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Characteristics and dominant microbial community structure of granular sludge under the simultaneous denitrification and methanogenesis process

Xiao-Hui Yi[a,b], Jinquan Wan[a,b,c,*], Yongwen Ma[a,b,c], Yan Wang[a,b]

[a] School of Environment and Energy, South China University of Technology, Guangzhou Higher Education Mega Centre, Guangzhou 510006, PR China
[b] The Key Lab of Pollution Control and Ecosystem Restoration in Industry Clusters, Ministry of Education, South China University of Technology, Guangzhou Higher Education Mega Centre, Guangzhou 510006, PR China
[c] State Key Laboratory of Pulp and Paper Engineering, South China University of Technology, Guangzhou 510640, PR China

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ABSTRACT

Batch experiments under different COD/NO_3^--N ratios were carried out to investigate physicochemical characteristics and microbial community structure of granular sludge under the simultaneous denitrification and methanogenesis (SDM) process. COD/NO_3^--N ratio of 8.0 was proved to be a critical point of the SDM process and sludge at this ratio was selected for analysis. BET, SEM, FTIR and zeta potential measurement were used to characterize the micro-structure, functional groups and surface charge of the granular sludge related to nitrate addition. SEM observation showed that rod-shaped bacteria were predominant at the surface of granules and FTIR spectrum (1745 cm^{-1}) presented an evidence for the carboxyl group protonation upon reduction of the cytochrome *c* oxidase. Furthermore, high-throughput sequencing technology was used to analyze the microbial structure and diversity. Archaea was found to be accounted for 3.33% of the total microbial communities and *Methanosaeta* and *Methanobacterium* were the dominant archaeas. Otherwise, *Proteobacteria* (63.00%), *Bacteroidetes* (21.79%) and *Firmicutes* (9.73%) phyla were identified to be the three dominant bacterial communities. *Enterobacteriaceae* was detected with a content of 50.24% of the total bacterial sequences and might be the core bacterium contributed to the SDM process. The results would provide vital guidances for the design and stable operation of nitrate-containing wastewater treatment.

1. Introduction

High levels of nitrogen emissions were reported due to the intensive industrial and agricultural activities in China and nitrate was one of the major inorganic nitrogen contaminants [1,2]. Excess discharge of nitrate into the water bodies has caused serious environmental problems such as eutrophication of lakes, contamination of groundwater and deterioration of aquatic ecosystem, moreover, it also contributes to human and aquatic animals' health problem [3]. To protect lakes, groundwater and other natural water bodies from contamination or deterioration, stringent nitrate level is set for the effluent from wastewater treatment plants.

Biological denitrification is the most common process for nitrogen removal from wastewater. In the denitrification process, microorganisms used organic carbon as electron donor to reduce nitrate to gaseous nitrogen under anoxic condition. However, carbon sources of the influent are usually deficient to sustain biological denitrification, therefore, exogenous carbon sources such as methanol and acetate are usually chosen to add into the treatment systems [4], leading to increased costs. Combined treatment technologies, including anaerobic digestion and biological denitrification, for the simultaneous removal of carbon and nitrogen together have received considerable attentions due to advantages such as substrates supplement, energy recovery and cost and space reduction [5,6] and been widely applied in the treatment of piggery wastewater [7], aniline wastewater [8] and so on.

COD/NO_x^--N ratio of the influent has been reported to be a vital factor for the simultaneous denitrification and methanogenesis (SDM) and a suitable range of COD/NO_x^--N ratio was reported. Del Pozo and Diez [9] suggested the minimum bsCOD/NO_x^--N based on the following Eq. (1) and calculated a theoretical COD/NO_x^--N

* Corresponding author at: School of Environment and Energy, South China University of Technology, Guangzhou Higher Education Mega Centre, Guangzhou 510006, PR China. Fax: +86 20 87114970.
E-mail address: ppjqwan@scut.edu.cn (J. Wan).

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ratio, 3.21, according to the typical stoichiometric parameters from activated sludge model No. 3 (ASM 3).

$$COD/NO_3^- - N = \frac{2.86}{\left(1 - Y_{H,NO_x}\right) + (1 - f_{Xi})\, Y_{H,NO_x}} \quad (1)$$

where $Y_{H,NOx}$ is the anoxic yield coefficient and f_{Xi} is the fraction of inert COD generated in biomass lysis.

Considering practical conditions (biochemical reaction heat loss, cell growth and others), the actual required COD/NO_x^--N ratio is more than theoretical value (3.21). Ruiz et al. [10] noted that the process could not be maintained when the COD/N ratio lower than 5. Akunna et al. [5] observed the simultaneous denitrification and methane production at the COD/NO_x^--N ratio from 8.86 to 53 with glucose as the carbon source and Xie et al. [11] found that the suitable COD/NO_3^--N ratio was higher than 7. Suitable COD/NO_x^--N ratio for the SDM was also related to the nature of the substrates [8,12]. With glucose as carbon sources, COD/NO_x^--N ratio around 8.0 could be treated as the minimum threshold for the SDM under the anaerobic methanogenesis culture from aforementioned results.

Granular sludge have been known to be microbial conglomerates with organized spatial structure and the core of anaerobic treatment [13,14]. Since the nitrate amended in the anaerobic methanogenesis culture, denitrification process happened simultaneously and brought unique features into the system, such as rise of the environmental factors (pH, ORP), inhibition effects of the nitric oxides [15] and competition of substrates and habitat between the microorganisms [16]. Due to aforementioned factors, the simultaneous denitrification and methanogenesis granular sludge (SDMGS) was found to be light brown, fluffy and less dense. Shifts of microbial community structure and diversity under the integration of methanogenesis with denitrification were thought to be attributed to these features [17,18]. Karim and Gupta [17] suggested that growth of denitrifiers and facultative anaerobes on the surface of granular sludge could be the crux of the SDM and the granules. Sun et al. [19] investigated the microbial community structure of the SDMGS with the 16S rDNA clone libraries method and indicated that *Eubacteria* was the majority and *Methanosaeta* and *Methanobacteria* were the dominant methanogens. While, information about the SDMGS such as micro-structure, functional groups and other chemical characteristics, which determined the adsorption and mass transfer of the granules, have not been addressed, moreover, comprehensive microbial community structure analysis and functional microbial groups of the SDMGS is still deficiency and rarely reported. High-throughput sequencing is a more sensitive technique than others (as clone library method) for sequencing analysis and has been used for microbial analysis of different mixed cultures [20–22]. Due to the huge amounts of DNA sequences generated by means of high-throughput sequencing technology, information regarding the species, abundance and physiology based on 16S rDNA genes involved in the denitrification and methanogenesis can be elucidated.

In the present study, suitable COD/NO_3^--N ratio for the SDM process was studied and different methods (BET, SEM, FTIR and zeta potential measurement) were used to characterized unique features of the granule sludge. Furthermore, microbial community structure and diversity of the SDMGS were determined using high-throughput sequencing technology. Identification of the characteristics and microbial community structure of the SDMGS would help to figure out the core bacterium accounted for the SDM process and realize better treatment for the nitrate-containing wastewater.

Table 1
Sequencing information, numbers of OTUs, Chao1 and Shannon diversity index.

	SDMGS	MGS
Sequencing information		
Sequencing region	V4	V4
Effective tag number	58022	55620
Effective ratio (%)	74.93	70.6
Numbers of OTUs, Chao1 and Shannon Index (H′) under the level 0f 97%		
OTUs[a]	907	1627
Chao1[b]	1785	3578
Simpson[c]	0.038	0.207
Shannon[c] (total)	2.736	4.239
Shannon (archaea)	1.824	1.977
Shannon (bacteria)	2.616	4.131

[a] OTUs: operational taxonomic units (calculated by Mothur at the 3% distance limit).
[b] Chao1 richness estimators: the total number of OTUs estimated by infinite sampling. A higher number represents higher richness.
[c] Simpson/Shannon diversity index: an index to characterize species diversity. A higher number represents more diversity.

2. Material and methods

2.1. Seed sludge and media preparation

Granular sludge was obtained from a full scale internal circulation (IC) anaerobic reactor treating pulping wastewater (Guangzhou, China) and used as seed sludge. The volatile suspended solids (VSS) and volatile to total suspended solids (VSS/TSS) ratio of the seed sludge were 50.45 ± 3.2 g/L and 0.81, respectively. The artificial wastewater with the following composition was prepared with sterile water (L^{-1}): glucose 4.0 g; NH_4Cl 0.42 g; KH_2PO_4 0.2 g; $MgSO_4{\cdot}6H_2O$ 0.4 g; $CaCl_2$ 0.15 g; L-cysteine 0.1 g. Trace metals solution and resazurin solution (1.0 g L^{-1}) were also prepared as described by Wagner et al. [23]. All the media solutions were stored at 4 °C before usage.

2.2. Experimental setup and procedure

Series of identical 250 mL serum bottles were used as batch bioreactors. Seed sludge (20 g wet weight), artificial wastewater (150 mL), trace metals solution (1 mL) and resazurin solution (1 mL) were respectively added into each bottle. COD/NO_3^--N ratios of bioreactors were adjusted with sodium nitrate and the control group was prepared without nitrate. The initial pH of the culture media was 7.4 ± 0.1 with the adjustment of sodium bicarbonate. The mixtures and culture bottles were flushed with 99.995% argon gas for 10 min until the liquid became colorless, then closed with butyl rubber septum and sealed with aluminum cap immediately. Cultivation was conducted in an automatic shaker at a constant temperature of 35 ± 0.2 °C and all the COD/NO_3^--N ratios' experiments were performed in triplicate.

2.3. Sampling and chemical analysis

Liquid samples were collected with sterile syringe, and centrifuged at 10,000 × *g* for 10 min at 4 °C (3-18k, Sigma, Germany), then used for analysis. COD, NH_4^+-N, VSS and TSS were determined according to standard methods [24]. NO_3^--N and NO_2^--N were measured using an ion chromatography (ICS-90, Dionex, USA) equipped with IonPac AS14 (4 × 250 mm) column with an eluent of 3.5 mM Na_2CO_3 and 1.0 mM $NaHCO_3$. pH and ORP were monitored by pH meter (LeiCi PHS-25, China) equipped with a pH electrode and an oxidation reduction potential electrode.

Composition of the volatile fatty acids (VFAs) was measured using a gas chromatograph (A90, Echrom Inc., China) equipped with a capillary column (DB-FFAP, 30 m × 0.32 mm × 0.5 μm) and