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Characterization of a combined batch-continuous procedure for the culture of anammox biomass

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ABSTRACT

Interest in autotrophic nitrogen (N) removal through anaerobic ammonium oxidation (anammox) is high in the field of wastewater treatment as a more economic and sustainable alternative than conventional nitrification-denitrification. However, anammox biomass is difficult to enrich, and this can hinder the start-up of new applications. We carried out experimental work to characterize a combined batch-continuous procedure for the enrichment and culture of anammox biomass. In the first stage (time span: 120 d), the enrichment was started in batch mode (sealed vial) using suspended activated sludge as inoculum. Anammox activity was clearly developed since the specific ammonium (NH_4^+) conversion rate increased from 0 to 118 ±1 mg NH_4^+-N/(g VS d) (VS, volatile solids); i.e., 560 ±11 mg N/(L d) in terms of N-conversion rate (NCR). Subsequently, the sludge was transferred into a continuous upflow reactor packed with a polyester non-woven material to promote the attached growth of the biomass. Such bioreactor was operated without interruption during 400 d. Under an appropriate feeding regime, the anammox activity increased fast, and a sustained NCR of 1183 ±100 mg N/(L d) was reached according to the N-loading rate applied. Evolution of the microbial community structure was characterized using high-throughput DNA sequencing. The overall procedure prompted the selection of a community enriched in the anammox bacterial species Candidatus *Brocadia sinica* (up to ~70% of the total DNA sequences). Other coexisting microbial groups belonged to *Rhodocyclaceae* (class β-*Proteobacteria*), *Anaerolineae* (phylum *Chloroflexi*) and *Ignavibacteriaceae* (phylum *Chlorobi*).

1. Introduction

The anaerobic ammonium oxidation (anammox) is an interesting bioprocess for the removal of nitrogen (N) from wastewaters since it allows more economic and sustainable treatment than conventional approaches based on heterotrophic denitrification (Ma et al., 2016; Siegrist et al., 2008). In engineered systems, its coupling with partial nitritation results in a complete autotrophic deammonification, which reduces by 60% the oxygen requirement, 100% the organic carbon requirement, and 90% the biosolids production with respect to classical nitrification-denitrification. In addition, it offers the chance of working with more compact reactors at higher loading rates (Magrí et al., 2013; Van Hulle et al., 2010).

The anammox process is mediated by chemolithoautotrophic bacteria that oxidize ammonium (NH_4^+) into dinitrogen gas (N_2) using nitrite (NO_2^-) as the electron acceptor (Strous et al., 1998). Nitric oxide (NO) and hydrazine (N_2H_4) are two known intermediates of such reaction (Kartal et al., 2011). This bioconversion takes place under absence of oxygen and presence of inorganic carbon. A small amount of nitrate (NO_3^-) is also produced due to the oxidation of nitrite linked to the fixation of inorganic carbon in anabolism (de Almeida et al., 2011). According to the full stoichiometry pro-

Abbreviations: anammox, anaerobic ammonium oxidation; DO, dissolved oxygen; HRT, hydraulic residence time; N, nitrogen; NCE, nitrogen conversion efficiency; NCR, nitrogen conversion rate; NLR, nitrogen loading rate; NMDS, non-metric multidimensional scaling; OTU, operational taxonomic unit; PCR, polymerase chain reaction; SBR, sequencing batch reactor; VS, volatile solids; WWTP, wastewater treatment plant.

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posed by Strous et al. (1998) –Eq. (1)–, ammonium and nitrite are converted into dinitrogen gas and nitrate under the molar ratios 1.00/1.32/1.02/0.26 for NH_4^+ consumption, NO_2^- consumption, N_2 production, and NO_3^- production, respectively –values obtained through mass balance in a sequencing batch reactor (SBR) running under stable conditions-.

$$1.00NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (1)$$

To date, six "Candidatus" anammox bacterial genera have been enriched from samples collected in wastewater treatment facilities and natural environments such as freshwater and marine zones; i.e., Ca. *Brocadia* (Strous et al., 1999), Ca. *Kuenenia* (Schmid et al., 2000), Ca. *Scalindua* (Kuypers et al., 2003), Ca. *Anammoxoglobus* (Kartal et al., 2007), Ca. *Jettenia* (Quan et al., 2008), and Ca. *Anammoximicrobium* (Khramenkov et al., 2013). All these genera belong to the same phylum *Planctomycetes*. In spite of this fact, while the first five aforementioned genera form a deeply branched monophyletic group (family *Brocadiaceae*), the sixth is closely related to the genus *Pirellula* (family *Planctomycetaceae*). In physiological terms, the anammox bacteria feature a specific cytoplasmatic membrane-bound organelle known as anammoxosome, which is the locus of the anammox catabolism (van Niftrik and Jetten, 2012). They are also characterized by a low growth rate, with doubling times (at ~30 °C) of 2.1–11 days (Lotti et al., 2015; Strous et al., 1998). Owing to this slow biomass development and the specialized metabolism, the anammox bacteria may be difficult to culture.

The anammox bacteria have not been isolated in pure culture yet. Otherwise, such microorganisms have been enriched from various environments up to a culture purity degree of about 80–95% (van Niftrik and Jetten, 2012) –maximum value found in the literature is 98 ±1% in a suspended cell anammox culture (Lotti et al., 2014)–. Frequently, those strategies used to enrich anammox biomass consist on the utilization of different types of continuously operated reactors such as the SBR, rotating biological contactor, membrane bioreactor, upflow anaerobic sludge blanket reactor, upflow fixed-bed biofilm reactor, or stirred-tank moving-packed-bed reactor, among others (Egli et al., 2003; Strous et al., 1998; Tsushima et al., 2007; Wang et al., 2009; Xiong et al., 2013; Bae et al., 2015). Alternatively, anammox enrichments have also been performed in batch mode using sealed vials (Bae et al., 2010; Connan et al., 2016; Yasuda et al., 2011). In all cases, appropriate selection of the environmental conditions such as temperature, pH, and levels of ammonium, nitrite, organic carbon, dissolved oxygen (DO), and other nutrients and inhibitors, is critical for a successful enrichment and mass culture (Carvajal-Arroyo et al., 2013). The enrichment of anaerobic ammonium-oxidizing biomass from conventional sludge is time-consuming and may take from several months to years depending on the seeding source, reactor setup, and operational conditions applied (Ibrahim et al., 2016). Thus, biomass enrichment usually is the critical step for the start-up of new anammox applications (especially when pre-enriched sludge is not available).

The objective of this study is to characterize a culture of anammox biomass obtained from activated sludge using a combination of batch and continuous procedures. The enrichment was started under suspended biomass batch mode (sealed vial) and subsequently continued using a continuous upflow reactor packed with a polyester non-woven material to promote the attached growth of the biomass. The evolution of the microbial community structure was characterized throughout the process by means of 16S rRNA gene high-throughput sequencing.

2. Material and methods

2.1. Biomass sources: collection and pretreatment

Activated sludge collected in a municipal wastewater treatment plant (WWTP) that combine the use of a Modified Ludzack-Ettinger bioreactor unit and a membrane filtration loop to perform N-removal was used as inoculum for the enrichment of anammox biomass. Such treatment facility is located in Betton (France). Before starting with the anammox enrichment procedure, denitrification was favored during the first days after sampling in order to promote biodegradation of residual organic matter. Such pretreatment was carried out at room temperature by adding a nitrate source (KNO_3) in pulses equivalent to 100 mg N/L, similarly to Casagrande et al. (2011), and controlling the pH within the range 7.0-8.0 (HCl). The anammox batch enrichment was started after 4 weeks, once denitrification activity decreased. For microbial characterisation purposes, an alternative anammox biomass sample was obtained from a lab-scale 10 L jacketed upflow fixed-bed biofilm reactor running at the USDA-ARS laboratory in Florence, South Carolina, USA (Vanotti et al., 2011). At the time of sludge collection, the reactor was fed with mineral medium containing 153 mg NH_4^+-N/L and 153 mg NO_2^--N/L, and was operated with a hydraulic residence time (HRT) of 4 h, N-loading rate (NLR) of about 1800 mg N/(L d) and water temperature of 30 °C, analogously as detailed by Magrí et al. (2012b). Once received at the Irstea laboratory in Rennes, the external sample (E) was stored for 2 years under freezing conditions before proceeding with DNA extraction.

2.2. Batch stage

The enrichment in batch mode was performed using a glass vial which contained the inoculum and mineral medium (working volume of 0.5 L). Such vial was sealed with a rubber stopper plus an aluminium cap and placed into an incubator shaker (model KS4000i control, IKA, Germany) at 150 rpm, 35 °C, and in dark conditions. Initial solids content inside the vial was adjusted to 1.50 g VS (VS, volatile solids). Biomass settling was allowed weekly to withdrawn the supernatant and, subsequently, to refill the vial with new mineral medium (avoiding the eventual accumulation of inhibitory compounds). The mineral medium was initially prepared with low amount of nitrite and ammonium (25 mg NO_2^--N/L + 25 mg NH_4^+-N/L). Once the anammox activity was detected, a second weekly addition of nitrogenous substrates was performed targeting a progressive increase in the concentration of nitrite from 25 to 150 mg NO_2^--N/L (ammonium was added at a ratio of 1.2 g NO_2^--N/g NH_4^+-N). The pH within the vial was controlled in the range from 7.0 to 8.0 (HCl 2 M). N_2 flushing was used to displace air in the vial headspace every time it was opened. Liquid samples were taken before and after each feeding event and filtered using 0.45 μm polypropylene membrane filters. A biological sample was taken once per month (B0-B4), centrifuged at 10,000*g* for 4 min, and the pellet was stored at −20 °C (after discarding the supernatant). This enrichment step lasted 120 days (4 months), and final anammox activity was evaluated in a batch test (in duplicates), as described elsewhere (Connan et al., 2016).

2.3. Continuous stage

The biomass enriched following the aforementioned method was seeded in a continuous upflow column reactor (0.94 g VS). This was a jacketed cylindrical reactor made of glass (Trallero & Schlee, Spain) with inner diameter of 9 cm and column height (to the effluent port) of 66 cm. Similarly to other works (Furukawa et al., 2003; Vanotti et al., 2011), a support made of polyester non-woven material coated with pyridinium-type polymer (Japan Vilene Co., Japan)