Bioresource Technology 181 (2015) 207–213

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

# Microbial community shifts on an anammox reactor after a temperature shock using 454-pyrosequencing analysis

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## HIGHLIGHTS

- Pyrosequencing was used to assess microbial community shifts in an anammox reactor.
- Microbial diversity decreased as anammox reactor recovered from temperature shock.
- N-removal results were consistent with 454-pyrosequencing results.
- Anammox population shifted from *Candidatus Kuenenia* to *Brocadia anammoxidans*.

## ARTICLE INFO

*Article history:*
Received 18 November 2014
Received in revised form 9 January 2015
Accepted 14 January 2015
Available online 22 January 2015

*Keywords:*
454-Pyrosequencing
*Brocadia anammoxidans*
*Candidatus Kuenenia*
Microbial diversity

## ABSTRACT

To explore the changes in the microbial community structure during the recovery process of an anammox reactor after a temperature shock, the 454-pyrosequencing technique was used. The temperature shock reduced the nitrogen removal rate up to 92% compared to that just before the temperature shock, and it took 70 days to recover a similar nitrogen removal rate to that before the temperature shock (ca. 0.30 g N $L^{-1}$ $d^{-1}$). Pyrosequencing results indicated that microbial diversity in the reactor decreased as the reactor progressively recovered from the temperature shock. Anammox bacteria were accounted as 6%, 35% and 46% of total sequence reads in samples taken 13, 45 and 166 days after the temperature shock. These results were in agreement with N-removal performance results and anammox activity measured in the reactor during the recovery process. An anammox specific primer was used to precisely determine the anammox species in the biomass samples.



## 1. Introduction

The anaerobic ammonium oxidation (anammox) is widely considered as the most economical and sustainable process for nitrogen removal from wastewater, due to its high nitrogen removal potential at reduced operating costs (Kartal et al., 2010; Lackner et al., 2014). Compared to conventional nitrification–denitrification process, the anammox process does not require organic matter for nitrogen removal, and oxygen requirements are reduced since only ca. half ammonium needs to be oxidized into nitrite in a previous partial nitritation step (Lackner et al., 2014).

The main disadvantage of the anammox process is, however, the very slow growth rate of the microbes responsible for this process, i.e. the "anammox bacteria". Due to this slow growth rate, anammox bacteria doubling times around 10–14 days have been typically reported at their optimum growth temperature, i.e. 35–40 °C (Dosta et al., 2008; Strous et al., 1999). Anammox growth rate is reduced ca. 30–40% every 5 °C of temperature decrease (Dosta et al., 2008; Strous et al., 1999). Perhaps for this reason, early anammox process implementations were generally at temperatures close to the optimum (Abma et al., 2010; van der Star et al., 2007), in order to minimize the slow growing characteristics of anammox bacteria. For some applications, anammox reactors are even heated to enhance the anammox growth, such as in some lab-scale rectors (Hu et al., 2013) or industrial reactors (Wett et al., 2013).

Although in natural environments, anammox growth has been reported to occur at temperatures as low as −2.5 °C in sea ice (Rysgaard and Glud, 2004) and as high as 70 °C in hot springs and hydrothermal vent areas (Byrne et al., 2009), in wastewater treatment applications anammox growth is limited to a maximum

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http://dx.doi.org/10.1016/j.biortech.2015.01.064
0960-8524/

temperature of 43 °C (Strous et al., 1999). In fact, Toh et al. (2002) could not cultivate thermophilic anammox biomass at 55 °C. Dosta et al. (2008) observed that the exposure of anammox biomass at a temperature of 45 °C during a few hours resulted in a complete loss of activity and evidences of cell lysis. Similarly, Liu et al. (2015) found that an accidental overheating at 48 °C for 1 h caused anammox bacteria damage and death in an irreversible process. After that short temperature shock, they needed two weeks to recover a measurable anammox activity. Accordingly, temperature in anammox reactors should be carefully controlled below ca. 40 °C, especially in those reactors using heaters for temperature control (as abovementioned), or in reactors used for the treatment of industrial hot effluents (Lopez-Vazquez et al., 2014), to avoid the risk of overpassing the maximum permissible temperature of anammox bacteria which would lead to an anammox reactor failure. However, to the best of the authors' knowledge, there is lack of information in the literature about the impact of a long term temperature shock over the performance of an anammox reactor.

Changes in environmental conditions, such as a temperature shock, have a direct impact in microbial communities. The study of these microbial communities and their changes provides valuable information to better understand the nutrient removal processes occurring in wastewater treatment ecosystems. Different molecular biology techniques, such as fluorescence *in situ* hybridization (FISH) (Lotti et al., 2014), denaturing gradient gel electrophoresis (DGGE) (Park et al., 2010) or clone library (Hu et al., 2010) have been widely used for this purpose. The second generation of 454-pyrosequencing has been recently developed. This technique offers a high-throughput, fast and economical sequencing platform, with exceptional accuracy (Droege and Hill, 2008). With 454-pyrosequencing, thousands of operational taxonomic units (OTUs) can be identified in a reasonable period of time. Therefore, this technique can provide wider and more complete information about microbial community structures than previous conventional molecular biology techniques. For these reasons, 454-pyrosequencing has gained interest in the study of complex microbial communities such as those of wastewater treatment environments (Hu et al., 2012; Xie et al., 2013). Despite the potential of 454-pyrosequencing, the use of this novel technique with anammox reactors is still scarce (Costa et al., 2014; Pereira et al., 2014).

Hence, the performance of an anammox reactor before and after the occurrence of a high and long term temperature shock (46 °C for 8 days) is presented. The research was focused on exploring the microbial community structure changes during the N-removal recovery process of the anammox reactor. With that purpose, 454-pyrosequencing technique was used.

## 2. Methods

### 2.1. Experimental set-up description

The anammox process was carried out in a sequencing batch reactor (SBR) with a working volume of 10 L, a diameter of 20 cm and a height of 61 cm. The SBR was operated in cycles of 6 h, divided in four phases: mixed filling (300 min), mixing (30 min), settling (20 min) and effluent withdrawal (10 min). The volumetric exchange ratio was of 25%. Biomass was mixed using a mechanical stirrer operated at rotating speed of 72 rpm. Nitrogen gas was flushed into the headspace at an average flow rate of 300 mL $min^{-1}$. The pH was not controlled but ranged between 7.5 and 8.5 during one cycle. The temperature before and after the temperature shock was maintained constant at 35 °C.

The reactor was fed with a mineral medium containing (mg $L^{-1}$): $KHCO_3$ (100); $H_2PO_4$ (50); $CaCl_2{\cdot}2H_2O$ (100); $MgSO_4{\cdot}7H_2O$ (200); $FeSO_4$ (6.3); EDTA (6.3) and 1.25 mL $L^{-1}$ of a trace elements solution (van de Graaf et al., 1996). The required amounts of nitrite and ammonium in the form of $NaNO_2$ and $(NH_4)_2SO_4$ were added to this mineral medium (as specified in Section 3, Fig. 1).

The SBR was operated for more than 700 days previously to the temperature shock with nitrogen loading rate (NLR) ranging from 0.20 to 0.55 g N $L^{-1}$ $d^{-1}$. The biomass had an average Specific Anammox Activity (SAA) of 0.40 ± 0.05 g N $g^{-1}$ VSS $d^{-1}$.

The temperatures shock lasted 8 days (from days −8 to 0 in Fig. 1) and consisted in a step change of the temperature in the anammox SBR from 35 to 46 °C.

### 2.2. Analytical methods

Ammonium was measured by means of an ammonium analyzer (AMTAX, Hach Lange, Germany). Nitrate and nitrite concentrations were analyzed with ionic chromatography using an ICS-2000 Integrated Reagent-Free IC system (DIONEX Corporation, USA) which performs ion analyses using suppressed conductivity detection. Mixed liquor total suspended solids (TSS), mixed liquor volatile suspended solids (VSS) were measured according to Standard Methods (APHA, 2008).

SAA was determined in batch assays, measuring the overpressure produced by di-nitrogen gas production of anammox bacteria (adapted from Dapena-Mora et al., 2007).

Nitrifying activity was determined in an aerobic batch assay, measuring the ammonium, nitrite and nitrate concentration over time. Dissolved oxygen (DO) concentration, pH and temperature were maintained in this assay at 0.5–1.0 mg $O_2$ $L^{-1}$, 7.5 ± 0.1 and 34 ± 1 °C, respectively.

### 2.3. Granular sludge morphology

The Anammox granules for scanning electron microscope (SEM) observation were washed three times with water to remove impurities. For fixation, cells were immersed in 2.5% (vol/vol)

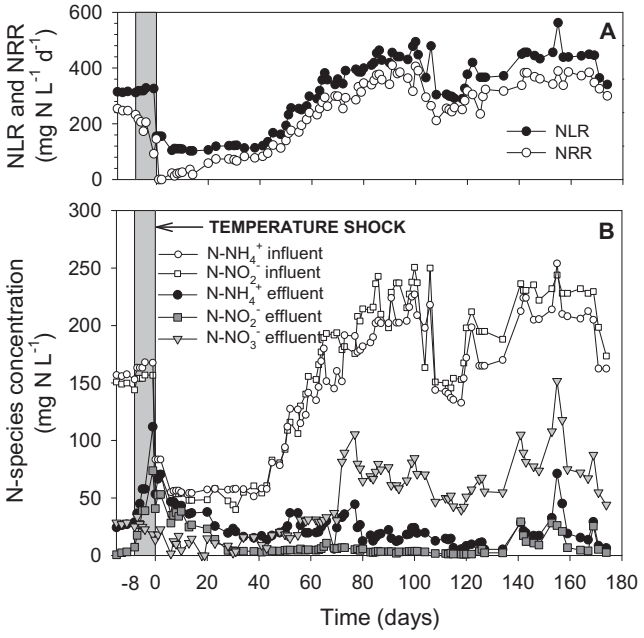


**Fig. 1.** Time course of nitrogen removal performance measured in the anammox reactor before and after the temperature shock event (highlighted with a gray band). (A) nitrogen loading and removal rate; (B) influent and effluent ammonium, nitrite and nitrate concentration.