., Cheng, Z., Li, Y. Y. & Tang, J. L. 2016b Effects of additional fermented food wastes on nitrogen removal enhancement and sludge characteristics in a sequential batch reactor for wastewater treatment. *Environmental Science and Pollution Research* **23** (13), 12890–12899.

Zhang, R.-C., Xu, X.-J., Chen, C., Xing, D.-F., Shao, B., Liu, W.-Z., Wang, A.-J., Lee, D.-J. & Ren, N.-Q. 2018 Interactions of functional bacteria and their contributions to the performance in integrated autotrophic and heterotrophic denitrification. *Water Research* **143**, 355–366.

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