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Effects of residual organics in municipal wastewater on hydrogenotrophic denitrifying microbial communities

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ABSTRACT

Hydrogenotrophic denitrification is promising for tertiary nitrogen removal from municipal wastewater. To reveal the influence of residual organics in municipal wastewater on hydrogenotrophic denitrifiers, we adopted high-throughput 16S rRNA gene amplicon sequencing to examine microbial communities in hydrogenotrophic denitrification enrichments. Using effluent from a municipal wastewater treatment plant as water source, COD, nitrate and pH were controlled the same except for a gradient of biodegradable carbon (*i.e.*, primary effluent (PE), secondary effluent (SE), or combined primary and secondary effluent (CE)). Inorganic synthetic water (IW) was used as a control. *Hydrogenophaga*, a major facultative autotroph, accounted for 17.1%, 5.3%, 32.7% and 12.9% of the sequences in PE, CE, SE and IW, respectively, implicating that *Hydrogenophaga* grew well with or without organics. *Thauera*, which contains likely obligate autotrophic denitrifiers, appeared to be the most dominant genera (23.6%) in IW and accounted for 2.5%, 4.6% and 8.9% in PE, CE and SE, respectively. *Thermomonas*, which is related to heterotrophic denitrification, accounted for 4.2% and 7.9% in PE and CE fed with a higher content of labile organics, respectively. In contrast, *Thermomonas* was not detected in IW and accounted for only 0.6% in SE. Our results suggest that *Thermomonas* are more competitive than *Thauera* in hydrogenotrophic denitrification with biodegradable organics. Moreover, facultative autotrophic denitrifiers, *Hydrogenophaga*, are accommodating to residual organic in effluent wastewater, thus we propose that hydrogenotrophic denitrification is amenable for tertiary nitrogen removal.

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Introduction

Nitrogen pollution is a major contributing nutrient to eutrophication and may pose potential hazards to humans, livestock and the environment (Breisha and Winter, 2010; Ghafari et al., 2008). Total nitrogen (TN) control has become one of the most challenging and important targets of wastewater treatment plants (WWTPs). In recent years, the standard for TN control has become increasingly rigid, particularly for water reuse purposes (Zhao et al., 2013). However, nitrate removal by conventional secondary treatment in WWTPs can hardly fulfil the strict TN control standard (Boltz et al., 2012). Thus, tertiary nitrogen removal units are needed to remove nitrate in the secondary effluent by applying the principle of heterotrophic denitrification, which utilizes organic carbon sources as electron donors for microbial reduction of nitrate. However, the secondary effluent from WWTPs contains insufficient biodegradable organics; hence, dosing with additional organics, such as methanol, ethanol and acetate, is implemented to provide sufficient amounts of carbon source for heterotrophic denitrification (Breisha and Winter, 2010). However, in heterotrophic denitrification, nitrite can accumulate which is toxic to humans, if carbon sources are depleted. In contrary, excessive organic residue may remain if organics are overdosed (Her and Huang, 1995).

As an alternative to heterotrophic denitrification, hydrogenotrophic denitrification has elicited much attention. It involves the autotrophic metabolism of microorganisms, which uses hydrogen as inorganic electron donors and inorganic carbon (carbon dioxide or bicarbonate) as carbon sources (Karanasios et al., 2010). This process is environment friendly because hydrogen is non-toxic and the byproduct is water. In addition, autotrophic metabolism generates less biomass compared to heterotrophic denitrification, and the cost of excess sludge treatment can be saved (Ghafari et al., 2008). Thus, hydrogenotrophic denitrification has attracted the interest of many researchers, especially in the fields of nitrate removal from drinking water (Lee and Rittmann, 2002; Park and Yoo, 2009). However, only a few studies have investigated the application of hydrogenotrophic denitrification in tertiary nitrogen removal from wastewater, which focused on process performance and gas diffusion (Celmer et al., 2008; Li et al., 2013).

Primary wastewater treatment focuses on the removal of suspended solids using physical and chemical technologies and the remaining effluent contains relatively high content of labile organics. Secondary treatment aims to remove most biodegradable organics by applying biological technologies so effluent contains organic residues with low biodegradability (Tchobanoglous et al., 2002). When investigating autotrophic denitrification under diffident C/N ratios by using methanol or acetate as organic carbons, some researchers reported that organics can enhance heterotrophic denitrification and thus improve nitrate removal efficiency in simultaneously heterotrophic and autotrophic denitrification (Kiskira et al., in press; Zhao et al., 2012). Along with the increasing C/N ratio, the proportion of heterotrophic denitrifying bacteria increases and that of autotrophic denitrifying bacteria decreases (Hao et al., 2016). For industrial application of hydrogenotrophic denitrification in tertiary wastewater treatment, it is important to reveal whether residual organics in effluents of wastewater can stimulate the growth of heterotrophic bacteria in hydrogenotrophic denitrifying process. Moreover, given the fluctuation of organic loading in municipal wastewater, it is also crucial to reveal the competition of facultative autotrophic, obligate autotrophic and heterotrophic denitrifiers in hydrogenotrophic denitrification. The understanding of microbial community can facilitate good controls of facultative autotrophic denitrifiers *versus* heterotrophic denitrifiers common in WWTPs, thus improve the stable and highly efficient performance of hydrogenotrophic denitrification.

High-throughput sequencing of the 16S rRNA gene is an efficient technology to obtain in-depth information of microbial communities (MacLean et al., 2009). It has been utilized in a few recent studies (Chen et al., 2015; Mao et al., 2013) to reveal the microorganisms in autotrophic denitrification process with inorganic synthetic water (IW). However, no study has investigated the influence of residual organics in municipal wastewater effluents on hydrogenotrophic denitrifier communities. In this study, microbial communities in hydrogenotrophic denitrification enrichments cultivated in three types of effluents from a WWTP (*i.e.*, primary effluent (PE), secondary effluent (SE), or 1:1 combined primary and secondary effluent (CE)) were examined by sequencing, with IW serving as a control. All of the key parameters (chemical oxygen demand (COD), nitrate concentration, pH, *etc.*) were maintained identical except for biodegradable carbon proportion. The aim is to reveal the diversity of hydrogenotrophic denitrifiers, and to resolve the influence of labile and low-biodegradable organics on hydrogenotrophic denitrifier community. The results can provide microbiological support to determine the feasibility of hydrogenotrophic denitrification in tertiary nitrogen removal from municipal wastewater.

1. Materials and methods

1.1. Hydrogenotrophic denitrifying enrichments

Hydrogenotrophic denitrifiers were cultivated in four 1000 mL serum bottles with tight caps. The apparatus and research flow are shown in Fig. 1. Three hundred fifty millilitres of medium was placed in each bottle, and the headspace was filled with hydrogen gas. To determine the effects of residual organics on hydrogenotrophic denitrification, three effluent sources (PE, CE, and SE) from Xiaojiahe (XJH) WWTP in Beijing and one inorganic control (IW) were used. According to the effluent analysis in XJH WWTP during the experiment, the average COD concentration in PE was 220 mg/L and that in SE was 40 mg/L. The value of five-day biochemical oxygen demand / chemical oxygen demand (BOD_5/COD), which represents the biodegradability of wastewater, was 0.45 in PE, and only 0.05 in SE. Thus BOD_5/COD was calculated as 0.25 in CE according to the 1:1 volume rate of PE and SE. For all effluent types, media COD was adjusted to the same concentration of 40 mg/L by dilution with deionized water, and NO_3^-–N was adjusted to 30 ± 1.0 mg/L by adding KNO_3. In each serum bottle, H_2 was excess for hydrogenotrophic denitrification of 30 mg/L NO_3^-–N. Since heterotrophic denitrifiers rely on

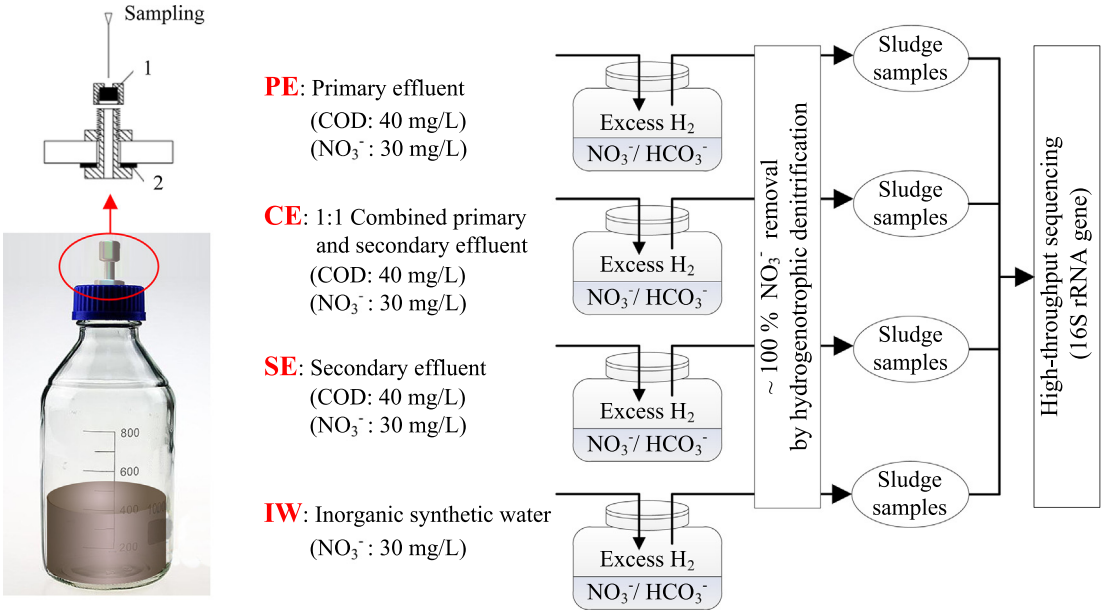


Fig. 1 – The apparatus and research flow for hydrogenotrophic denitrifying enrichments (1 — silicone pad and 2 — polytetrafluoroethylene gasket were installed to keep good sealing when sampling.)

high organic carbon demand, with COD/N ratio above 3–5 (Lee et al., 2001), the organic carbon source of 40 mg/L COD is insufficient for heterotrophic denitrification. Therefore, the competition of autotrophic and heterotrophic denitrifiers is expected in serum bottles fed with PE, CE and SE. As a control, the medium for IW only included KNO_3 (NO_3^-–N concentration of 30.0 ± 1.0 mg/L). $NaHCO_3$ (500 mg/L) was used as the inorganic substrate for all cultures. A microelement concentrated solution (1 mL/L), which was added to all substrate to support microbial growth, was prepared in advance as follows: $ZnSO_4{\cdot}7H_2O$: 0.22 g/L, $CaCl_2{\cdot}2H_2O$: 7.3 g/L, $MnCl_2{\cdot}4H_2O$: 2.5 g/L, $CoCl_2{\cdot}6H_2O$: 0.5 g/L, $FeSO_4{\cdot}7H_2O$: 5 g/L, $CuSO_4{\cdot}5H_2O$: 0.2 g/L, and $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$: 0.5 g/L.

All the four serum bottles were inoculated with activated sludge from XJH WWTP, which was also the municipal wastewater source in the experiments. To avoid the interference of other environmental or operating parameters, the four enrichment cultures were cultivated under the same operational condition as following. Seed sludge (SS, 100 mg MLSS/L) was inoculated in a shaking incubator (HZQ-F100, Donglian Instrument Co., China) operated at 30°C and 150 r/min. pH values were maintained at 7.2 by adding a phosphate buffer solution with 2.18 g/L of $NaH_2PO_4{\cdot}2H_2O$ and 8.45 g/L of $K_2HPO_4{\cdot}3H_2O$. The bottles were sealed to ensure the anoxic environment during the operation. The medium was replaced with 300 mL fresh substrate every 12 hr after 1 hr of prior settlement without shaking. After the medium replacement, the headspace was refilled with H_2 for 5 min at a flow of 500 mL/min.

1.2. Analytical methods and sludge sampling

Based on our previous experiences about hydrogenotrophic denitrifier cultivation under similar conditions, the first 25 days of cultivation are considered as an acclimation period. After 25 days of operation, water samples were collected from four serum bottles routinely before medium replacement, and the concentrations of NO_3^-–N (ultraviolet spectrophotometry method) and NO_2^-–N (spectrophotometry method) were analysed according to APHA (2005) standard methods. The four enrichments were cultivated in the same way for 50 days in all to achieve stability. At the end of cultivation, three replicate sludge samples from each bottle were obtained for DNA extraction and molecular analysis.

1.3. DNA extraction and polymerase chain reaction (PCR) amplification

Genomic DNA of the five sludge samples (PE, CE, SE, IW and SS) were extracted in triplicate using the Power Soil™ DNA Isolation Kit 12888-50 (Mobio, USA) according to the manufacturer's instructions. The nucleic acid purity and concentration were assessed by measuring the absorbance ratios of 260/280 and 260/230 using an ultraviolet–visible spectrophotometer (NanoDrop 2000c, Thermo Scientific, USA). DNAs of triplicate samples were pooled for each sludge sample for further analysis. The V4 hypervariable regions of 16S rRNA were amplified with primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCT AAT-3′) (Caporaso et al., 2012). Triplicate amplicons were pooled and purified with Qiagen Gel Extraction Kit (Qiagen, USA). The details for PCR amplification have been described in a previous study (Li et al., 2016b).

1.4. High-throughput sequencing and statistical analysis

Sample libraries were prepared according to the MiSeq™ Reagent Kit Preparation Guide (Illumina, San Diego, CA, USA) and run on MiSeq Illumina at the Institute for Environmental Genomics, University of Oklahoma. Pre-processing of reads

and statistical analyses were performed with the IEG pipeline (http://ieg.ou.edu). After quality filtration, 43,403 effective sequences were selected from each sample for microbial analysis. Operational taxonomic units (OTUs) were divided and clustered with 97% similarity using UPARSE (http://drive5.com/uparse/). Heat maps were generated using R-code, and the Bray–Curtis dissimilarity and Euclidean distance were employed to cluster the different samples. Venn diagram was also applied using R-code, and a phylogenetic tree was constructed with the neighbour-joining algorithm by using the software package Molecular Evolutionary Genetics Analysis (MEGA 6.0).

1.5. *Data accessibility*

All reads of high-throughput MiSeq sequencing were archived at the NCBI Sequence Read Archive Database with accession numbers SRR3105306–SRR3105310.

2. Results and discussion

2.1. *Nitrate removal performance of hydrogenotrophic denitrification*

After 25 days cultivation, the NO_3^-–N and NO_2^-–N concentrations of all effluent samples were almost zero or below their detection limits, suggesting the successful enrichment of hydrogenotrophic denitrifiers with the four different water sources. The high efficiencies achieved are consistent with our speculation. According to our previous study under similar conditions, NO_3^-–N reduction rate with hydrogenotrophic denitrifiers fed by IW could achieve 0.6 kg N/(kg MLSS·day) (Li et al., 2016a). Considering the sludge concentration (100 mg MLSS/L) and NO_3^-–N concentration in the medium (30 NO_3^-–N mg/L), the speculative time to remove all nitrate added each time is calculated as 12 hr in IW. In PE, CE and SE, the time needed to remove all nitrate could be shortened, when organics provided part of the electron donors. As medium was replaced every 12 hr, nitrate removal efficiencies reached 100% in this study. To focus on the hydrogenotrophic denitrifying communities with easily controlled parameters, simply designed apparatus were used in this study. This is also a common approach in the studies on influence factors in hydrogenotrophic denitrification (Ghafari et al., 2009; Zhang et al., 2009). However, hydrogen transfer and utilization efficiency, as well as the reactor design for efficiently and securely hydrogen supplying, should be further studied when hydrogenotrophic denitrification is applied to practical application.

2.2. *Microbial diversity and hierarchical clustering analysis*

Microbial communities in the four enrichment cultures of hydrogenotrophic denitrifier (PE, CE, SE and IW), in addition to the SS sample, were analysed by high-throughput sequencing. The rarefaction and rank-abundance distribution curves are shown in Appendix A Fig. S1. The recovered sequences effectively represented the diversity of the microbial communities in the five samples, with coverages of 99.0% (PE), 98.4% (CE), 98.8% (SE), 99.3% (IW) and 95.8% (SS). The species richness of SS was much higher than those of the other four samples, since the diversity decreased dramatically under anoxic condition. The second highest was that of CE because of its labile and complicated substrate contributed by combined PE and SE. By contrast, the low microbial diversities of IW and SE were mainly because of their organic-free substrate and low-biodegradable-organic substrate, respectively.

Hierarchical clustering analysis showed that the SS sample was dissimilar from the other samples. PE and CE samples were grouped together, suggesting that the microbial communities of these two samples were similar. Microbial community in SE showed the highest similarity to IW. This is probably due to the fact that SE contained more low-biodegradable organics than PE and CE, though their COD concentrations were the same. This result suggests that biodegradable organics are influential for microbial community.

2.3. *Microbial community compositions at phylum and class levels*

The relative abundance of dominant microorganisms at phylum and class levels in all the samples is shown in Fig. 2. In all the samples, the phylum Proteobacteria was the most abundant, accounting for 40.9%–60.3% of the 43,403 sequences. This result is consistent with other researcher's study indicating that most reported hydrogenotrophic denitrifiers belong to Proteobacteria (Karanasios et al., 2010). The relative abundance of phylum Bacteroidetes, which is also related to hydrogenotrophic denitrification, ranked the second in samples CE, SE, IW and SS. However, the relative abundance of phylum Euryarchaeota was the second abundant in PE as a result of the increase of genus *Methanobrevibacter*. In the sample SS, significantly more sequences were distributed in the phyla Gemmatimonadetes, Actinobacteria, Acidobacteria and Planctomycetes, compared to the other four samples.

At the class level, the community structures were different among four enrichments cultivated with different water sources, although all the other environmental and operational conditions were controlled the same. β-Proteobacteria and Flavobacteria, which contain hydrogenotrophic denitrifiers (Karanasios et al., 2010), were dominant in SE and IW; their abundance was 51.3% and 35.1% in SE and 44.4% and 18.6% in IW, respectively. In samples PE and CE with a higher content of labile organics, β-Proteobacteria was the dominant class as well. Besides, a remarkable portion of microorganisms in PE was distributed in the classes of Methanobacteria. This is probably as a result of methanogenesis occurred from H_2/CO_2. As discussed in Section 2.1, nitrate in IW was exhausted after 12 hr, according to the concentration of sludge and NO_3^-–N. Since medium was replaced every 12 hr, nitrate probably exists in IW for most of the time. Thus, methanogens cannot survive for a higher oxidation reduction potential (ORP) in the serum bottle. Referring to PE with a higher content of labile organics, NO_3^-–N reduction rate could be increased by the combination of hydrogenotrophic and heterotrophic denitrifiers, so that the serum bottles were shifted to strict anaerobic condition from anoxic condition after 100% nitrate removal and benefited the growth of methanogens. These results

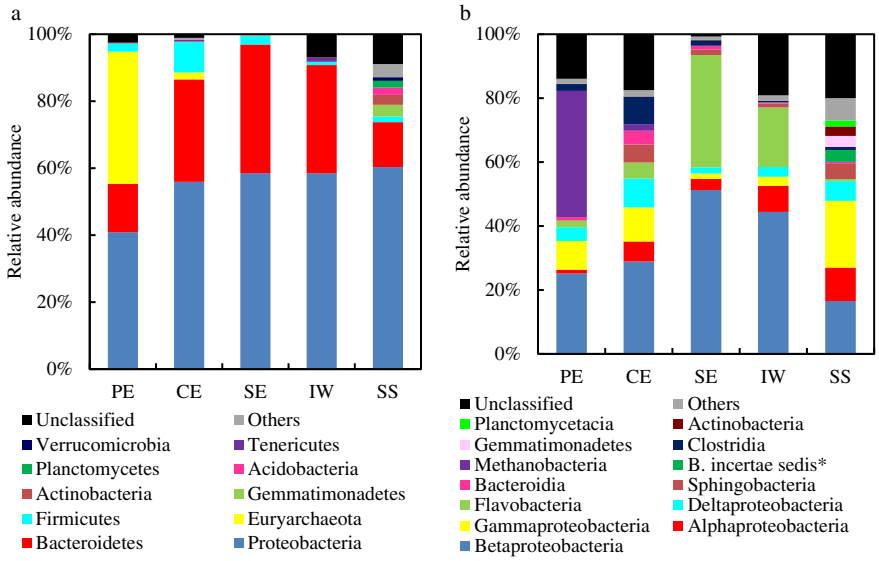


Fig. 2 – Distributions at (a) phylum and (b) class levels derived by MiSeq sequencing of 16S rRNA genes in different samples. * denotes the section of "Bacteroidetes incertae sedis".

indicate that the presence of labile organics lead to competition between denitrifiers and methanogens. Although nitrate removal efficiencies remained high in PE, operating parameters such as hydraulic retention time (HRT) and ORP are crucial for stable and successful hydrogenotrophic denitrification process.

2.4. Microbial communities at the genus level

As shown in Fig. 3, the 39 most dominant genera (including unclassified genera) accounted for 95.4%, 88.3%, 92.7%, 96.6% and 68.0% of all sequences in PE, CE, SE, IW and SS, respectively. In the samples PE, CE, SE and IW, *Hydrogenophaga* was the common dominant genus, accounting for 17.1%, 5.3%, 32.7% and 12.9%, respectively. In sample IW, hydrogenotrophic denitrification is the only major reaction since nitrate removal efficiency achieved 100% under organic-free condition. Only six classified genera were observed more than 1% of all sequences (Appendix A Fig. S2). *Thauera* (23.6%), *Hydrogenophaga* (12.9%) and *Paracoccus* (7.2%) were the most dominant genus. In the samples PE and CE with labile organics, the abundance of *Thauera* decreased to 2.5% and 4.6%, respectively, whereas that of *Thermomona* (not detected in IW and accounted for only 0.6% in SE) increased to 4.1% and 7.9%, respectively.

Studies on physiological property have indicated that bacteria can be divided into heterotrophs, facultative autotrophs (mixotrophs) and obligate autotrophs (Kuenen et al., 1982; Liessens et al., 1992). Facultative autotrophs can utilize both organics and inorganics as electron donors and are capable of transforming to heterotrophic growth when biodegradable organics is available, and back to autotrophic growth under organic-free conditions (Kuenen et al., 1982). The result that *Hydrogenophaga* can outcompete successfully with residual organics or organic-free condition is in accordance with a previous study which reported that most

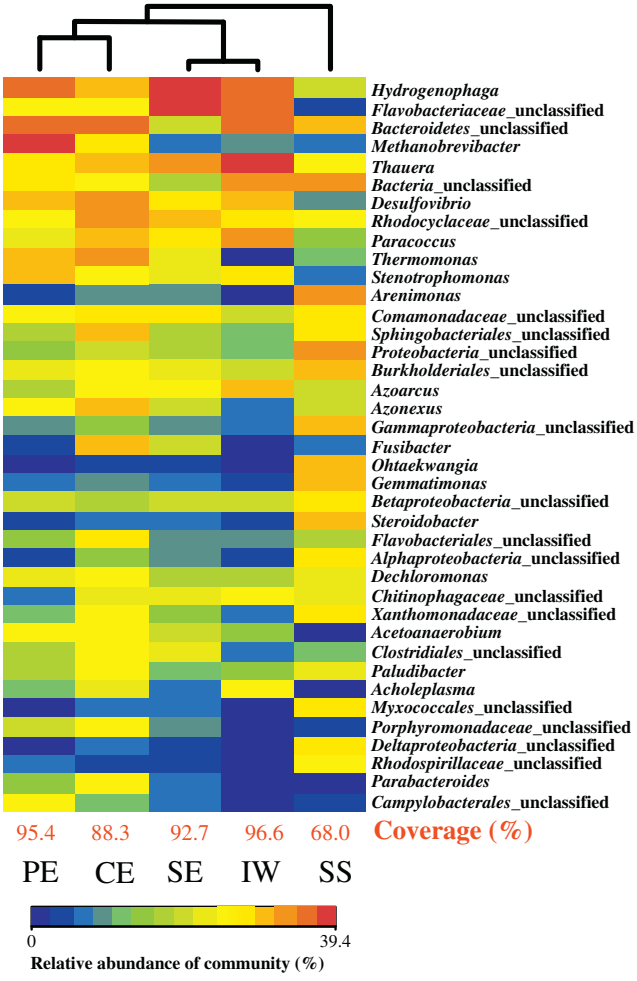


Fig. 3 – Heat maps of the relative abundance of the genera (including unclassified genera) derived by MiSeq sequencing of 16S rRNA gene in the five different samples. Only the 39 most dominant genera are shown.

hydrogenotrophic bacteria (including *Hydrogenophaga*) are actually facultative autotrophs (Liessens et al., 1992). Given the fluctuation of organic loading in municipal wastewater, facultative autotrophs *Hydrogenophaga* could be the major contributors to the application of the hydrogenotrophic denitrification process in tertiary wastewater treatment. The result that *Thauera* was the most dominant genera in IW but decreased markedly in PE and CE indicated that *Thauera* contains likely obligate autotrophic denitrifiers. In a previous study, *Thauera* was also found dominant in hydrogenotrophic denitrification with IW (Mao et al., 2013). The genus *Thermomonas* was confirmed to be one of the core active denitrifiers in WWTPs (McIlroy et al., 2016). According to previous reports, some species in *Thermomonas*, such as *Thermomonas fusca* and *T. brevis* isolated from a nitrate removal reactor with polyplastic, possess the ability of heterotrophic denitrification (Mergaert et al., 2003). The variation on the relative proportions of *Hydrogenophaga*, *Thauera* and *Thermomonas* presented the growth and decline of typical facultative autotrophic, obligate autotrophic and heterotrophic denitrifiers in different organic conditions, well supporting their competitive relationship in hydrogenotrophic denitrification.

2.5. Venn diagram analysis and key OTUs

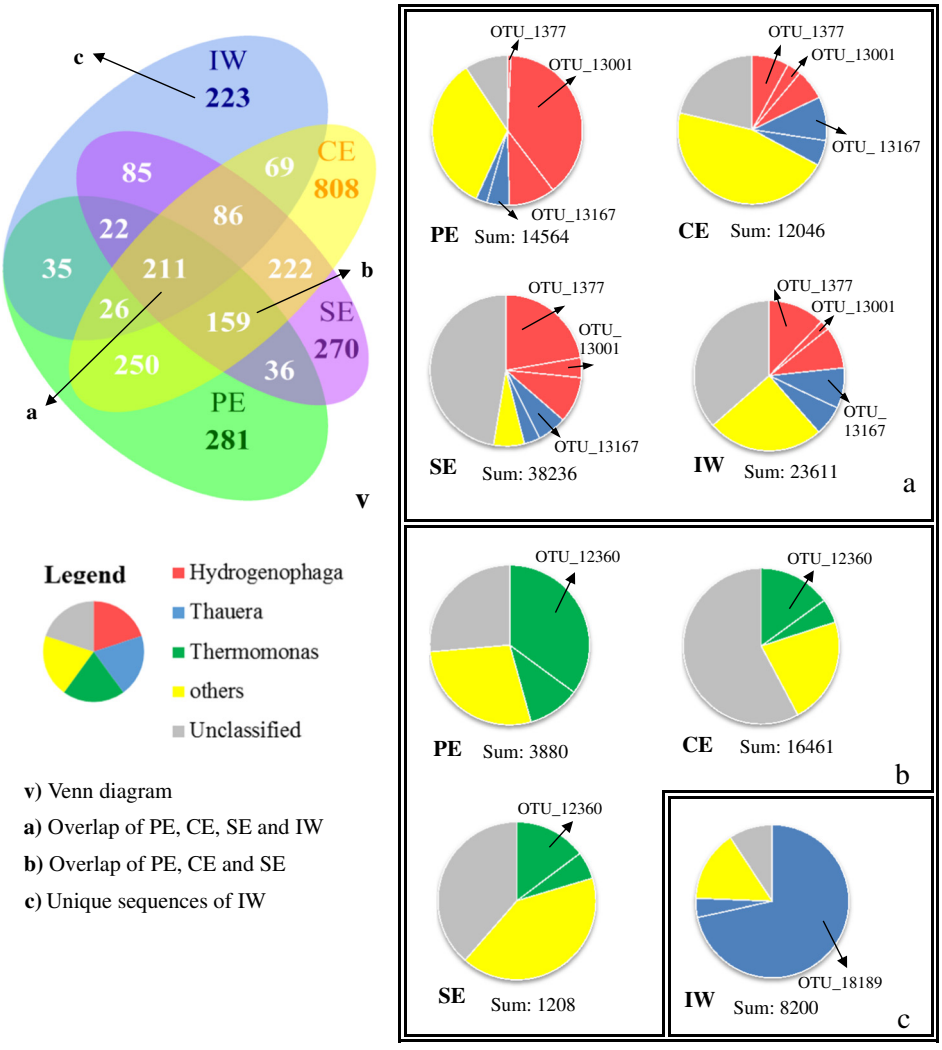
A Venn diagram was constructed as shown in Fig. 4. A total of 211 OTUs were shared in four samples (Fig. 4a). According to the common OTUs, the genus *Hydrogenophaga* prevailed in each sample. Among the genus *Hydrogenophaga*, OTU_1377 covered significant portions in samples SE and IW, and OTU_13001 presented a much larger portion in sample PE. Aside from *Hydrogenophaga*, *Thauera* (represented by OTU _13167) was also an important shared genera in all the four samples. A total of 159 OTUs were shared in samples PE, CE and SE rather than IW (Fig. 4b), in which the genus *Thermomonas* (especially OTU_12360) encompassed remarkable portions. Besides, each of the four samples possessed a large number of specific OTUs. Among the 223 specific OTUs that appeared only in IW (Fig. 4c), the genera *Thauera* (especially OTU_18189) accounted for large portions. The analysis of OTU proportion is in accordance with the results that *Hydrogenophaga* can keep the predominant position with residual organics in municipal wastewater, while *Thauera* declines and *Thermomonas* are more competitive, as discussed in Section 2.4.

The phylogenetic analysis of key OTUs is shown in Fig. 5. Eight OTUs were most dominant in IW, accounting for 68.2% of all sequences. Since hydrogenotrophic denitrification is the only major reaction in IW, these eight OTUs are closely related to hydrogenotrophic denitrifiers. OTU_18189 and OTU_13167 belong to genus *Thauera*, which are highly similar to *Thauera linaloolentis* and *Thauera mechernichensis*. OTU_1377 showed 98% sequence similarity to *Hydrogenophaga pseudoflava*, which has been verified as a hydrogenotrophic denitrifier together with several other species in the genus *Hydrogenophaga* (Willems et al., 1989). OTU_3323 also showed 98% sequence similarity to a model hydrogenotrophic denitrifier, *Paracoccus denitrificans* (Rainey et al., 1999). OTU_1040 showed 98% sequence similarity to *Stenotrophomonas nitritireducens*, which can reduce nitrite to nitrous oxide (Finkmann et al., 2000). The other three dominant OTUs had a low similarity with all the cultured bacteria. However, they presented a high similarity to several uncultured bacterium clones, such as the uncultured clones in microbial fuel cells (99% similarity) (Song et al., 2012; Xia et al., 2012; Yates et al., 2012). Besides above eight OTUs which were dominant in IW, OTU_12360 (belong to genus *Thermomonas*) and OTU_13001 (belong to genus *Hydrogenophaga*) were presented a larger portion in enrichments fed with organics, especially in PE. These two OTUs probably represented heterotrophic or mixotrophic denitrifiers, which can successfully compete with autotrophs under a higher content of labile organics.

The results obtained in this study reveal that residual organics significantly affect denitrifying microbial community structures, even with 100% nitrate removal among all samples. Particularly, inorganic or organic carbon sources, as well as different degradabilities of organic carbon sources are important factors affecting the denitrifying microbial community structure in class, genus and OTU levels. On the basis of competition among facultative autotrophic, obligate autotrophic and heterotrophic denitrifiers, facultative hydrogenotrophic denitrifiers with *Hydrogenophaga* as a representative could be cultivated with biodegradable organics in wastewater. Thus, hydrogenotrophic denitrifiers are accommodating to residual biodegradable organics in effluent wastewater and hydrogenotrophic denitrification is amenable for tertiary nitrogen removal from municipal wastewater. However, denitrifying bacteria present high diversity in both biochemical and taxonomical perspectives (Mousavi et al., 2012) and information on hydrogenotrophic denitrifiers is still limited. It is relatively difficult to identify the real autotrophic or heterotrophic function through a taxonomical analysis of the bacteria during denitrification processes. Further investigation combined with a phylogenetic analysis of this topic and the physiological function is still required.

3. Conclusions

Hydrogenotrophic denitrification is promising for tertiary nitrogen removal from municipal wastewater. Nevertheless, the competition between heterotrophic and autotrophic denitrifiers in the presence of residual organics remains unknown, hampering its industrial application in wastewater treatment. In this study, microbial communities in four hydrogenotrophic denitrifying enrichments, which were fed with three types of effluent wastewater as well as IW, were investigated by high-throughput MiSeq sequencing. The results showed that *Hydrogenophaga* (a major facultative hydrogenotrophic denitrifier) was the common dominant genus, accounting for 17.1%, 5.3%, 32.7% and 12.9% in PE, CE, SE and IW, respectively. This suggests that *Hydrogenophaga* can keep the predominant position without or with residual biodegradable organics in effluent wastewater, thus benefitting hydrogenotrophic denitrification in tertiary nitrogen removal from municipal wastewater. However, residual organics can significantly affect the denitrifying microbial communities. The likely obligate autotrophic denitrifier *Thauera* was less competitive than *Thermomonas*, when fed with higher contents of labile organics. These findings



v) Venn diagram

a) Overlap of PE, CE, SE and IW

b) Overlap of PE, CE and SE

c) Unique sequences of IW

Fig. 4 – Venn diagram evaluating the influence of residual organics on hydrogenotrophic denitrification. (v) Venn diagram showing unique and shared operational taxonomic units (OTUs) in each sludge sample. (a) Shared sequence analysis of samples primary effluent (PE), combined primary and secondary effluent (CE), secondary effluent (SE), and inorganic synthetic water (IW). (b) Shared sequence analysis of samples PE, CE and SE. (c) Unique sequence analysis of samples IW. The sum under each pie chart refers to the total sequences of the unique or shared parts in each sample. The arrows show the specific OTUs with significant portions.

revealed the competition among different denitrifiers with or without biodegradable organics, thus providing understanding of hydrogenotrophic denitrifying communities, and facilitating improvements in stable and highly efficient performance of hydrogenotrophic denitrification.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2017.03.001.

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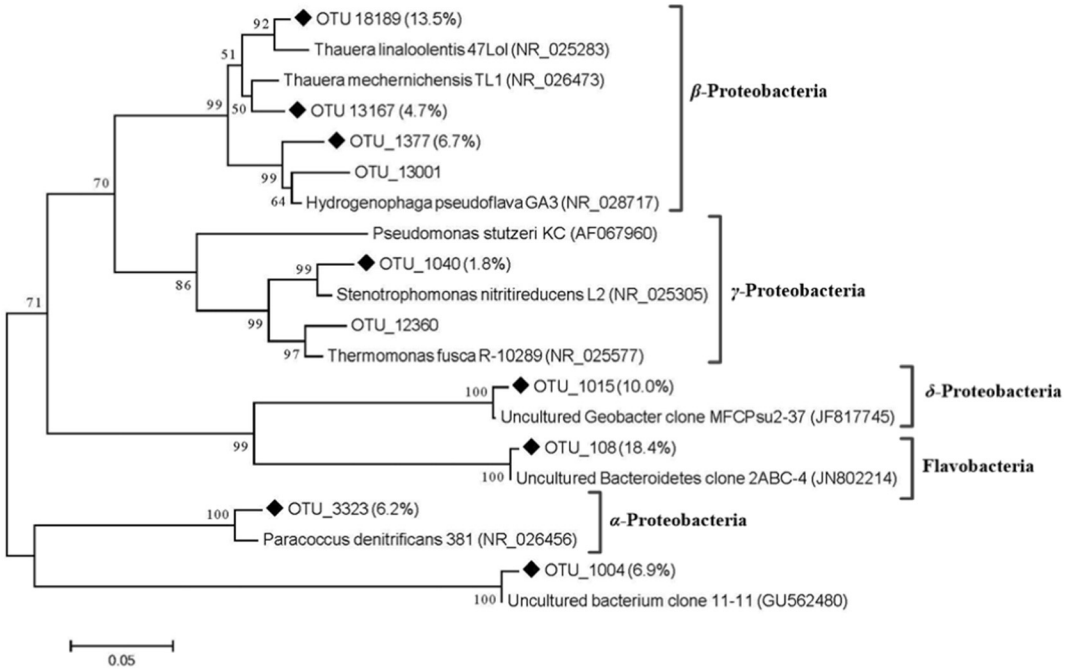

Fig. 5 – Phylogenetic relationships of the key OTUs derived by MiSeq sequencing of 16S rRNA gene. The eight most dominant OTUs in sample IW are denoted by diamonds (♦), and the numbers in parentheses present their proportions in sample IW.

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