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Effect of trace hydrazine addition on the functional bacterial community of a sequencing batch reactor performing completely autotrophic nitrogen removal over nitrite

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HIGHLIGHTS

- AOB population decreased under long-term trace N_2H_4 addition.
- An AOB population increased under long-term trace N_2H_4 addition.
- NOB suffered more significant inhibition from trace N_2H_4 addition than AOB.

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ABSTRACT

A sequencing batch reactor (SBR) was conducted to perform completely autotrophic nitrogen removal over nitrite (CANON). The effect of long-term trace N_2H_4 addition on ammonium oxidizing bacteria (AOB) and anaerobic AOB (AnAOB) in the CANON system was investigated. AOB and AnAOB primarily related to *Nitrosococcus*, *Nitrosomonas* and *Candidatus scalindua*, respectively. Before and after trace N_2H_4 addition, the estimates of AOB population decreased from 1.03×10^7 to 6.25×10^4 copies/g (dry sludge), but that of AnAOB increased from 3.14×10^9 to 5.86×10^{10} copies/g (dry sludge). Despite there was a partially negative impact on AOB growth, the trace N_2H_4 addition exerted a stronger inhibition on nitrite oxidizing bacteria (NOB) and promoted AnAOB growth, which improved the nitrogen removal of the CANON system. Sludge granules enriched under long-term trace N_2H_4 addition were spherical and ellipsoidal, and the aerobic AOB were mainly located on the outer layers while AnAOB occupied most of the interior parts.

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1. Introduction

The completely autotrophic nitrogen removal over nitrite (CANON) (Dijkman and Strous, 1999) system, where the aerobic ammonium oxidizing bacteria (AOB) partially oxidize NH_4^+ to NO_2^-, and the anaerobic ammonium oxidizing (ANAMMOX) bacteria (AnAOB) convert the resulting NO_2^- and the remaining NH_4^+ to N_2, such that biological nitrogen removal can be achieved without the need for organic carbon sources, has been used in engineering applications (Joss et al., 2011; Siegrist et al., 2008). AOB and AnAOB (Liu et al., 2012) are both chemoautotroph and grow slowly. Due to the difficulty of maintaining pure culture of such environmental microbes as AnAOB and the possible difference between the cultured microbes and these in environmental samples, the utility of the traditional purified culture method for studies of the physiological and ecological characteristics of CANON granular sludge (biofilm) is limited. Benefited from the development of molecular biotechnology (Bae et al., 2010; Kindaichi et al., 2007; Penton et al., 2006; Schmid et al., 2007; Tsushima et al., 2007), there has been progress in studies on the functional microbes in CANON sludge. Xiao et al. (2009) used the polymerase chain reaction denaturing gradient gel electrophoreses (PCR-DGGE) technique to identify the nitrifying bacteria, denitrifying bacteria and ANAMMOX bacteria as well as the stability of their bacterial community structures in a sequencing batch biofilm reactor (SBBR). By analyzing the gene sequence of 16SrDNA, Liu et al. (2011) discovered that

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the AOB in the CANON system primarily belonged to the genus *Nitrosomonas*, while the AnAOB primarily related to the order *Planctomycetes*; quantitative analysis using quantitative real-time PCR (qPCR) revealed that the quantities of these microbes increased slightly with gradual stabilization of the CANON system. Based on the fluorescence in situ hybridization (FISH) technique, Zhang et al. (2014a) quantified the relative amounts of the functional microbes in the granular sludge in a CANON reactor at different concentrations of NH_4^+-N; they discovered that the effect of the concentration of NH_4^+-N on the abundances of the AOB and AnAOB was insignificant, but high concentration of NH_4^+-N were beneficial for eliminating the nitrite oxidizing bacteria (NOB) in the CANON system. The FISH technique has also revealed that the AOB and AnAOB in CANON granular sludge exhibit significantly aggregated distributions: the AOB are primarily located on the top surfaces of the granules, while the AnAOB are primarily located inside the granules (Nielsen et al., 2005).

Hydrazine (N_2H_4) is a strong reducing agent with biological toxicity. However, despite partial inhibition on aerobic ammonia oxidation, adding trace N_2H_4 to the CANON reactor could improve the nitrogen removal performance by effectively strengthening ANAMMOX and significantly inhibiting the oxidization of nitrite, and consequently reduce the generation of nitrate (Yao et al., 2013). In order to provide microbiological evidence for the possible strengthening mechanism mentioned above and microscopic theoretical supports for the optimization of CANON system, based on the functional gene of the AOB, the α-subunit of ammonia monooxygenase (amoA), and the functional gene of the AnAOB, the α-subunit of hydrazine synthase (hzsA), the following work were accomplished in this paper: (1) the community structures of functional bacteria (AOB and AnAOB) in the CANON system were qualitatively analyzed; (2) the AOB and AnAOB were quantitatively determined through qPCR before and after the addition of trace N_2H_4; (3) the superficial morphology of the granular sludge in the CANON system with long-term addition of trace N_2H_4 was detected by using scanning electron microscope (SEM) and spatial distribution patterns of the AOB and AnAOB in the granular sludge were revealed via using FISH technique.

2. Methods

2.1. Enrichment of CANON granular sludge

The CANON process was started under an alternating limited oxygen-limited/anaerobic condition in a sequencing batch reactor (SBR). The reactor (Fig. 1) was made from polymethyl methacrylate. The internal diameter, height, and working volume of the reactor were 14 cm, 35 cm and 3 L, respectively. The reactor was inoculated with CANON sludge from an aerated upflow sludge bed reactor (Zhang et al., 2010) and were operated with a 4 h cycle (aeration: 2.5 h, including 0.2 h for feeding (volume 1 L); mixing: 1.2 h, no aeration; settling: 0.1 h; effluent withdrawal: 0.2 h). The DO concentration was maintained at 0.3 ± 0.1 mg/L by controlling the aeration rate with a gas flow meter during the aeration phase of the SBR, and the temperature of the reactors was maintained at 31 ± 1 °C. $(NH_4)_2SO_4$ and $NaHCO_3$ were used to prepare synthetic wastewater. The concentration of $NaHCO_3$ was 1.5 g/L, and 1.25 mL trace element solution (Yao et al., 2013) was added in 1 L synthetic wastewater. The solids retention time and the hydraulic retention time were 20 days and 11.8 h, respectively. N_2H_4 (in the form of $N_2H_4 \times H_2SO_4$) at a concentration of approximately 4 mg/L was added for a long period of time into the influent. The whole running time of the CANON SBR was classified into the initial start-up stage without N_2H_4 addition(stage I), the middle stable operation stage with 4 mg/L N_2H_4 addition(stage II), and the late stage when granular sludge emerged (stage III) (Fig. 2). Sludge samples ZY1, ZY2, and ZY3 were taken from these 3 stages, respectively, for molecular biological determination and analysis.

2.2. DNA extraction

The collected CANON sludge sample (0.1 g) was placed in a sterile Eppendorf vial, and 1.5 mL DNA lysis buffer was dripped into the vial. DNA extraction was performed by using a Soil DNA Extraction Kit (OMEGA BIO-TEK, Norcross, GA, USA) following the instruction described by Zhou et al. (1996). Subsequently, 1% agarose electrophoresis (EP) was used to test the obtained DNA sample. A micro-ultraviolet spectrophotometer was used to determine the concentration and purity of the sample. The DNA was used for functional genes amplification and quantification, Miseq high throughout sequencing and analysis.

2.3. PCR amplification and sequencing of functional genes

Functional genes of amoA corresponding to AOB in the CANON sludge were amplified by using the primer set of amoA1F/amoA2R with the sequence (5′–3′) of GGGGTTTCTACTGGTGGT and CCCCTCKGSAAAGCCTTCTTC, at the cycling conditions of 95 °C for 5 min followed by 30 cycles (denaturation for 1 min at 94 °C; annealing for 1 min at 56 °C; elongation for 3 min) (Purkhold et al., 2000).

Functional genes of hzsA corresponding to AnAOB in the CANON sludge were amplified by using the primer set of hzsA-526F/hzsA-1857R with the sequence (5′–3′) of TAYTTTGAAGGDGACTGG and AAABGGYGAATCATARTGGC, at the cycling conditions of 95 °C for

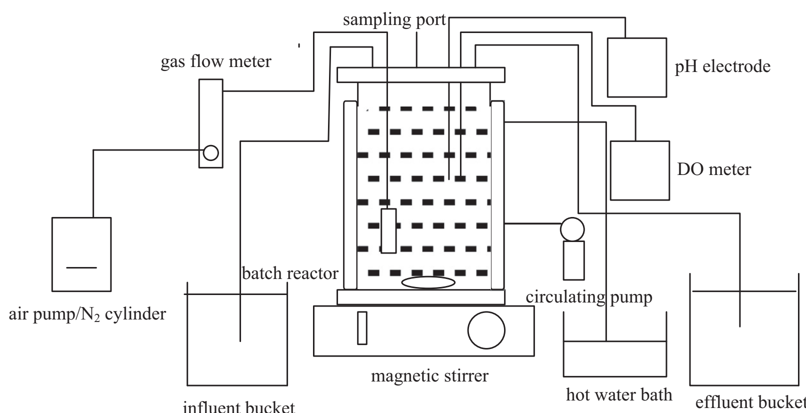

Fig. 1. Schematic diagram of the CANON SBR.

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