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Biodiversity and quantification of functional bacteria in completely autotrophic nitrogen-removal over nitrite (CANON) process

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HIGHLIGHTS

- We realize a high NH_4^+–N removal loading in relatively low NH_4^+–N concentration.
- We examine the biodiversity and species identification of functional microbes.
- Population of denitrify-related bacteria changes with NH_4^+–N concentration's change.
- We propose some approaches in CANON's application in low NH_4^+–N concentration.

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ABSTRACT

The research was conducted to investigate the microbial diversity and population with the different concentration of NH_4^+–N in a biofilm reactor filled with volcanic filter for completely autotrophic nitrogen-removal over nitrite (CANON) process. The reactor had an excellent performance with the decreasing of NH_4^+–N concentration from 400 to 200 mg L^{-1} while NH_4^+–N removal loading reduced at the NH_4^+–N concentration of 100 mg L^{-1}. Biodiversity analysis indicated that *Nitrosomonas* related aerobic ammonia oxidizing bacteria (AOB) and *Planctomycetales*-like anaerobic ammonia oxidizing (anammox) bacteria were dominant functional bacteria. Despite the different influent NH_4^+–N concentration, anammox bacteria had a low and stable biodiversity, which was not the same to AOB. With the concentration reduction of influent NH_4^+–N, the estimates of total bacteria population ranged between 2.29×10^{11} and 1.44×10^{12} copies mg^{-1} total DNA, and the quantity of AOB decreased while anammox bacteria kept stable. The population of *Nitrospira* increased and little *Nitrobacter* was detected during the experiment.

1. Introduction

The completely autotrophic nitrogen-removal over nitrite (CANON) process has been considered as one of the most efficient and economical approaches to remove ammonia from wastewater with high ammonia loading and low organic carbon content for consuming 63% less oxygen and nearly 100% less reducing agent than traditional nitrogen removal systems (Sliekers et al., 2003). CANON process has been used in several biofilm systems (Helmer et al., 2001; Third et al., 2005), which relies on the harmonious and balanced interaction between the aerobic and the anaerobic ammonia oxidizing bacteria that performs two sequential reactions simultaneously (Windey et al., 2005). In oxygen-limited condition, ammonia is first oxidized to nitrite by aerobic ammonia oxidizing bacteria (AOB), which consumes oxygen and creates an anoxic environment, and then nitrite is utilized with the remainder of ammonia and converts into dinitrogen gas by anaerobic ammonia oxidation (anammox) bacteria (Nielsen et al., 2005; Sliekers et al., 2003). During the process, accumulation of nitrite is regarded as a key point, and the further oxidation of nitrite to nitrate by nitrite oxidizing bacteria (NOB) should be prevented. Consequently, some process controlling strategies are urgently required to accumulate sufficient nitrite and prevent the production of nitrate (Yang et al., 2008).

However, most CANON process has been used to treat industrial sewage like sludge digestion and landfill leachate with high temperature (>30 °C) and high ammonia (>400 mg L^{-1}) nowadays (van der Star et al., 2007), and there are still some problems and limitations in treating domestic sewage. Consequently, understanding of the functional bacteria can be valuable to microbial

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engineering efforts aimed at a widely use of CANON process (Yang et al., 2008). Previous studies mainly focused on microbe ecology, spatial distribution and species identification, and the results showed that AOB belonged to *β-proteobacteria* was active in the outer aerobic region while *Planctomycetales*-like anammox bacteria existed in the inner anoxic region of both biofilm and aggregates (Helmer et al., 1999; Liu et al., 2008a; Nielsen et al., 2005). Besides, identification of anammox bacteria in CANON process indicated a various species like *Candidatus Brocadia anammoxidans*, *Candidatus Kuenenia stuttgartiensis*, anammox bacteria KSU-1 and some others (Hu et al., 2010). But unfortunately, there are currently few reports of their success as well-established guidelines for process monitoring that aiming at realizing an efficient CANON process in treating domestic sewage so far. Thus, it is vital and crucial to adopt proper tactics and strategies to reverse the current situation of CANON's limited application.

Influent NH_4^+–N concentration and temperature are regarded as two important factors for CANON treating domestic sewage because of the feature of wastewater. The decreasing of NH_4^+–N concentration and temperature can affect certain species, which may leads to either an acclimation of existing bacterial population to new conditions or a significant shift of microbial community and population (Liu et al., 2008a). And recent studies also indicated that seeding sludge, temperature and the type of wastewater were regarded as the most important factors that might control the community composition in anammox-like reactors (Hu et al., 2010). Moreover, a study showed the growth of a bacterial population depended on influent nitrogen loading (Isaka et al., 2006). Liu et al. studied the effect of salinity on functional microbiological community in a relatively low NH_4^+–N concentration (200 mg L^{-1}) in CANON process (Liu et al., 2008a).

No one specific study had been done on the relationship between the shift of bacterial community and population and the change of NH_4^+–N concentration in the light of previous studies. In this study, a lab-scale CANON biofilm reactor was started up in high NH_4^+–N concentration (400–480 mg L^{-1}) at room temperature, followed by a decreasing of influent NH_4^+–N concentration from 400 to 100 mg L^{-1} successively. Total DNA was extracted from the biofilm on the surface of the volcanic filled in the reactor, and used to evaluate the biodiversity of the two functional microorganisms (AOB and anammox bacteria) and the quantitative feature of total bacteria, AOB, anammox bacteria, *Nitrobacter*-like and *Nitrospira*-like NOB. The information related with microbial ecology and population in CANON reactor is essential for a better understanding about the microorganisms, which, may further lead to the application of CANON process in treating domestic sewage.

2. Methods

2.1. Reactor and operational parameters

A lab-scale CANON reactor filled with volcanic filter was used in this study with the diameter, height, total volume and working volume of 150 mm, 700 mm, 8.15 and 1.8 L respectively. Before start-up of the system, the volcanic filter was inoculated by sludge from another CANON reactor that operated at high ammonia (400–480 mg L^{-1}) and high temperature (30–35 °C) in the laboratory. The synthetic wastewater using tap water mixed with various amount of $NaHCO_3$, $(NH_4)_2SO_4$ and KH_2PO_4 was pumped continuously from the bottom of the reactor and output from the upper outlet.

The reactor started up successfully and run steadily for 150 days in high NH_4^+–N concentration (400–480 mg L^{-1}), with temperature and pH value of 12–23 °C and 8.0 respectively, and NH_4^+–N removal rate and removal loading were detected when influent NH_4^+–N concentration decreased from 400 to 100 mgL^{-1} gradually.

2.2. Chemical analysis of water quality

NH_4^+–N and NO_2^-–N were measured using different colorimetric methods and NO_3^-–N was analyzed by using ultraviolet spectrophotometric method. The pH value was detected by a digital, portable pH meter (OAKTON Waterproof pH Testr 10BNC) while DO and temperature were measured by a multi-line dissolved oxygen meter (WTW inoLab Oxi197i + StirrOx-G).

2.3. Sampling and DNA extraction

Some pieces of volcanic filter were collected and stored in 50 mL sterile plastic test tubes at −20 °C when the reactor was in a stable operation with different influent NH_4^+–N concentrations. Biofilm was removed from the volcanic filter with sterile brush and collected into 10 mL sterile plastic test tube. DNA extraction liquid (100 mM Tris–HCl, 100 mM EDTA, 1.5 M NaCl, 100 mM Na_3PO_3, 1% CTAB, pH 8.0) was added with an amount of 2.7 mL together with 50 μL proteinase K (30 g L^{-1}), 50 μL lysozyme (20 g L^{-1}) and some glass beads (0.1 mm in diameter), and incubated at 37 °C for 30 min. SDS (200 g L^{-1}) was added with an amount of 1.5 mL and incubated at 65 °C for 2 h with a brief vortexing every 15–20 min. The suspension was then centrifuged at 8000 g for 10 min and the supernatant was transferred to a new sterile tube. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol/vol) was added and centrifuged as described above and the upper phase was transferred to a fresh sterile tube. DNA was then precipitated with 0.6 volume of isopropanol and stored in −20 °C overnight. Precipitated DNA was collected by centrifugation at 12000*g* for 5 min and the supernatant was discarded, and then centrifuged at 12000*g* for 3 min again. Finally the total DNA was suspended in 50 μL of 1 × TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and detected by 0.8% (w/V) agarose gel electrophoresis. Resulting DNA concentration and quality were measured by UV spectrophotometry.

2.4. PCR amplification and DGGE

The primers used for PCR-DGGE in this study were listed in Table 1. To amplify 16S rDNA of *β-Proteobacteria* ammonia oxidizing bacteria for DGGE, a nested PCR approach was adopted. For the first round PCR, 465 bp DNA fragments were obtained with the primers CTO 189fA/B and CTO 189fC of a 2:1 ratio, together with the reverse primer CTO654r. The conditions and the procedure were described previously (Kowalchuk et al., 1997). The PCR products were purified with the universal DNA purification kit (Tiangen, China) according to the manufacturer's instructions, which were used as templates for a second amplification with universal primers F338 (with a GC-clamp) and R518 (Muyzer et al., 1993). The protocol was as follows: 94 °C for 5 min; 30 cycles consisting of 94 °C for 40 s, 55 °C for 40 s; 72 °C for 60 s; and 72 °C for 10 min, the annealing temperature dropped by 0.1 °C after each cycle.

Primers Amx368f (with a GC-clamp) and Amx820r were used to amplify 16S rDNA of anammox bacteria. The protocol was as follows: 94 °C for 5 min; 30 cycles consisting of 94 °C for 60 s, 60 °C for 60 s; 72 °C for 90 s; and 72 °C for 10 min.

Thermocycling was performed in Takara PCR Thermal Cycler Dice with a total volume of 50 μL PCR mixture which contained 5 μL 10 × PCR buffer (with 1.5 mM $MgCl_2$), 4 μL deoxynucleoside triphosphate solution (2.5 mM each dATP, dCTP, dGTP and dTTP), 2 μL of each primer (10 mM), and 1.25 U of DNA polymerase (Takara, Japan). The total amount of template DNA added to PCR mixtures was approximately 10–100 ng. All products were detected by 1.5% (w/V) agarose gel electrophoresis to confirm the product size and then purified with the TIANgel midi purification kit