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Effect of temperature on microbial diversity and nitrogen removal performance of an anammox reactor treating anaerobically pretreated municipal wastewater

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ABSTRACT

The effects of temperature reduction (from 35 °C to 20 °C) on nitrogen removal performance and microbial diversity of an anammox sequencing batch reactor were evaluated. The reactor was fed for 148 days with anaerobically pretreated municipal wastewater amended with nitrite. On average, removal efficiencies of ammonium and nitrite were high (96%) during the enrichment period and phases 1 (at 35 °C) and 2 (at 25 °C), and slightly decreased (to 90%) when the reactor was operated at 20 °C. Deep sequencing analysis revealed that microbial community structure changed with temperature decrease. Anammox bacteria (*Ca.* Brocadia and *Ca.* Anammoximicrobium) and denitrifiers (*Burkholderiales*, *Myxococcales*, *Rhodocyclales*, *Xanthomonadales*, and *Pseudomonadales*) were favoured when the temperature was lowered from 35 °C to 25 °C, while *Anaerolineales* and *Clostridiales* were negatively affected. The results support the feasibility of using the anammox process for mainstream nitrogen removal from anaerobically pretreated municipal wastewater at typical tropical temperatures.

1. Introduction

For decades, aerobic nitrification followed by anoxic denitrification has been used to remove nitrogen from wastewaters. Anaerobic ammonium oxidation (anammox) is a biological process and a very promising alternative for nitrogen removal owing to its sustainable characteristics (low or even no oxygen consumption, no addition of external carbon source, and CO_2 consumption), and therefore presents the possibility of wide application (Kartal et al., 2013). Anammox bacteria are chemolithoautotrophic microorganisms that can oxidize ammonium (NH_4^+) into dinitrogen gas (N_2) using nitrite (NO_2^-) as an electron acceptor under anoxic conditions (Strous et al., 1998). To date, seven genera capable of anammox metabolism have been described in the literature. They belong to the phylum *Planctomycetes* and orders *Brocadiales* and *Planctomycetales* (Pereira et al., 2017).

The anammox process has been applied mainly to remove nitrogen from ammonium-rich wastewaters with low chemical oxygen demand (COD)/N ratios (Ali and Okabe, 2015; Shen et al., 2012; Tang et al., 2010). Nevertheless, some studies have shown that it can be applied to remove nitrogen from anaerobic effluents with high COD/N ratios (Leal et al., 2016; Vlaeminck et al., 2012).

Temperature is a key factor for microorganism growth and metabolism that directly affects their abundance (Ali and Okabe, 2015; Ma et al., 2016). Higher temperatures (35 °C–40 °C) were usually associated with the maximum anammox biomass activity and cell doubling time; however, extreme conditions (above 45 °C) may irreversibly inhibit cell activity because of cell lysis (Dosta et al., 2008). Gao and Tao (2011) and Strous et al. (1998) reported that the anammox process can occur in the temperature range of 20 °C to 43 °C, with the optimum activity at 40 ± 3 °C. Additionally, Ali and Okabe (2015) reported that 37 °C is the optimum temperature for the anammox process. Yet, Zhu et al. (2008) reported that 26 °C–28 °C is the optimum temperature range and anammox metabolism decreased considerably at temperatures below 15 °C and above 40 °C. Dosta et al. (2008) also noticed a decrease in metabolism when the temperature was lowered from 20 °C to 15 °C.

Currently, the anammox and partial nitritation (PN)/anammox

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processes have been used to treat warm and concentrated effluents, such as anaerobic sludge digestate, at wastewater treatment plants (Lackner et al., 2014), which is known as sidestream treatment. Nevertheless, these processes have not been applied for mainstream treatment (more diluted effluents), taking into account anaerobically pretreated municipal wastewater.

The performance of anammox reactors for nitrogen removal at temperatures under 25 °C has been extensively investigated for ammonium-rich wastewaters (with 500 mg N·L^{-1}) (Hendrickx et al., 2012; Vázquez-Padín et al., 2010). However, higher ammonium removal efficiency (90%) was reported by Hu et al. (2013) in a nitration-anammox bioreactor at 12 °C fed with synthetic pretreated municipal wastewater (70 mg·L^{-1} of ammonium). Additionally, low ammonium concentrations and total nitrogen removal efficiencies around 40% were reported in a moving bed biofilm reactor with PN/anammox operated at 13 °C that was treating diluted anaerobic digestate (at decreasing ammonium concentrations from 496 mg·L^{-1} to 43 mg·L^{-1}). In this case, anammox bacteria outcompeted nitrifying bacteria (Persson et al., 2016). Moreover, Gonzalez-Martinez et al. (2015) investigated the performance and bacterial community dynamics of a completely autotrophic nitrogen removal over nitrite (CANON) bioreactor that was treating anaerobic digestate at decreasing temperatures (from 35 °C to 15 °C) and decreasing ammonium concentrations (from 466 mg N·L^{-1} to 100 mg N·L^{-1}). When the system acclimated from 35 °C to 25 °C, nitrogen removal efficiency showed a moderate decrease, affecting the bacterial community structure by selecting *Candidatus* Brocadia and *Candidatus* Anammoxoglobus and increasing the abundance of some genera (*Anaerolinea*, *Acidobacterium*, *Chloroflexi*, *Fluviicola*, and *Prosthecobacter*). An additional biomass acclimation step from 25 °C to 15 °C sharply decreased the nitrogen removal efficiency in the CANON bioreactor.

Despite previous studies, little is known about the microbial community composition and dynamics in anammox reactors under mainstream conditions, i.e. treating real anaerobic effluent. Furthermore, few studies have dealt with temperature variation impacts on microbial community diversity and process performance. In this sense, Leal et al. (2016) reported that high COD, nitrite, and ammonium removal efficiencies (80%, 90%, and 95%, respectively) were reached with addition of real anaerobically pretreated municipal wastewater (supplemented with nitrite) to a SBR. Moreover, the bacterial community structure changed and DNA sequences related to *Ca.* Brocadia sinica, *Ca.* Brocadia caroliniensis and *Chloroflexi* were identified. Nevertheless, the long-term effects of adding real anaerobic effluent to an SBR were not investigated in this study, and the bacterial community structure was investigated via PCR-denaturing gradient gel electrophoresis (DGGE), which detects dominant members of a bacterial community.

Therefore, in the present study, the effect of typical tropical temperature variation (20–35 °C) on the microbial community and nitrogen removal efficiency of an anammox SBR fed with anaerobically pretreated municipal wastewater over 148 days was investigated. The microbial community structure and diversity were investigated via PCR-DGGE and high-throughput amplicon sequencing (Ion Torrent), which provide more detailed information about microbial communities. Quantitative PCR (qPCR) was also applied to determine the abundance of denitrifiers and anammox bacteria.

2. Methods

2.1. Experimental setup and monitoring

The inoculum used to enrich anammox bacteria was obtained from excess sludge in an activated sludge plant located in the Brazilian city of Belo Horizonte. According to previous studies, anammox bacteria have been enriched successfully from this sludge (Leal et al., 2016). A 2.0-L glass reactor (Benchtop Fermentor & Bioreactor BioFlo/CelliGen 115, New Brunswick Scientific Co., Enfield, CT, USA) was used for anammox bacteria cultivation. This reactor comprised dissolved oxygen and pH probes, as well as acid and base in-flow tubes for pH control. The temperature was maintained at 35 °C (or reduced to 25 °C and 20 °C, see Table 1) via a water jacket, and the pH was maintained at 7.5. Anaerobic conditions were assured by bubbling N_2 gas (99.99%) through the liquid (in the enrichment period). This gas was also flushed into the feed vessel to maintain anaerobic conditions in the synthetic wastewater. When real anaerobic effluent was used, nitrogen was not flushed in feed vessel or in the reactor.

Table 1
Mean values of physico-chemical parameters and experimental phases in the SBR.

Experimental phase	Duration (days)	Temperature (°C)	NO_2 (mg·L^{-1})	NH_4 (mg·L^{-1})	COD (mg·L^{-1})
Anammox enrichment period[a]	0–160	35	47	32	0
Phase 1[b]	40	35	78	42	130
Phase 2	63	25	72	41	100
Phase 3	45	20	70	32	97

[a] During the enrichment period, the reactor was fed with autotrophic medium.
[b] In phases 1, 2, and 3, the reactor was fed with anaerobically pretreated municipal wastewater supplemented with nitrite.

The reactor was monitored for 308 days under different operational conditions regarding the applied temperature (Table 1) and was operated in sequencing batch mode with two cycles, one of 7 h (short cycle) and the other of 17 h (long cycle). Each cycle had four phases: (i) continuous feeding period (40 min for both cycles), (ii) anaerobic reaction period (420 min for the short cycle and 1020 min for the long cycle), (iii) settling period (30 min for both cycles), and (iv) withdrawal period (40 min for both cycles).

In the enrichment period, the SBR was fed an autotrophic medium (Dapena-Mora et al., 2004) and operated at 35 °C. Concentrations of ammonium and nitrite in the synthetic medium ranged from 40 mg·L^{-1} to 80 mg·L^{-1} and from 30 mg·L^{-1} to 60 mg·L^{-1}, respectively. The nitrite to ammonium ratio was kept around 1.32. The anammox enrichment period lasted 160 days. In the following periods (shown in Table 1), the reactor was fed with anaerobically pretreated municipal wastewater containing on average 109 mg COD·L^{-1}, 38.4 mg N-NH_4^+·L^{-1} and 170 mgCaCO$_3$·L^{-1}, when operated at 35 °C, 25 °C, and 20 °C (phases 1, 2, and 3, respectively). The characteristics of the anaerobic effluent (from a demo-scale upflow anaerobic sludge blanket (UASB) reactor) were previously described by Leal et al. (2016). In phases 1, 2, and 3, the reactor was fed with real anaerobic effluent amended with nitrite (from 60 mg·L^{-1} to 80 mg·L^{-1}, in order to keep the nitrite to ammonium ratio around 1.4 to 1.8 therefore providing sufficient nitrite for anammox and denitrifying bacteria. Considering the stoichiometry of the anammox reaction (reported by Strous et al., 1998), it is possible to estimate an alkalinity consumption of 0.28 mg HCO_3^- (0.23 mg $CaCO_3$)/mgNH_4^+-N oxidized in the anammox reaction. Thus, the alkalinity present in the real anaerobic effluent was high enough to support the growth of anammox bacteria.

The temperature range (20–35 °C) was chosen to simulate the typical climate conditions prevailing in tropical regions (e.g. Brazil). In addition, the annual average temperature found in the city of Belo Horizonte, MG, Brazil, where this research was conducted, is around 23 °C. Therefore, the results could further support the implementation of the anammox process at ambient temperatures found in tropical regions worldwide.

Influent and effluent samples were collected four times a week to monitor the concentrations of ammonium, nitrite, and COD. Analyses were performed according to Standard Methods for the Examination of Water and Wastewater (APHA, 2012). Biomass samples were taken from the reactor at the end of each operational phase to investigate the