Environmental Research 212 (2022) 113403

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Comparison of primary and secondary sludge carbon sources derived from hydrolysis or acidogenesis for nitrate reduction and denitrification kinetics: Organics utilization and microbial community shift

Yiding Guo[a,b], Liang Guo[b,c,*], Chunji Jin[b], Yangguo Zhao[b], Mengchun Gao[b], Junyuan Ji[b,**], Zonglian She[b], John P. Giesy[d,e]

[a] *College of Environmental Science and Engineering, Tongji University, Shanghai, 200092, China*
[b] *College of Environmental Science and Engineering, Ocean University of China, Qingdao, 266100, China*
[c] *Key Laboratory of Marine Environmental and Ecology, Ministry of Educatin, Ocean University of China, Qingdao, 266100, China*
[d] *Department of Veterinary Biomedical Sciences and Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan, Canada*
[e] *Department of Environmental Science, Baylor University, Waco, TX, USA*

ARTICLE INFO

Keywords:
Denitrification kinetics
Proteins
Carbohydrates
Volatile fatty acids (VFAs)
Microbial community

ABSTRACT

Seeking available and economical carbon sources for denitrification process is an intractable issue for wastewater treatment. However, no study compared different types of waste sludge as carbon source from denitrification mechanism, organics utilization and microbial community aspects. In this study, primary and secondary sludge were pretreated by thermophilic bacteria (TB), and its hydrolysis or acidogenic liquid were prepared as carbon sources for denitrification. At C/N of 8–3, the variations of NO_3^--N and NO_2^--N were profiled in typical cycles and denitrification kinetics was analyzed. Primary sludge achieved a competitive NO_X-N removal efficiency with less dosage than secondary sludge. Fourier transform infrared (FTIR) spectroscopy was introduced to analyze organic composition from functional-group perspective and the utilization of organic matters in different sludge carbon sources was investigated. To further analyze the microbial community shift in different denitrification systems, high-throughput sequencing technology was applied. Results showed that denitrifier *Thauera*, belonging to *Proteobacteria*, was predominant, and primary sludge acidogenic liquid enriched *Thauera* most intensively with relative abundance of 47.3%.

1. Introduction

For a long time, it has been a common issue that lack of available carbon sources restricts denitrification in WWTPs (wastewater treatment plants). Denitrification is the microbial process by which fixed nitrate and nitrite are reduced to gaseous forms of nitrogen, by use of electrons and energy provided by carbon sources. Various, alternative carbon sources, including chemical pure carbon sources, solid carbon (Kiani et al., 2020) and concentrated organic wastewater (Fernandez-Nava et al., 2010; Zheng et al., 2018b) have been identified. However, the disadvantages of the carbon sources mentioned above, such as high cost, secondary pollution, requirements for long retention time and acute change of wastewater characteristics, impede their extensive application. Use of fermentation liquid formed during treatment of organic wastes is an attractive, alternative carbon source for denitrification, due to not only enhancement of nitrogen removal, but also reduction of wastes that need to be treated or disposed of to minimize their release into the environment (Frison et al., 2013; Lee et al., 2014; Zheng et al., 2018b). Large amounts of waste sludge need to be treated annually, which occupies 60% cost of operation for WWTPs (Coma et al., 2013). Fortunately, organic substances in waste sludge can be degraded through anaerobic digestion to simple molecules, that can be carbon sources to drive denitrification (Sun et al., 2016).

Primary and secondary sludge are two main by-products during operation of WWTPs. Primary sludge, in which the organics are contained in large separate particles, is produced in the primary settling tank (Ji et al., 2010; Wu and He, 2012). Due to the limited removal of organic matters in primary settling tank in WWTPs, it is often assumed

---

* Corresponding author. College of Environmental Science and Engineering, Ocean University of China, Qingdao, 266100, China.
** Corresponding author.
E-mail addresses: geletu@ouc.edu.cn (L. Guo), ouc_jjy@163.com (J. Ji).

https://doi.org/10.1016/j.envres.2022.113403
Received 14 February 2022; Received in revised form 23 April 2022; Accepted 28 April 2022
Available online 5 May 2022

that the organic content of primary sludge is small (Li et al., 2020). However, particulate material in primary sludge can be hydrolyzed into more readily biodegradable organics and serve as carbon source during removal of nutrient. Hydrolyzed primary sludge afforded similar removal efficiency of nitrogen with pure methanol (Gali et al., 2006). Addition of primary sludge fermentation liquor was also proved to improve nitrogen removal efficiency (Huang et al., 2020). Secondary sludge, in which a great deal of biomass exists with organic matter wrapped by cell walls, is produced during biological treatment (Ji et al., 2010; Song et al., 2010). Many pretreatments were applied to strengthen the degradation of glycan strands of cell walls to release more organics (Kim et al., 2009; Ruiz-Hernando et al., 2014). These released substances exhibited an excellent enhancement of heterotrophic denitrification (Kim et al., 2009; Tong and Chen, 2009). In our previous research, the utilization of organics in secondary sludge carbon source was analyzed and the optimal operation conditions were selected (Guo et al., 2017, 2018). However, specific differences between primary and secondary sludge as carbon source during denitrification remained unclear. Specifically, the denitrification dynamics, utilization of various organic compounds in the sludge carbon source and characteristics of the microbial community remained to be elucidated.

During denitrification, availability of sludge carbon source has a close affinity with denitrification rates (Zhang et al., 2016). Thermophilic bacteria (TB) treatment was applied to enhance the availability of sludge carbon source. It is a novel process that the thermostable protease excreted from thermophilic stains is used to enhance the sludge solubilization and accelerate the pathogens removal (Lee et al., 2009; Yang et al., 2015). It exhibited much superiority in inactivating pathogen, releasing organics from sludge, reducing solid content and producing biogas (Guo et al., 2012; Kavitha et al., 2016). In our previous study, a bacterial strain *Bacillus* sp. *AT07-1* was isolated to enhance sludge solubilization and hydrogen fermentation (Guo et al., 2012), but was not further applied in denitrification. To investigate the optimum condition of denitrification when using the sludge carbon source pretreated by TB, two factors were selected. First one is amounts of carbon present, measured as the C/N ratio. It is recognized that denitrification intermediate NO_2^--N accumulates when C/N is low and the effluent COD increases when excess carbon source is supplied (Krishna Mohan et al., 2016; Sahinkaya et al., 2013). The amount of carbon supplied influences not only the electron donor for denitrification, but also growth of microbes. Secondly, carbon generated from hydrolysis or acidogenesis of sludge also affects its availability. Hydrolysis and acidogenesis are the first two steps of anerobic fermentation (Zamri et al., 2021). VFAs, which are regarded as the preferable electron donor for denitrification, is generated primarily during acidogenesis (Yuan et al., 2019; Zamri et al., 2021). However, organic matters, including proteins and carbohydrates, released during hydrolysis can also act as carbon source for denitrification, although they were utilized after VFAs had been exhausted (Guo et al., 2017, 2018). Consequently, C/N and the origin of sludge carbon source were selected as two influencing factors during this study.

In this study, primary and secondary sludge hydrolysis or acidogenic liquid, which were pretreated by TB, were selected as carbon sources. With carbon source derived from different types of waste sludge, the nitrogen variations at different C/N (8–3) were profiled. Parameters characterizing kinetics of denitrification, including the NO_3^--N to NO_2^--N transformation ratio (NTR), rate of denitrification (V_{DN}), potential for denitrification (P_{DN}) and heterotroph anoxic yield (Y_H), were determined. Fourier transform infrared (FTIR) spectroscopy was used to characterize functional groups of various carbon sources. The utilization of organic matters such as chemical oxygen demand (COD), proteins, carbohydrates and volatile fatty acids (VFAs) was quantified. To further explore functional groups of bacteria during denitrification, microbial communities in reactors were characterized by use of high-throughput sequencing.

2. Materials and methods

2.1. Sludge carbon source

Waste sludge, which was used as carbon source and seeding sludge for denitrification, was collected from Haibohe WWTP (Qingdao, China). Sludge was concentrated by settling for 24 h, at 4 °C and then the supernatant was discarded. Sludge characteristics are listed in Table 1. The thermophilic bacterial strain *Bacillus* sp. AT07-1 (registration number: FJ231108), which was used to pretreat sludge, was inoculated in broth medium at 65 °C, 120 rpm for 24 h in a shaking bath (Guo et al., 2012).

Primary or secondary sludge was mixed 50:1 (v/v) with bacteria suspension and then hydrolyzed at 65 °C, 120 rpm for 12 h in a shaking bath in microaerobic environment. The mixture was centrifuged at 2862×*g* for 10 min, and the supernatant collected was defined as the hydrolysis liquid. In order to obtain the acidogenic liquid, the hydrolyzed sludge was fermented at 35 °C, 120 rpm for 60 h in a shaking bath in anaerobic environment, and then centrifuged at 2862×*g* for 10 min. The hydrolysis or acidogenic liquid were stored at 4 °C for use. The characteristics of sludge carbon source are shown in Table 2.

2.2. Operation of reactors

Simulated nitrate-containing wastewater, which contained potassium nitrate (50 mg/L NO_3^--N), dipotassium hydrogen phosphate (COD/phosphorus was 20:1), sodium bicarbonate (pH 7.0 to 7.5), trace element, and sludge carbon source, was synthesized in this study. The trace element comprised $MgSO_4$, $FeSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, $MnCl_2$, $NiCl_2{\cdot}6H_2O$, H_3BO_3, $ZnSO_4{\cdot}7H_2O$, $CuSO_4{\cdot}5H_2O$ and $CoCl_2{\cdot}6H_2O$ (Sun et al., 2016). The C/N, which ranged from 8 to 3, was adjusted by the amount of sludge carbon source added. Nitrogen gas was pumped for 5 min to build anoxic environment in synthetic wastewater. Denitrification was carried out in four plastic cylinders (2.0 L working volume) operated as a sequencing mode. R_1, R_2, R_3 and R_4 represented reactors with primary sludge hydrolysis liquid, secondary sludge hydrolysis liquid, primary sludge acidogenic liquid and secondary sludge acidogenic liquid as carbon source, respectively. All reactors were operated with the following cycles controlled by a timer: filling (7 min); mixing (7 h); settling (40 min); decanting (3 min) and idle (10 min). One liter supernatant was discharged during the decanting period with an equal volume of fresh synthetic wastewater added to reactors during filling phase. The whole operating process was conducted for 60 days. Samples, to determine whether the reactors become stable at each C/N, were collected every two days. When reactors become stable, a specific operating cycle was chosen to measure variations in concentrations of NO_3^--N and NO_2^--N, during which the samples were taken every 15 min in the first 90 min, and every 30 min in the rest time. Samples were taken in triplicate.

2.3. Determination of chemical properties

Concentrations of NO_3^--N, NO_2^--N and NH_4^+-N were determined according to the standard methods (APHA, 2005). Concentrations of proteins and carbohydrates were detected by Lowry's method and

Table 1
Characteristics of sludges.

Sludge type	COD/ (mg/L)	Protein/ (mg/L)	Carbohydrate/ (mg/L)	TSS/ (g/L)	VSS/ (g/L)	pH
Primary sludge	3567.2 ± 125.3	565.4 ± 37.5	60.7 ± 5.4	12.5 ± 0.3	7.5 ± 0.4	7.2 ± 0.1
Secondary sludge	3326.9 ± 132.1	605.6 ± 54.3	40.5 ± 6.7	11.8 ± 0.2	7.2 ± 0.2	7.1 ± 0.2

Table 2
Characteristics of sludge carbon sources.

Carbon source	COD/ (mg/L)	Protein/ (mg/L)	Carbohydrate/ (mg/L)	VFAs/ (mg/L)
Primary sludge hydrolysis liquid	7399.7 ± 313.2	2704.2 ± 108.9	308.6 ± 10.7	NA
Secondary sludge hydrolysis liquid	6931.8 ± 324.6	2454.2 ± 124.4	268.0 ± 13.6	NA
Primary sludge acidogenic liquid	6463.9 ± 297.8	1995.8 ± 98.6	270.3 ± 12.2	3121.4 ± 140.4
Secondary sludge acidogenic liquid	5929.1 ± 288.6	2120.8 ± 105.8	283.8 ± 11.9	2678.6 ± 129.6

phenol-sulfuric acid method, respectively (Sun et al., 2016). Suspended solid (SS) and volatile suspended solid (VSS) were quantified gravimetrically (He, 1998). COD was estimated after digestion as described by (Knechtel, 1978). VFAs were identified and quantified by use of a Gas Chromatography (GC 2010, Shimadzu, Japan), which was equipped with a capillary column (DB-FFAP, 30 mm × 0.25 mm × 0.25 mm) and a flame ionization detector (FID). The sample was acidified with 3% H_3PO_4 to pH 3.0 and the injection volume was 1.0 μL. The parameters set has been described previously (Guo et al., 2017). All assays were performed in triplicate.

To further characterize and compare organic constituents, among sludge carbon sources, FTIR spectroscopy was applied. The sludge carbon source was freeze-dried to powder via a vacuum freeze-drier (Labconco7670530, USA) Tensor 27, and then was mixed with KBr at 100:1 in an agate grinder. Samples were then examined by use of FTIR spectrometer (Bruker Optics, Ltd, Germany) after being pressed into tablets. The scanning wavenumber ranged from 4000 to 400 cm^{-1}.

2.4. Kinetics of denitrification

During denitrification, NO_3^--N is first transformed to NO_2^--N and then reduced to N_2. NO_2^--N is an intermediate product and its toxic potency is greater than NO_3^--N (Zhang et al., 2020a). NTR is often used to measure NO_3^--N to NO_2^--N transformation ratio, which is calculated as following equation (Du et al., 2016):

$$\mathrm{NTR} = \left(\mathrm{NO_2^-{-}N_t} - \mathrm{NO_2^-{-}N_i}\right)/\left(\mathrm{NO_3^-{-}N_i} - \mathrm{NO_3^-{-}N_t}\right) \times 100\% \tag{1}$$

Where: NO_3^--N_i and NO_2^--N_i are the initial NO_3^--N and NO_2^--N concentration, respectively (mg/L); NO_3^--N_t and NO_2^--N_t are the NO_3^--N and NO_2^--N concentration at sampling time, respectively (mg/L).

To comprehensively describe removal of NO_3^--N and NO_2^--N, NO_X-N, which was calculated by NO_3^--N+0.6 NO_2^--N theoretically (Sage et al., 2006), was introduced to evaluate denitrification performance in the presence of various sludge carbon sources. According to NO_X-N removal curve (Sage et al., 2006), deduced some denitrification parameters that could be used to characterize kinetics of denitrification, including V_{DN} (mg NO_X-N/g VSS·h), P_{DN} (g NO_X-N/g COD) and Y_H (g COD/g COD):

$$\mathrm{V_{DN}} = (\mathrm{NO_X{-}N_i} - \mathrm{NO_X{-}N_e})/(\mathrm{VSS} \times \mathrm{t}) \tag{2}$$

$$\mathrm{PDN} = (\mathrm{NO_X{-}N_i} - \mathrm{NO_X{-}N_e})/(\mathrm{COD_i} - \mathrm{COD_e}) \tag{3}$$

$$1 - \mathrm{Y_H} = 2,86\mathrm{P_{DN}} \tag{4}$$

Where: NO_X-N_i and COD_i are the initial NO_X-N and COD concentration, respectively (mg/L); NO_X-N_e and COD_e are the NO_X-N and COD concentration at the end of denitrification reaction, (mg/L); t is the denitrification reaction time (h); VSS is the concentration of volatile suspended solids in reactors (g/L).

2.5. Analysis of microbial community

Samples of sludge were collected at the end of every C/N stage to ensure the bacteria adaption. Eight samples of sludge R_{1opt}, R_{2opt}, R_{3opt}, R_{4opt}, R_{1low}, R_{2low}, R_{3low} and R_{4low} were selected to investigate microbial community. The markers 'opt' and 'low' represented the optimal C/N (5 for primary sludge carbon source and 6 for secondary sludge carbon source) and the lowest C/N (C/N = 3), respectively. The analysis included extraction and detection of DNA, amplification by use of Polymerase Chain Reaction (PCR), purification of PCR products, library preparation, test on Illumina HiSeq and analysis of sequence data. Briefly, DNA was extracted by PowerSoil DNA® Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) and the quality was monitored by 1% agarose gel electrophoresis. The primers 515 F and 806 R in V4 region were chosen for amplification of 16 S rRNA gene by PCR and the amplified products were monitored with 2% agarose gel electrophoresis. Sequencing of PCR products was carried out on the HiSeq 2500 PE 250 platform in Novogene (Beijing, China).

3. Results and discussion

3.1. Denitrification performance with different sludge carbon sources

3.1.1. The removal of NO_X-N during the whole process

Efficiency of removal of NO_X-N with various carbon sources derived from different types of waste sludge was analyzed (Fig. 1). Initially (0–18 d), at C/N of 8 or 7, approximately 98% of NO_X-N was removed, without fluctuation in four reactors, except that the effluent NO_X-N accumulated accidently in R_4 at 8 d and R_1 at 16 d. These results were superior to those of a previous study in which denitrifiers achieved 98.1% NO_3^--N removal after 40 d accommodation with alkaline fermentation liquid as carbon source (Sun et al., 2016). This indicated that denitrification bacteria adapted faster to the TB pretreated carbon source. When it is introduced to be carbon source, pretreatment of sludge not only influences the content of released organics, but also denitrification. Results of previous study had shown that the liquid product of alkaline fermentation of sludge contained significant amounts of soluble organics, but resulted in lesser removal of nitrate than when the fermentation liquid was added without pretreatment (Cao et al., 2019a). As C/N decreased to 6, the effluent NO_X-N initially increased to 12.9, 6.4 and 20.6 mg/L, respectively, in R_1, R_2 and R_4. Then, the NO_X-N was removed completely with reactors operating. This result suggested that at C/N of 6, denitrification bacteria with primary sludge hydrolysis liquid, secondary sludge hydrolysis liquid and secondary sludge acidogenic liquid needed cultivation before achieving excellent denitrification. In comparison, using primary sludge acidogenic liquid as carbon source (R_3), excellent and stable denitrification was obtained in the range of C/N from 8 to 5. This demonstrated that, in terms of stable complete denitrification, primary sludge acidogenic liquid was the best of four sludge carbon sources.

As C/N further decreased to 4 and 3, serious deterioration in NO_X-N removal was observed in R_1 and R_3. This result demonstrated that there were insufficient electron donors for complete denitrification at C/N of 4 and 3 with primary sludge carbon sources. Thus, the optimal C/N was 5 for primary sludge carbon sources. In R_2 and R_4, complete denitrification was obtained at C/N of 6 or higher, whereas significant amounts of residual NO_X-N remained in effluents of reactors with C/N ratios of 5 or less. Thus, the optimal C/N was 6 for secondary sludge carbon sources. The different optimum C/N ratios between the primary and secondary sludge carbon source were caused by the structural distinction. The organic substances in primary sludge exist in separate large particles and are more available, while the secondary sludge is mainly formed by microorganisms and organic matters are primarily in the form of polymers (Ruiz-Hernando et al., 2014; Ucisik and Henze, 2008).

3.1.2. The variations of NO_X-N during the specific cycle

In the profile of NO_3^--N and NO_2^--N during a specific operating cycle, regardless of which carbon source was used, NO_3^--N was rapidly removed in the initial 75 min at optimal C/N or higher (Fig. 2). It was

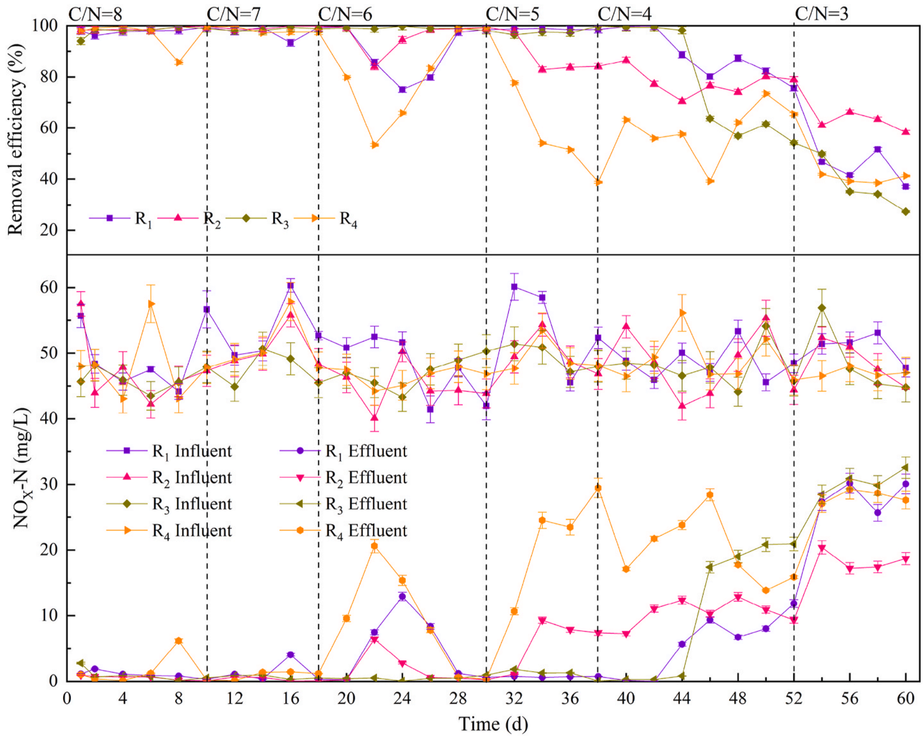

Fig. 1. Removal of NO_x-N with different sludge carbon sources as C/N decreasing
(R_1: Primary sludge hydrolysis liquid; R_2: Secondary sludge hydrolysis liquid; R_3: Primary sludge acidogenic liquid; R_4: Secondary sludge acidogenic liquid).

faster than with thermal fermentation liquid, which required 180 min to remove all the NO_3^--N at optimal C/N of 7 (Guo et al., 2018). This indicated that sludge carbon source pretreated by TB could enhance NO_3^--N removal more efficiently with less dosage.

In all reactors, obvious accumulation of NO_2^--N occurred temporarily (at optimal C/N or higher) or permanently (at low C/N) during a typical operating cycle. This was due to the presence of nitrate respiring bacteria, which were not true denitrifiers and only converted NO_3^--N to NO_2^--N (Ge et al., 2012). The over-competition of nitrate reductase, which inhibited nitrite reductase activity for substrate electrons, also caused the imbalance in reduction of NO_3^--N and NO_2^--N (Ge et al., 2012). As C/N decreased, accumulation of NO_2^--N increased and time required to remove nitrogen increased. This result demonstrated that competition between nitrate reductase and nitrite reductase was more intense when carbon was scarce, and that nitrite reductase was more sensitive to decreases in the amount of carbon. Regardless of whether the carbon source was through hydrolysis or acidogenesis, primary sludge carbon source achieved a lower NO_2^--N accumulation and a shorter removal time. This result indicated that primary sludge carbon source more efficiently provided electrons for reduction of NO_2^--N than secondary sludge carbon source.

3.1.3. *Denitrification kinetics*

In order to compare the carbon sources derived from different sludge, avoiding the influence of insufficient carbon source supply, denitrification parameters were calculated at optimal C/N or greater (Eqs. (1)–(4)) (Table 3). NTR at NO_2^--N accumulation peak, calculated by Eq. (1), represented the ratio of NO_3^--N transformed to NO_2^--N without further reduction at the maximal NO_2^--N accumulation point. At each C/N, NTR in R_1 and R_3 were lower than that in R_2 and R_4, respectively. Especially at C/N of 7, the NTR at NO_2^--N accumulation peak in R_3 was 16.6 percentage points lower than in R_4. This indicated that with primary sludge carbon source, there was more NO_2^--N immediately reduced after conversion from NO_3^--N. Primary sludge minimized accumulation of NO_2^--N more efficiently than did secondary sludge. This result was consistent with the results mentioned above. Some researchers also found that primary sludge carbon source showed a desirable NO_2^--N reduction capacity at optimal dosage (Cao et al., 2020a).

Comprehensive reduction of NO_3^--N and NO_2^--N was represented by denitrification rate (V_{DN}), which was calculated by Eq. (2). V_{DN} in R_1 was 1.4-, 2.0- and 4.3-fold of that in R_2 at C/N of 8, 7 and 6, respectively, and V_{DN} in R_3 was 2.8-, 1.9- and 2.5-fold of that in R_4. Carbon derived from primary sludge increased V_{DN} more efficiently than did secondary sludge. This was due to readily available organics in the primary sludge rather than the carbon being present as polymers in the secondary sludge (Ucisik and Henze, 2008). In addition, the V_{DN} with acidogenic liquid was higher than with hydrolysis liquid, which was due to the more degradable organic matter in acidogenic liquid.

P_{DN}, represented denitrification potential, was deduced by Eq. (3) to evaluate denitrification ability (measured by NO_X-N removal) with equivalent amounts of carbon source (measured by COD) (Sage et al., 2006). That is, with the same amount of different sludge carbon sources, greater NO_X-N removal resulted in higher P_{DN}. Compared with optimal C/N ratio, P_{DN} decreased by 0.02–0.09 g NO_X-N/g COD at high C/N with different sludge carbon sources. This result demonstrated that excess carbon did not enhance denitrification. Some researchers have suggested that surplus COD would support some other pathways in denitrifiers, such as assimilation, or some other bacteria, such as methanogens (Ge et al., 2012; Zhang et al., 2020a). Heterotroph anoxic yield (Y_H), which represented the "lost" COD consumed in other pathways rather than used for denitrification (Sage et al., 2006), showed coherent results. Thus, it was unwise to raise C/N blindly.

3.2. *Utilization of organic matter in different sludge carbon sources*

3.2.1. *Consumption of COD*

During denitrification, simultaneous consumption of COD from various sludge with C/N from 8 to 3 was observed (Fig. 3). Initially (0–6 d at C/N = 8), efficiency of utilization of COD in R_1-R_4 fluctuated, which

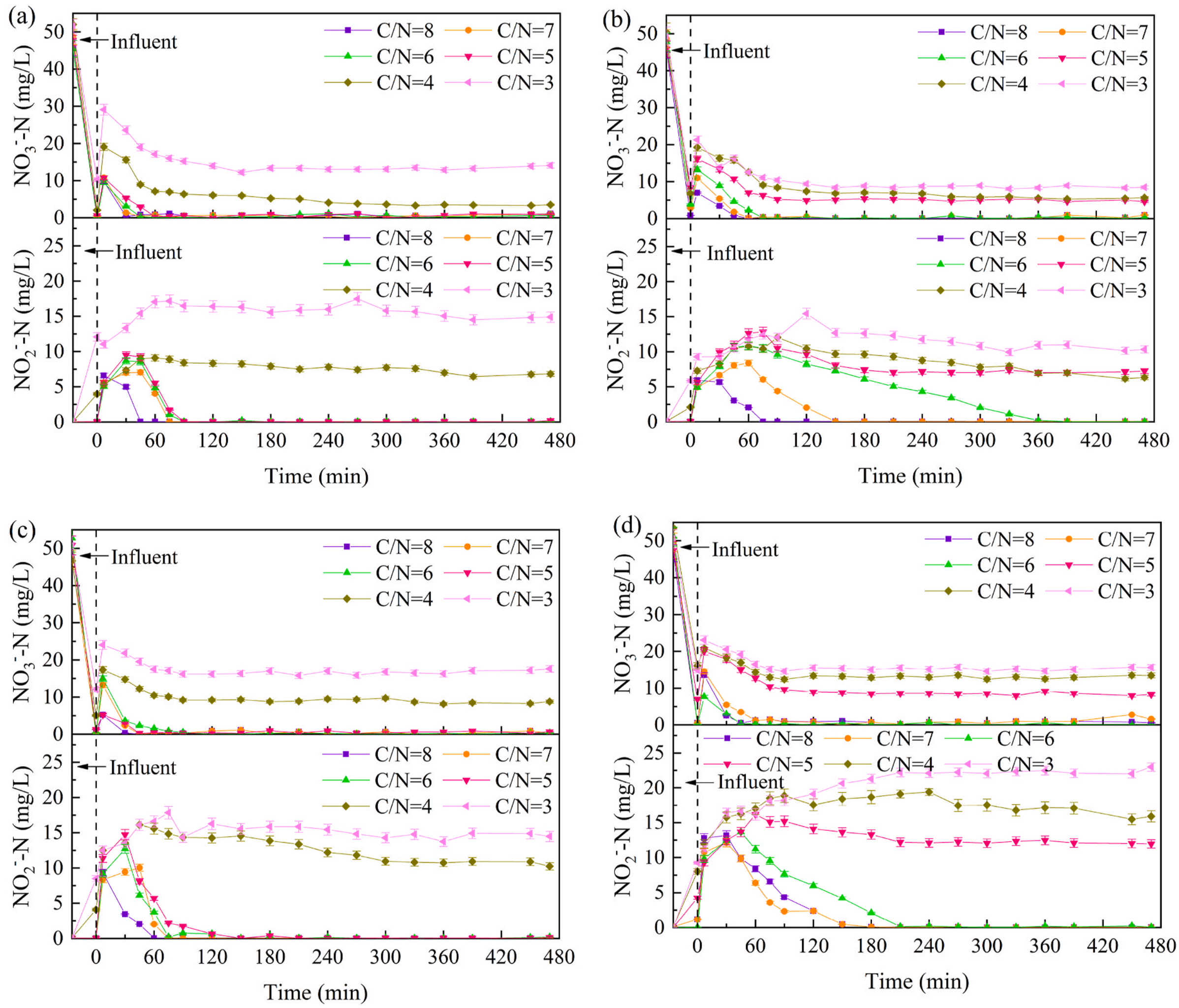

Fig. 2. Reduction curves of NO_3^--N and NO_2^--N in typical cycles with different sludge carbon sources with range of C/N of 8 to 3
(a: Primary sludge hydrolysis liquid; b: Secondary sludge hydrolysis liquid; c: Primary sludge acidogenic liquid; d: Secondary sludge acidogenic liquid).

Table 3
Denitrification parameters with different sludge carbon sources at optimal C/N or higher.

Parameters	Reactor[a]	C/N			
		8	7	6	5
NTR (%)	R_1	21.9	29.1	43.9	50.0
	R_2	36.2	32.6	45.9	–
	R_3	50.3	42.2	55.2	63.5
	R_4	59.4	58.8	53.8	–
V_{DN} (mg NO_X-N/g VSS·h)	R_1	10.37	8.55	8.42	7.13
	R_2	7.62	4.16	1.95	–
	R_3	15.62	10.17	10.51	7.20
	R_4	5.61	5.28	4.15	–
P_{DN} (g NO_X-N/g COD)	R_1	0.16	0.18	0.20	0.24
	R_2	0.16	0.20	0.25	–
	R_3	0.15	0.18	0.24	0.23
	R_4	0.18	0.20	0.20	–
Y_H (g COD/g COD)	R_1	0.55	0.47	0.42	0.31
	R_2	0.56	0.42	0.28	–
	R_3	0.56	0.47	0.32	0.33
	R_4	0.48	0.44	0.43	–

[a] R_1: Primary sludge hydrolysis liquid; R_2: Secondary sludge hydrolysis liquid; R_3: Primary sludge acidogenic liquid; R_4: Secondary sludge acidogenic liquid.

indicated unstable reactor performance. However, it didn't affect NO_X-N removal (Fig. 1) due to the excess carbon source supply at C/N of 8. With reactors operating (6–30 d at C/N = 8, 7 and 6), utilization of COD tended to be similar and stable no matter with which carbon source. As C/N further decreased to 5, efficiency of utilization of COD in R_1 and R_3 was 9.1 and 6.8 percentage points higher than in R_2 and R_4, respectively. This facilitated greater removal of NO_X-N with primary sludge carbon source at C/N of 5 (Fig. 1). At C/N of 4, COD was further utilized in R_2 and R_4 while its utilization efficiency decreased in R_1 and R_3. As C/N decreased to 3, efficiency of utilization of COD decreased in all reactors, but R_2 and R_4 was still higher than R_1 and R_3. This was attributed to that some refractory organics in secondary sludge carbon source such as building blocks were degraded when the carbon source was insufficient (Cao et al., 2019b). However, due to the extreme scarcity of carbon at C/N of 4 and 3, removal of NO_X-N in R_2 and R_4 was unsatisfactory.

3.2.2. Analysis of specific organic matters

Fig. 4a shows the organic components in different sludge carbon sources at functional-group level by FTIR. The broad absorption region with peaks at 3150-3186 cm^{-1} and 2968-2972 cm^{-1}, which are due to O–H stretch, N–H stretch and C–H antisymmetric stretch of carboxyl, amide and alkyl group, respectively, represent several types of organic. The weak peak at 2353-2360 cm^{-1} is related to the CO_2 absorbed from surrounding environment (Kumar et al., 2006). The sharp absorption at 1568-1570 cm^{-1} is attributed to the N–H deformation vibration in

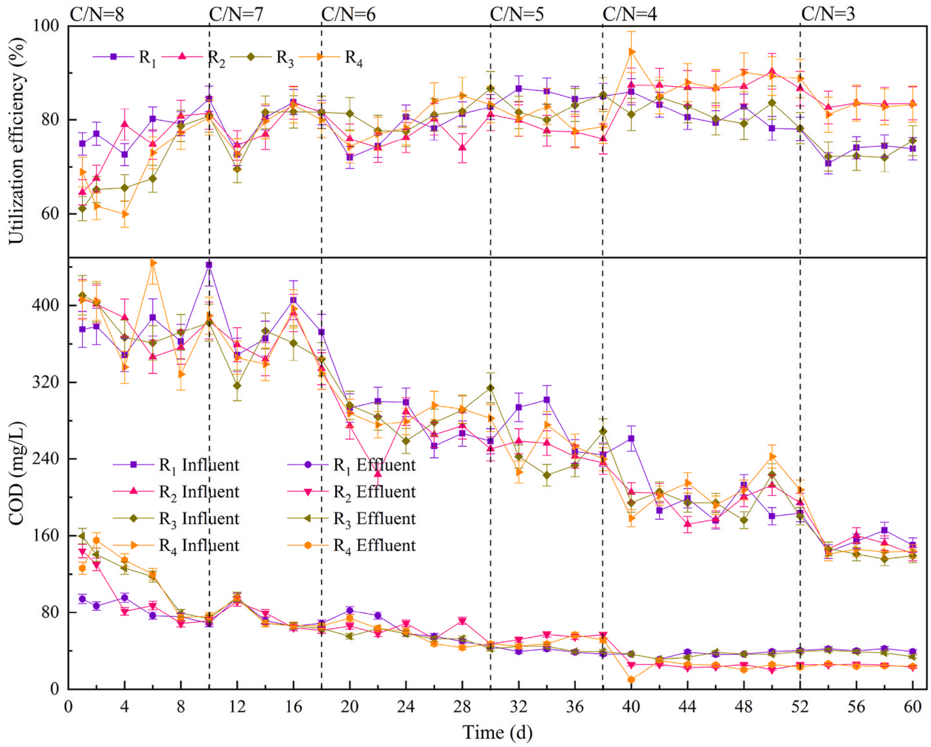

Fig. 3. Utilization of COD with different sludge carbon sources as C/N decreasing
(R_1: Primary sludge hydrolysis liquid; R_2: Secondary sludge hydrolysis liquid; R_3: Primary sludge acidogenic liquid; R_4: Secondary sludge acidogenic liquid).

amide II (Zhang et al., 2015). Another strong absorption appeared at 1406-1412 cm^{-1}, which is formed by C–N stretch in amide and C–H deformation vibration in alkyl or allyl group. Pronounced peaks near 3150, 1570 and 1410 cm^{-1} demonstrate the existence of proteins. C–O stretching vibrations in carbohydrates cause the absorption at 1020-1057 cm^{-1}. Bands lower than 1000 cm^{-1} in the "fingerprint region", are attributed to unsaturated bonds. Characteristic peaks were located at similar wavenumbers in R_1-R_4 (Fig. 4a), which was indicative of presence of similar organic constituents in various sludge carbon sources. These results further illustrated that it was not the type, but proportions of various organic matters influenced denitrification in the presence of different carbon sources.

Various organic components in sludge, including proteins (Fig. 4b), carbohydrates (Fig. 4c) and VFAs (Fig. 4d), can be used as carbon sources. A remarkable increase in protein and carbohydrate utilization was observed in R_4 at C/N lower than 5, which was higher than R_3. It demonstrated that proteins and carbohydrates in secondary sludge acidogenic liquid could be further degraded at low C/N, which caused the increasing utilization of COD at low C/N. It has been reported that proteins and high-molecular-weight carbohydrates in alkaline fermentation liquid of secondary sludge could be further reduced during prolonged hydraulic retention time (HRT>12 h) (Cao et al., 2019b). The HRT in this study was 16 h. This could explain why the efficiency of removal of NO_X-N in R_4 at C/N of 4 and 3 (65.4 and 41.3%) was higher than in R_3 (54.3 and 27.4%) as shown in Fig. 1. Nevertheless, better denitrification was observed in R_3 than R_4 at C/N of 5, which was due to sufficient available carbon, without further utilizing refractory organic matter, in primary sludge acidogenic liquid at C/N of 5. Compared with acidogenic liquid (R_3 and R_4), hydrolysis liquid (R_1 and R_2) exhibited greater utilization of proteins and carbohydrates at C/N higher than 5. This was due to the fact that more preferred organic matter, such as VFAs, for denitrification bacteria were available in acidogenic liquid (Fig. 4d). More than 90.0% of VFAs was utilized in R_3 and R_4, which indicated that VFAs in acidogenic liquid were preferentially utilized by denitrifiers than proteins and carbohydrates.

3.3. *Microbial community analysis*

The major phyla and patterns of relative proportions of genera varied among sludge carbon sources (Fig. 5). *Proteobacteria*, which contains a number of denitrification bacteria (Cao et al., 2020a), was the most dominant phylum and accounted for 48.6–68.7% among sludge samples (Fig. 5a). The genus *Thauera*, belonging to the phylum *Proteobacteria*, was predominant with relative abundance of 22.2–47.3% among sludge samples (Fig. 5b). It had been reported that *Thauera* can act as denitrifiers (Liu et al., 2017). Primary sludge acidogenic liquid (R_{3opt}) intensively stimulated growth of *Thauera* (47.3%) compared with other carbon sources (Fig. 5b and c). Sample R_{2low} showed a higher relative abundance of *Paracocccus* (3.5%) and *Defluviimonas* (2.9%), both of which were also denitrifiers (Sun et al., 2018; Zhou et al., 2017). It indicated that *Paracocccus* and *Defluviimonas* had a low threshold for C/N with secondary sludge hydrolysis liquid as carbon source. *Chryseobacterium*, *Shinella* and *Hyphomicrobium* with mean, relative abundances less than 1.0% could also contribute to denitrification (Chen et al., 2018; Guo and Liu, 2020; Li et al., 2016). From the above results, it can be concluded that *Thauera* was absolutely dominant. In addition, the total relative abundance of the denitrifying bacteria varied from 28.2 to 51.1% among different sludge samples and the maximum value was also obtained with primary sludge acidogenic liquid. The relative abundance of denitrifying bacteria determines the denitrification performance. That was why the primary sludge acidogenic liquid achieved the most efficient denitrification.

In addition to denitrifing bacteria, fermentative bacteria were also observed. For example, *Thermobrachium* and *Anaerolinea* can utilize proteins and carbohydrates as energy sources and transform them (Qiu et al., 2011; Zhang et al., 2017). *Trichococcus*, *Smithella*, *Proteiniclasticum*, *Petrimonas* and *Acetoanaerobium*, which were able to degrade complex organic substrates into more simple molecules, have been reported to be acid-forming bacteria (He et al., 2018; Mielcarek et al., 2016; Zheng et al., 2018a). The existence of these fermentative bacteria was caused by the addition of sludge carbon source and fermentation

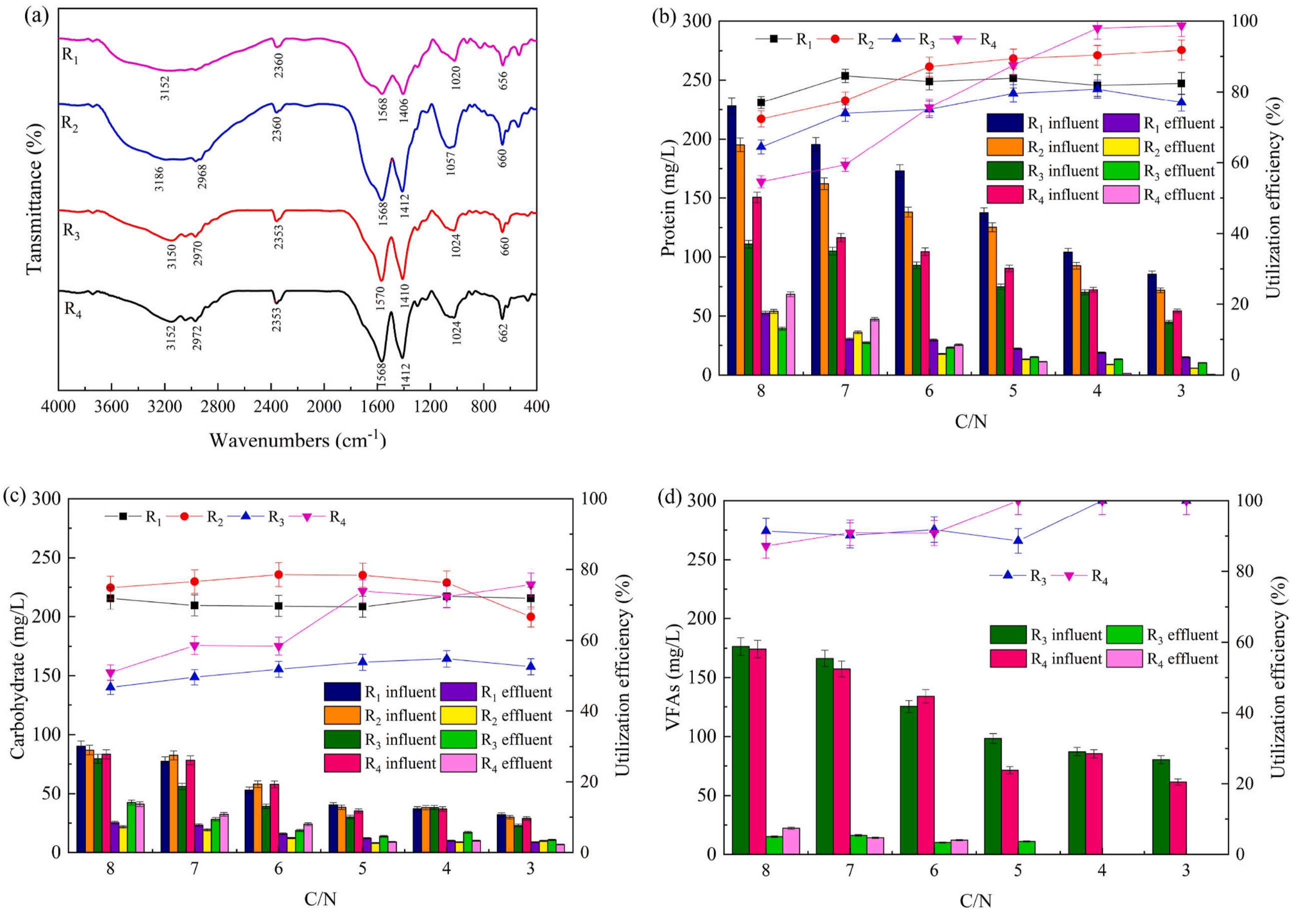

Fig. 4. FTIR spectra of sludge carbon source (a) and the organic matters utilization with different sludge carbon sources as C/N decreasing (b, c and d) (R_1: Primary sludge hydrolysis liquid; R_2: Secondary sludge hydrolysis liquid; R_3: Primary sludge acidogenic liquid; R_4: Secondary sludge acidogenic liquid).

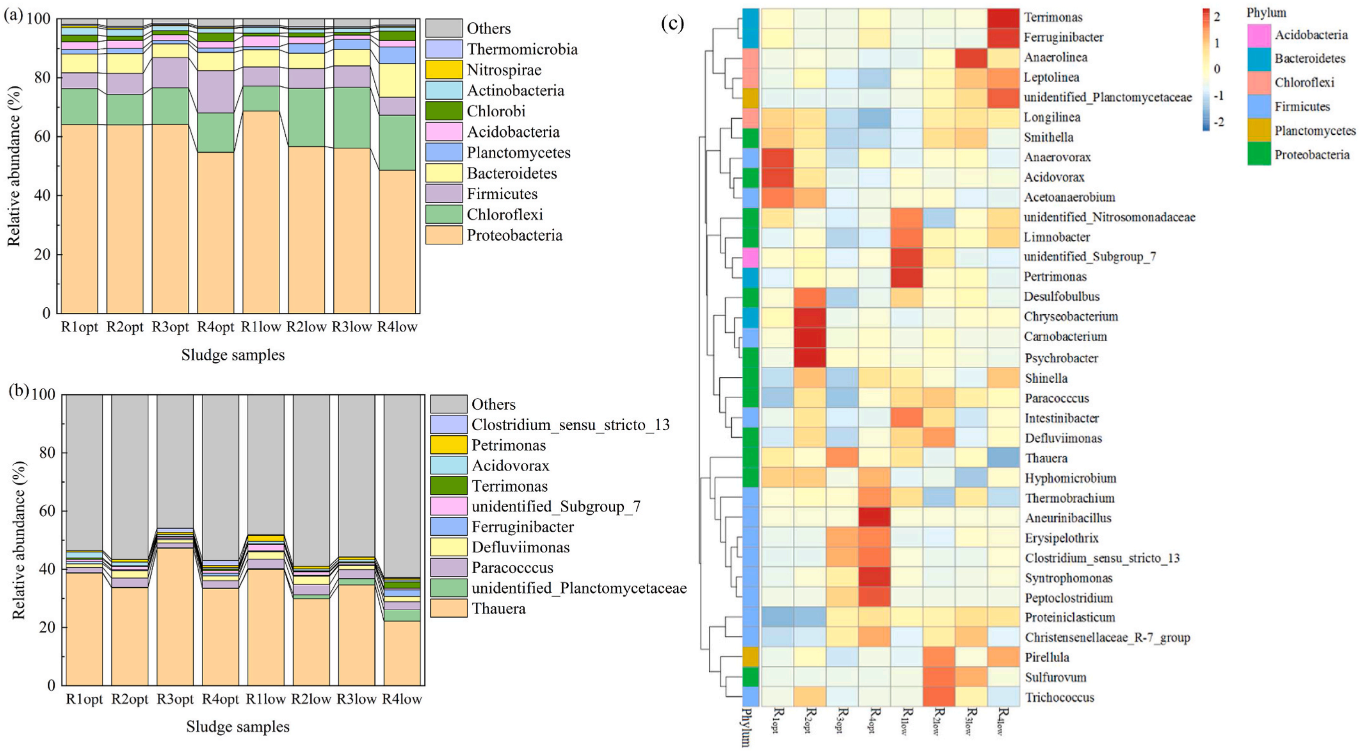

Fig. 5. Predominant phyla (a) and genera (b) in four reactors at specific C/N and heatmap of microbial communities at genus level (c).

occurred simultaneously with heterotrophic denitrification under anoxic condition (Cao et al., 2019b). These fermentative genera accounted for 4.9 and 3.7% at low and optimal C/N, respectively. This indicated that fermentation of sludge would strengthen when the carbon source was not sufficient. Thus, deciding an optimal carbon source dosage is imperative.

3.4. Comparison of sludge carbon source with commercial carbon source

Commercial carbon sources, such as methanol, ethanol and acetate, have been studied deeply and applied widely in WWTPs to enhance heterotrophic denitrification (Wang et al., 2021). Sludge carbon source, as an alternative, is imperative to be compared with traditional commercial carbon sources. The differences between sludge and commercial carbon sources are elucidated from the perspective of structure, denitrification performance and microbial community.

Commercial carbon sources are simple in structure with micromolecular organics, while macromolecular substances in sludge require to be degraded into VFAs or other small molecular organic matters to afford the denitrification (Fu et al., 2022; Wang et al., 2021). Theoretically, smaller molecular organic matters are easily utilized by denitrifying bacteria, hence obtain better denitrification performance (Fu et al., 2022). However, sludge carbon source exhibited higher nitrogen removal rate than commercial carbon sources due to the more biodegradable organics and synergistic effects of different acids (Fu et al., 2022; Mahmoud et al., 2022; Wang et al., 2021). Various nutrients and specialized indigenous microorganisms in sludge carbon source can accelerate the start-up of reaction (Wang et al., 2021). Denitrification systems with multi-carbon organics own higher population diversity than with single-carbon organics, which is conducive to maintain the stability of the reaction system (Lu et al., 2014).

Primary sludge acidogenic liquid, which was selected as the best sludge carbon source in this study, was compared with acetate under salinity conditions in the subsequent experiment. Higher microbial richness and diversity was observed in the system with primary sludge acidogenic liquid than acetate, leading to a more stable respond to the salinity change (Zhang et al., 2020b). Consequently, sludge carbon sources have potential to achieve the sustainable concept with “turning waste into wealth” (Cao et al., 2020b; Fu et al., 2022).

3.5. Application potential

The ultimate purpose of all the laboratory experiments is to guide the practical application. In this study, the primary sludge carbon source, especially primary sludge acidogenic liquid exhibited the best denitrification performance based on a comprehensive evaluation, which indicated its potential in practical application. Firstly, the primary sludge carbon source could achieve comparable denitrification performance with less dosage than the secondary sludge carbon source (Section 3.1.1). With the same amount of primary and secondary sludge, the primary sludge carbon source could treat more wastewater than the secondary sludge carbon source, which would save the cost and time on acidogenic digestion. Secondly, the primary sludge carbon source could mitigate the accumulation of NO_2^--N (Section 3.1.2). It is vital to reduce the intermediate product NO_2^--N due to its toxic potency (Zhang et al., 2020a). Thirdly, primary sludge acidogenic liquid showed superiority in enriching denitrifying bacteria (Section 3.3). For these reasons, primary sludge acidogenic liquid is preferred in practical application.

However, some practical issues should be considered when the primary sludge acidogenic liquid is applied in WWTPs. The acidogenic liquid was prepared after hydrolysis under anaerobic condition, which was strict with the equipment and energy-cost. In contrast, the preparation of hydrolysis liquid was conducted in microaerobic environment without further treatment, which was not very strict with dissolved oxygen. Moreover, the higher Y_H with primary sludge carbon source (Section 3.1.3) meant the higher amount of biological sludge produced due to denitrification, hence extra cost of sludge treatment (Mahmoud et al., 2022). Consequently, it is necessary to seek trade-offs between practical cost and denitrification performance when the laboratory results are applied in practice.

4. Conclusions

It was deemed to be critical to determine the optimal carbon dosage for each sludge used as carbon source. Surplus carbon resulted in lower P_{DN}, while insufficient carbon strengthened fermentative bacteria. The optimal C/N ratio was 5 and 6 with primary and secondary sludge as carbon source, respectively, regardless of hydrolysis or acidogenic liquid. Primary sludge acidogenic liquid exhibited the best denitrification performance, with the stable and complete NO_X-N removal. Primary sludge alleviated NO_2^--N accumulation and elevated V_{DN} more efficiently than did secondary sludge. VFAs was utilized preferentially no matter in which carbon source. The denitrifier *Thauera* was the dominant genus using sludge carbon source and primary sludge acidogenic liquid exhibited a superior enrichment of *Thauera*.

Credit author statement

Yiding Guo: Methodology, Investigation, Data curation, Writing – original draft, Formal analysis. **Liang Guo**: Conceptualization, Methodology, Resources, Supervision, Project administration, Funding acquisition, Writing – review & editing. **Chunji Jin**: Resources. **Yangguo Zhao**: Resources. **Mengchun Gao**: Software. **Junyuan Ji**: Software. **Zonglian She**: Validation. **John P. Giesy**: Writing – review & editing.

Note

This research does not involve human subjects or animal research.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (52070173); Qingdao Science and Technology Benefit People Demonstration and Guidance Project (21-1-4-sf-12-nsh); Urban and Rural Housing Construction Science and Technology Project of Shandong (2021-K8-7); Fundamental Research Funds for the Central Universities (201964005). Prof. Giesy was supported by the Canada Research Chair program, the 2012 “High Level Foreign Experts” (#GDT20143200016) program, funded by the State Administration of Foreign Experts Affairs, the P.R. China to Nanjing University and the Einstein Professor Program of the Chinese Academy of Sciences and a Distinguished Visiting Professorship in the Department of Environmental Sciences, Baylor University in Waco, TX, USA.

References

APHA, 2005. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington, DC, USA.

Cao, S., Du, R., Zhou, Y., 2020a. Development of a denitrification system using primary sludge as solid carbon source - potential to couple with anammox process. Sci. Total Environ. 737, 140315. https://doi.org/10.1016/j.scitotenv.2020.140315.

Cao, S., Qian, T., Zhou, Y., 2019a. New insights on the sludge fermentation liquid driven denitrification: evaluation of the system performance and effluent organic matter (EfOM). Bioresour. Technol. 289, 121621. https://doi.org/10.1016/j.biortech.2019.121621.

Cao, S., Sun, F., Lu, D., et al., 2019b. Characterization of the refractory dissolved organic matters (rDOM) in sludge alkaline fermentation liquid driven denitrification: effect of HRT on their fate and transformation. Water Res. 159, 135–144. https://doi.org/10.1016/j.watres.2019.04.063.

Cao, S., Wang, L., Yan, W., et al., 2020b. Primary sludge as solid carbon source for biological denitrification: system optimization at micro-level. Environ. Res. 191, 110160. https://doi.org/10.1016/j.envres.2020.110160.

Chen, H., Zhao, X., Cheng, Y., et al., 2018. Iron robustly stimulates simultaneous nitrification and denitrification under aerobic conditions. Environ. Sci. Technol. 52, 1404–1412. https://doi.org/10.1021/acs.est.7b04751.

Coma, M., Rovira, S., Canals, J., et al., 2013. Minimization of sludge production by a side-stream reactor under anoxic conditions in a pilot plant. Bioresour. Technol. 129, 229–235. https://doi.org/10.1016/j.biortech.2012.11.055.

Du, R., Peng, Y., Cao, S., et al., 2016. Mechanisms and microbial structure of partial denitrification with high nitrite accumulation. Appl. Microbiol. Biotechnol. 100, 2011–2021. https://doi.org/10.1007/s00253-015-7052-9.

Fernandez-Nava, Y., Maranon, E., Soons, J., et al., 2010. Denitrification of high nitrate concentration wastewater using alternative carbon sources. J. Hazard Mater. 173, 682–688. https://doi.org/10.1016/j.jhazmat.2009.08.140.

Frison, N., Di Fabio, S., Cavinato, C., et al., 2013. Best available carbon sources to enhance the via-nitrite biological nutrients removal from supernatants of anaerobic co-digestion. Chem. Eng. J. 215–216, 15–22. https://doi.org/10.1016/j.cej.2012.10.094.

Fu, X., Hou, R., Yang, P., et al., 2022. Application of external carbon source in heterotrophic denitrification of domestic sewage: a review. Sci. Total Environ. 817, 153061. https://doi.org/10.1016/j.scitotenv.2022.153061.

Gali, A., Dosta, J., Mata-Alvarez, J., 2006. Use of hydrolyzed primary sludge as internal carbon source for denitrification in a SBR treating reject water via nitrite. Ind. Eng. Chem. Res. 45, 7661–7666.

Ge, S., Peng, Y., Wang, S., et al., 2012. Nitrite accumulation under constant temperature in anoxic denitrification process: the effects of carbon sources and COD/NO_3-N. Bioresour. Technol. 114, 137–143. https://doi.org/10.1016/j.biortech.2012.03.016.

Guo, F., Liu, H., 2020. Impact of heterotrophic denitrification on BOD detection of the nitrate-containing wastewater using microbial fuel cell-based biosensors. Chem. Eng. J. 394 https://doi.org/10.1016/j.cej.2020.125042.

Guo, L., Guo, Y., Sun, M., et al., 2018. Enhancing denitrification with waste sludge carbon source: the substrate metabolism process and mechanisms. Environ. Sci. Pollut. Res. Int. 25, 13079–13092. https://doi.org/10.1007/s11356-017-0836-y.

Guo, L., Zhao, J., She, Z., et al., 2012. Effect of S-TE (solubilization by thermophilic enzyme) digestion conditions on hydrogen production from waste sludge. Bioresour. Technol. 117, 368–372. https://doi.org/10.1016/j.biortech.2012.04.010.

Guo, Y., Guo, L., Sun, M., et al., 2017. Effects of hydraulic retention time (HRT) on denitrification using waste activated sludge thermal hydrolysis liquid and acidogenic liquid as carbon sources. Bioresour. Technol. 224, 147–156. https://doi.org/10.1016/j.biortech.2016.11.056.

He, Y.L., 1998. The Anaerobic Biological Treatment of Wastewater. China Light Industry Press, Beijing.

He, Z.W., Liu, W.Z., Gao, Q., et al., 2018. Potassium ferrate addition as an alternative pre-treatment to enhance short-chain fatty acids production from waste activated sludge. Bioresour. Technol. 247, 174–181. https://doi.org/10.1016/j.biortech.2017.09.073.

Huang, X., Dong, W., Wang, H., et al., 2020. Sludge alkaline fermentation enhanced anaerobic- multistage anaerobic/oxic (A-MAO) process to treat low C/N municipal wastewater: nutrients removal and microbial metabolic characteristics. Bioresour. Technol. 302, 122583. https://doi.org/10.1016/j.biortech.2019.122583.

Ji, Z., Chen, G., Chen, Y., 2010. Effects of waste activated sludge and surfactant addition on primary sludge hydrolysis and short-chain fatty acids accumulation. Bioresour. Technol. 101, 3457–3462. https://doi.org/10.1016/j.biortech.2009.12.117.

Kavitha, S., Stella, P.B.C., Kaliappan, S., et al., 2016. Enhancement of anaerobic degradation of sludge biomass through surfactant-assisted bacterial hydrolysis. Process Saf Environ 99, 207–215. https://doi.org/10.1016/j.psep.2015.11.009.

Kiani, S., Kujala, K., Pulkkinen J, T., et al., 2020. Enhanced nitrogen removal of low carbon wastewater in denitrification bioreactors by utilizing industrial waste toward circular economy. J. Clean. Prod. 254 https://doi.org/10.1016/j.jclepro.2020.119973.

Kim, T.H., Nam, Y.K., Park, C., et al., 2009. Carbon source recovery from waste activated sludge by alkaline hydrolysis and gamma-ray irradiation for biological denitrification. Bioresour. Technol. 100, 5694–5699. https://doi.org/10.1016/j.biortech.2009.06.049.

Knechtel, R., 1978. A more economical method for the determination of chemical oxygen demand. Water Pollut. Control 25–29.

Krishna Mohan, T.V., Nancharaiah, Y.V., Venugopalan, V.P., et al., 2016. Effect of C/N ratio on denitrification of high-strength nitrate wastewater in anoxic granular sludge sequencing batch reactors. Ecol. Eng. 91, 441–448. https://doi.org/10.1016/j.ecoleng.2016.02.033.

Kumar, M., Adham, S., Pearce, W., 2006. Investigation of seawater reverse osmosis fouling and its relationship to pretreatment type. Environ. Sci. Technol. 40, 2037–2044.

Lee, S.H., Chung, C.W., Yu, Y.J., et al., 2009. Effect of alkaline protease-producing Exiguobacterium sp. YS1 inoculation on the solubilization and bacterial community of waste activated sludge. Bioresour. Technol. 100, 4597–4603. https://doi.org/10.1016/j.biortech.2009.04.056.

Lee, W.S., Chua, A.S.M., Yeoh, H.K., et al., 2014. A review of the production and applications of waste-derived volatile fatty acids. Chem. Eng. J. 235, 83–99. https://doi.org/10.1016/j.cej.2013.09.002.

Li, Y., Lin, L., Li, X., 2020. Chemically enhanced primary sedimentation and acidogenesis of organics in sludge for enhanced nitrogen removal in wastewater treatment. J. Clean. Prod. 244 https://doi.org/10.1016/j.jclepro.2019.118705.

Li, Y., Xu, Y., Liu, L., et al., 2016. First evidence of bioflocculant from Shinella albus with flocculation activity on harvesting of Chlorella vulgaris biomass. Bioresour. Technol. 218, 807–815. https://doi.org/10.1016/j.biortech.2016.07.034.

Liu, J., Yuan, Y., Li, B., et al., 2017. Enhanced nitrogen and phosphorus removal from municipal wastewater in an anaerobic-aerobic-anoxic sequencing batch reactor with sludge fermentation products as carbon source. Bioresour. Technol. 244, 1158–1165. https://doi.org/10.1016/j.biortech.2017.08.055.

Lu, H., Chandran, K., Stensel, D., 2014. Microbial ecology of denitrification in biological wastewater treatment. Water Res. 64, 237–254. https://doi.org/10.1016/j.watres.2014.06.042.

Mahmoud, A., Hamza, R.A., Elbeshbishy, E., 2022. Enhancement of denitrification efficiency using municipal and industrial waste fermentation liquids as external carbon sources. Sci. Total Environ. 816, 151578. https://doi.org/10.1016/j.scitotenv.2021.151578.

Mielcarek, A., Rodziewicz, J., Janczukowicz, W., et al., 2016. Denitrification aided by waste beer in anaerobic sequencing batch biofilm reactor (AnSBBR). Ecol. Eng. 95, 384–389. https://doi.org/10.1016/j.ecoleng.2016.06.083.

Qiu, C., Wen, J., Jia, X., 2011. Extreme-thermophilic biohydrogen production from lignocellulosic bioethanol distillery wastewater with community analysis of hydrogen-producing microflora. Int. J. Hydrogen Energy 36, 8243–8251. https://doi.org/10.1016/j.ijhydene.2011.04.089.

Ruiz-Hernando, M., Martin-Diaz, J., Labanda, J., et al., 2014. Effect of ultrasound, low-temperature thermal and alkali pre-treatments on waste activated sludge rheology, hygienization and methane potential. Water Res. 61, 119–129. https://doi.org/10.1016/j.watres.2014.05.012.

Sage, M., Daufin, G., Gesan-Guiziou, G., 2006. Denitrification potential and rates of complex carbon source from dairy effluents in activated sludge system. Water Res. 40, 2747–2755. https://doi.org/10.1016/j.watres.2006.04.005.

Sahinkaya, E., Kilic, A., Calimlioglu, B., et al., 2013. Simultaneous bioreduction of nitrate and chromate using sulfur-based mixotrophic denitrification process. J. Hazard Mater. 262, 234–239. https://doi.org/10.1016/j.jhazmat.2013.08.050.

Song, Y.D., Hu, H.Y., Zhou, Y.X., 2010. Lysis of stationary-phase bacterial cells by synergistic action of lytic peptidase and glycosidase from thermophiles. Biochem. Eng. J. 52, 44–49. https://doi.org/10.1016/j.bej.2010.07.002.

Sun, J., Chen, L., Rene, E.R., et al., 2018. Biological nitrogen removal using soil columns for the reuse of reclaimed water: performance and microbial community analysis. J. Environ. Manag. 217, 100–109. https://doi.org/10.1016/j.jenvman.2018.03.088.

Sun, J., Sun, M., Guo, L., et al., 2016. The effects of denitrification with sludge alkaline fermentation liquid and thermal hydrolysis liquid as carbon sources. RSC Adv. 6, 72333–72341. https://doi.org/10.1039/c6ra11982d.

Tong, J., Chen, Y., 2009. Recovery of nitrogen and phosphorus from alkaline fermentation liquid of waste activated sludge and application of the fermentation liquid to promote biological municipal wastewater treatment. Water Res. 43, 2969–2976. https://doi.org/10.1016/j.watres.2009.04.015.

Ucisik, A.S., Henze, M., 2008. Biological hydrolysis and acidification of sludge under anaerobic conditions: the effect of sludge type and origin on the production and composition of volatile fatty acids. Water Res. 42, 3729–3738. https://doi.org/10.1016/j.watres.2008.06.010.

Wang, H., Chen, N., Feng, C., et al., 2021. Insights into heterotrophic denitrification diversity in wastewater treatment systems: progress and future prospects based on different carbon sources. Sci. Total Environ. 780, 146521. https://doi.org/10.1016/j.scitotenv.2021.146521.

Wu, J., He, C., 2012. The effect of settlement on wastewater carbon source availability based on respirometric and granulometric analysis. Chem. Eng. J. 189–190, 250–255. https://doi.org/10.1016/j.cej.2012.02.066.

Yang, Q., Luo, K., Li, X.M., et al., 2015. Solubilization of waste activated sludge and nitrogenous compounds transformation during solubilization by thermophilic enzyme (S-te) process. Appl. Biochem. Biotechnol. 176, 700–711. https://doi.org/10.1007/s12010-015-1605-3.

Yuan, R., Shen, Y., Zhu, N., et al., 2019. Pretreatment-promoted sludge fermentation liquor improves biological nitrogen removal: molecular insight into the role of dissolved organic matter. Bioresour. Technol. 293, 122082. https://doi.org/10.1016/j.biortech.2019.122082.

Zamri, M.F.M.A., Hasmady, S., Akhiar, A., et al., 2021. A comprehensive review on anaerobic digestion of organic fraction of municipal solid waste. Renew. Sustain. Energy Rev. 137, 110637. https://doi.org/10.1016/j.rser.2020.110637.

Zhang, P., Shen, Y., Guo, J.S., et al., 2015. Extracellular protein analysis of activated sludge and their functions in wastewater treatment plant by shotgun proteomics. Sci. Rep. 5, 12041. https://doi.org/10.1038/srep12041.

Zhang, S., Su, J., Zheng, Z., et al., 2020a. Denitrification strategies of strain YSF15 in response to carbon scarcity: based on organic nitrogen, soluble microbial products and extracellular polymeric substances. Bioresour. Technol. 314, 123733. https://doi.org/10.1016/j.biortech.2020.123733.

Zhang, W., Liu, X., Wang, D., et al., 2017. Effects of bamboo charcoal on fouling and microbial diversity in a flat-sheet ceramic membrane bioreactor. Bioresour. Technol. 243, 1020–1026. https://doi.org/10.1016/j.biortech.2017.07.084.

Zhang, Y., Ji, G., Wang, R., 2016. Drivers of nitrous oxide accumulation in denitrification biofilters with low carbon:nitrogen ratios. Water Res. 106, 79–85. https://doi.org/10.1016/j.watres.2016.09.046.

Zhang, Z., Guo, Y., Guo, L., et al., 2020b. Elucidating salinity adaptation and shock loading on denitrification performance: focusing on microbial community shift and carbon source evaluation. Bioresour. Technol. 305, 123030. https://doi.org/10.1016/j.biortech.2020.123030.

Zheng, W., Yu, Z., Xia, Y., et al., 2018a. Influence of polyaluminum chloride on microbial characteristics in anaerobic membrane bioreactors for sludge digestion. Appl.

Microbiol. Biotechnol. 102, 1005–1017. https://doi.org/10.1007/s00253-017-8613-x.

Zheng, X., Zhou, W., Wan, R., et al., 2018b. Increasing municipal wastewater BNR by using the preferred carbon source derived from kitchen wastewater to enhance phosphorus uptake and short-cut nitrification-denitrification. Chem. Eng. J. 344, 556–564. https://doi.org/10.1016/j.cej.2018.03.124.

Zhou, J., Li, H., Chen, X., et al., 2017. Cometabolic degradation of low-strength coking wastewater and the bacterial community revealed by high-throughput sequencing. Bioresour. Technol. 245, 379–385. https://doi.org/10.1016/j.biortech.2017.08.119.