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Effect of phenol on the nitrogen removal performance and microbial community structure and composition of an anammox reactor

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HIGHLIGHTS

- Phenol at 300 mg L^{-1} decreased the ammonium–N removal efficiency by half.
- The anammox performance recovered after 45 days without phenol addition.
- Phenol changed considerably the microbial community structure and composition.
- The phenol addition reduced the anammox population abundance from 14.7% to 10.1%.
- Phenol selected for some phenol-degrading genera under denitrifying conditions.

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ABSTRACT

The effects of phenol on the nitrogen removal performance of a sequencing batch reactor (SBR) with anammox activity and on the microbial community within the reactor were evaluated. A phenol concentration of 300 mg L^{-1} reduced the ammonium–nitrogen removal efficiency of the SBR from 96.5% to 47%. The addition of phenol changed the microbial community structure and composition considerably, as shown by denaturing gradient gel electrophoresis and 454 pyrosequencing of 16S rRNA genes. Some phyla, such as *Proteobacteria*, *Verrucomicrobia*, and *Firmicutes*, increased in abundance, whereas others, such as *Acidobacteria*, *Chloroflexi*, *Planctomycetes*, *GN04*, *WS3*, and *NKB19*, decreased. The diversity of the anammox bacteria was also affected by phenol: sequences related to *Candidatus* Brocadia fulgida were no longer detected, whereas sequences related to *Ca.* Brocadia sp. 40 and *Ca.* Jettenia asiatica persisted. These results indicate that phenol adversely affects anammox metabolism and changes the bacterial community within the anammox reactor.

1. Introduction

The anaerobic oxidation of ammonium (anammox) is a very promising technology for the removal of nitrogen from wastewaters. The anammox process is catalyzed by chemolithoautotrophic bacteria belonging to the phylum *Planctomycetes*, which can directly oxidize ammonium under anoxic conditions using nitrite as a terminal electron acceptor, converting ammonium to dinitrogen gas in the absence of oxygen (Strous et al., 1998). The anammox process has advantages over the traditional nitrification–denitrification process for nitrogen removal because it uses up to 60% less oxygen and does not require the addition of external carbon. Furthermore, the process produces less sludge and no CO_2 or N_2O emissions (Gao and Tao, 2011). The main drawbacks of the process include the slow growth of the anammox bacteria, which have a high doubling time of approximately 11 days (Strous et al., 1998), and the need for strict control of the process, because factors such as toxic organic compounds, including phenols, can inhibit the anammox bacteria (Jin et al., 2012).

Phenolic compounds are frequently found in industrial wastewaters that also contain nitrogen, such as coke-oven wastewater (Toh and Ashbolt, 2002), wastewater from petrochemical industries (Yang and Jin, 2012), and resin-producing wastewater (Amor et al., 2005). Therefore, before using the anammox process to treat these kinds of wastewater, it is vital to study the effect of phenol on the anammox process and on the microbial community present in anammox reactors. The effects of phenol

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on anammox activity have been studied previously, but the microorganisms involved were not identified (Toh and Ashbolt, 2002; González-Blanco et al., 2012; Jin et al., 2013; Yang et al., 2013). Anammox bacteria were acclimated in phenol-containing wastewater at low nitrogen-loading rates over a long period (Toh and Ashbolt, 2002). In a recent study (Yang et al., 2013), the short- and long-term effects of phenol on anammox activity were determined. Most of these studies investigated the effect of phenol on the anammox activity and on high-rate anammox processes. However, the effect of phenol on the microbial community structure and composition in an anammox reactor has not been investigated. A better understanding of the microbial community composition of an anammox reactor before and after the disruption caused by a toxic compound such as phenol may help elucidate the processes that occur inside the reactors under stress conditions, which is important for improving processes used to treat industrial wastewaters that contain toxic compounds.

Because of their high coverage, second-generation sequencing technologies provide more comprehensive information about microbial communities than the traditional Sanger-based methods. Pyrosequencing can detect organisms at low abundance. This technique has not previously been used to study changes in the microbial community structure of an anammox reactor subjected to phenol stress. Therefore, this study aimed to investigate the following: (1) the effects of increasing concentrations of phenol on the nitrogen removal performance of an anammox enrichment reactor (without biomass acclimatization) and (2) the effect of phenol on the microbial community structure and composition in an anammox reactor.

To achieve these objectives, a sequencing batch reactor (SBR) seeded with activated sludge was used to produce the anammox biomass. The reactor was monitored for 591 days, and phenol was added after 335 days of operation under anammox conditions. The microbial community structure and composition in the reactor were investigated using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis and 454 pyrosequencing of bacterial 16S rRNA genes.

2. Methods

2.1. Seeding sludge and experimental set-up

Activated sludge from a municipal wastewater treatment plant located in Belo Horizonte (Brazil) was used as an inoculum to cultivate anammox bacteria because a previous study successfully enriched anammox bacteria from this sludge (Araújo et al., 2011). A 2.0-L glass SBR (Benchtop Fermentor & Bioreactor Bio Flo®/CelliGen® 115, New Brunswick Scientific Co., Enfield, CT, USA) was used for enrichment of anammox bacteria. The reactor was fitted with a fermentor lid containing a feed in-flow tube, a dissolved oxygen probe, a pH probe, acid and base in-flow tubes for pH control, a gas line, a sampling line, and an effluent-withdraw line. The temperature was controlled and maintained at 34–35 °C via a water jacket, and the pH was controlled at 7.5. Anaerobiosis was maintained by bubbling N_2 gas (99.99%) through the liquid. This gas was also flushed in the mineral medium (feed vessel) in order to maintain anaerobic conditions in the synthetic wastewater. The reactor was monitored for 591 days and was operated as a continuously fed SBR with two cycles, one of 8 h (short cycle) and the other of 16 h (long cycle). Each cycle had four phases: (i) continuous feeding period (180 min in both cycles), (ii) anaerobic reaction period (240 min in the short cycle and 720 min in the long cycle), (iii) settling period (30 min in both cycles), and (iv) withdrawal period (30 min in both cycles).

2.2. Autotrophic medium

The reactor was fed autotrophic medium with a composition similar to that described previously (Van de Graaf et al., 1996). The final concentrations of ammonium and nitrite in the medium were increased gradually according to the evolution of anammox activity in the reactor (as described in Section 3).

2.3. Addition of phenol to the SBR

From days 336 to 377, phenol was introduced into the SBR during the long cycle (16 h) through a septum located at the top of the reactor using 60-mL syringes. Varying amounts of a concentrated solution of phenol (1 g L^{-1}) were added to the reactor in order to achieve the desired final concentration. The phenol concentration in the reactor was gradually increased every week to 10 (1st week), 25 (2nd week), 50 (3rd week), 100 (4th week), 200 (5th week), and 300 (6th week) mg L^{-1}.

Statistical analysis was performed to assess whether the different concentrations of phenol added to the SBR altered the nitrite and ammonium removal efficiency and the stoichiometric ratio of ΔNO_2-N:ΔNH_4^+-N). The Kruskal–Wallis test ($\alpha = 5\%$) was performed, followed by a multiple comparisons test between medians ($\alpha = 5\%$), using the Statistica 8 software. Periods in which phenol was added to the reactor were compared to the previous period, without phenol addition (291–335 days), in which the operating conditions were similar (ammonium and nitrite influent concentrations of 100 mg L^{-1} and 118 mg L^{-1}, respectively; temperature of 35 °C; agitation equal to 70 rpm; and hydraulic retention time of 24 h).

2.4. Analytical procedures

The concentrations of NH_4^+-N and NO_2^--N were determined colorimetrically by the phenol-hypochlorite (measured at 630 nm) and sulfanilic acid (measured at 520 nm) methods, 4500 NH_3F and 4500 NO_2^-B, respectively, according to the Standard Methods of the Examination of Water and Wastewater (APHA, 2005).

2.5. Analysis of the microbial community in the biomass from the anammox reactor

2.5.1. PCR-DGGE

The biomass in the SBR was sampled at day 0 (inoculum), days 110, 190, 270, and 330 (before phenol addition), and day 370 (period after phenol addition), which represent different stages of the SBR operation. Samples (20 mL) for molecular analyses were taken from the reactor and centrifuged at 14,000 rpm for 10 min, and the pellet was used for further studies. DNA was extracted using the protocol described by Egli et al. (2003). PCR-DGGE was performed using the primer set 1055F and 1392R, with a GC clamp, as described previously (Ferris et al., 1996). DGGE was performed at 60 °C in 0.5× TAE buffer at 80 V for 17 h with a Bio-Rad DCode Universal Mutation Detection System (Hercules, CA, USA), using an 8% polyacrylamide gel with a 45–75% (M/V) gradient of urea formamide denaturant. Gels were stained with a SYBR gold solution and visualized under UV transillumination. Specific gel bands were excised, re-amplified, purified, and sequenced. The PCR products were sequenced using a genomic service (Macrogen Inc., Seoul, Korea). Sequences were compared with sequences from the National Center for Biotechnology Information database using the Basic Local Alignment Search Tool (Altschul et al., 1990).

DGGE patterns were analyzed using the BioNumerics software version 2.5 (Applied Maths, Austin, TX, USA). Hierarchical cluster comparisons were carried out to group similar profiles and to generate a binary matrix of band classes. Whole profiles were