FEASIBILITY OF ENRICHING ANAMMOX BACTERIA USING ACTIVATED SLUDGE FROM TWO WASTEWATER TREATMENT PLANTS

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Abstract
Anaerobic ammonium oxidation (anammox) is a promising biotechnology suitable for the removal of nitrogen from wastewaters. A proper choice of the seeding sludge and operational conditions is critical when dealing with the start-up of a new anammox reactor. The objective of this research was to evaluate the feasibility of enriching anammox bacteria using two different sources of activated sludge. Both sludges were obtained from full-scale treatment plants targeting N-removal from high strength wastewater. The monitoring of the reactors was carried out according to N-compositional variables and also by polymerase chain reaction (PCR) detection of the functional gene encoding for the hydrazine-oxidizing enzyme (*hzo*), which is specific of anammox bacteria. The combined analysis of both macroscopic and microscopic data demonstrated that anammox bacteria could be enriched using activated sludge from a treatment plant processing pig slurry in Catalonia.

Keywords: anaerobic ammonium oxidation (anammox); autotrophic nitrogen removal; bioreactor start-up; biomass immobilization; *hzo* gene; wastewater treatment.

Introduction
Discovery in the early 1990's of the anaerobic ammonium oxidation (anammox) as a new pathway to biologically convert ammonium (NH_4^+) to dinitrogen gas (N_2) under absence of oxygen has arisen great expectations in the field of wastewaters treatment. This process, when coupled with partial nitritation, stands for a more sustainable N-removal alternative than other conventional treatments (Kartal *et al.*, 2010). The anammox-based deammonification process is especially suitable for the treatment of wastewaters containing high ammonium and low biodegradable organic matter. It consists of a chemolithoautotrophic bioconversion mediated by *Planctomycetes*-like bacteria that under anoxic conditions oxidize NH_4^+ using nitrite (NO_2^-) as the electron acceptor. According to the anammox reaction proposed by Strous *et al.* (1998) (Eq. 1), NH_4^+ and NO_2^- are converted to N_2 and nitrate (NO_3^-) under reaction molar ratios NH_4^+:NO_2^-:N_2:NO_3^- of 1.00:1.32:1.02:0.26, respectively.

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

Despite the anammox biotechnology is becoming popular, there are still relatively few full-scale reactors running worldwide. The selection of a proper seeding sludge is critical to the start-up of anammox reactors as the doubling time of these microorganisms is about 11 days at 32ºC (Strous *et al.*, 1998). This is equivalent to a slow maximum specific growth rate (μ_m = 0.065 d^{-1}), that will result in a prolonged bioreactor start-up and a significant functional disruption in the event of biomass washout. Hence, immobilization of microbial cells may be a helpful mean to retain the highly valuable anammox biomass, and increase the process robustness. The aim of this research was to characterize the feasibility of enriching anammox bacteria in bioreactors packed with synthetic carriers for enhanced

biomass retention. Two sources of sludge were considered, both collected from full-scale treatment plants aiming at N-removal by nitrification-denitrification from high strength wastewaters (i.e., liquid digestate of the organic fraction of municipal solid waste and liquid fraction of raw pig slurry).

Material and methods

Sources of activated sludge

Samples of sludge were obtained from two treatment plants targeting N-removal from high strength wastewaters in the area of Barcelona (Catalonia, Spain). In both cases, the configuration of the reactors is under continuous operation according to a Modified Ludzack-Ettinger (MLE) design. The activated sludge no. 1 (AS1) was obtained from a centralized plant for the treatment of municipal solid waste (Ecoparc-2, Montcada i Reixac). It consists of anaerobic digesters and the liquid digestate is further treated in an activated sludge unit for N-removal. The solids retention time (SRT) of this later unit was around 15 days. Sampling was performed in the anoxic reactor. The activated sludge no. 2 (AS2) was obtained from an on-farm treatment plant (SAT Caseta d'en Grau, Calldetenes). This facility processed the slurry generated in a sow-herd farm with 400 sows (Flotats *et al.*, 2009). The nominal loading rate is 250 mg N L^{-1} d^{-1} and the corresponding SRT was about 12 d (final pond not considered). The sludge used for performing the enrichment was obtained after mixing samples from the anoxic reactor, aerobic reactor, settler, and pond.

Experimental set-up

Un-drained reactors. The enrichment of both sludge samples was carried out in individual glass reactors (R) without drainage (10-L). These reactors (R1 for AS1 and R2 for AS2) were magnetically stirred into a heated water bath at 33ºC and covered with a black plastic sheet to avoid light. The initial solids concentration was adjusted to 4 g TSS L^{-1} (Kunz *et al.*, 2007) by diluting activated sludge with deionized water. The required N-substrates were added in pulses as chemical solutions -nitrate as KNO_3, nitrite as $NaNO_2$, and ammonium as $(NH_4)_2SO_4$-. A new pulse was added each time the N-concentrations became limiting for microbial growth. In the case of nitrate dosage followed a progressive decrease (maximum concentration after a new pulse from 85 to 0 mg NO_3^--N L^{-1}), whereas for nitrite a concomitant increase (maximum concentration from 15 to 150 mg NO_2^--N L^{-1}). If needed, ammonium was added at rates of 100 mg NH_4^+-N L^{-1}. The liquid supernatant was not renewed throughout the 8 months of experiment. Liquid and sludge samples were extracted periodically for chemical analysis and microbial characterization, respectively. Each time a reactor was opened, its headspace was flushed with N_2 and the dissolved oxygen (DO) concentration in the liquid bulk was verified to be lower than 0.5 mg L^{-1}. A collateral test with high substrate-to-biomass (*S*/*X*) ratio was carried out at the 7^{th} month in 1-L stirred reactor filled with synthetic wastewater (SWW) and sprinkled with mixed liquor from reactor R2 (10% v/v). The SWW was prepared as described by Magrí *et al.* (2012); i.e., 150 mg NO_2^--N L^{-1} + 150 mg NH_4^+-N L^{-1}.

Continuous reactors. The sludge from the un-drained reactor positive in anammox activity (R2) was split and partly used for seeding two continuous reactors made of glass, jacketed, and fed by a low-flow peristaltic pump. Process temperature was of 33ºC. These reactors were sheltered from light placed in a dark chamber. Both reactors were packed with synthetic carriers that have been described elsewhere as good for biomass retention (Furukawa *et al.*, 2003; Rouse *et al.*, 2005). The first reactor (2.4-L) was an up-flow column filled with polyester non-woven material, while the second reactor (1.3-L) was perfectly stirred and packed (23% v/v) with polyvinyl alcohol (PVA) gel beads -4 mm diameter-. Synthetic wastewater used as feeding was SWW.

Molecular techniques

The presence of anammox bacteria in the sludge samples was evaluated by means of conventional polymerase chain reaction (PCR) tools. Total DNA was extracted with the PowerSoil™ DNA Isolation Kit (MoBio Laboratories Inc., USA). The gene encoding specific enzyme hydrazine oxidoreductase (*hzo*) was used as functional biomarker. The primer set used to selectively amplify anammox bacterial fragment was hzoF1 (5′-TGT GCA TGG TCA ATT GAA AG-3′) and hzoR1 (5′-CAA CCT CTT CWG CAG GTG CAT G-3′) (Li *et al.*, 2010). The thermal profile had a first step of 95ºC, 5 min; 35 cycles of 95ºC, 1 min; 50ºC, 1 min; 72ºC, 1 min; and a final extension of 72ºC, 5 min. The amplicons were checked in 1% agarose gels by using positive and negative controls in all PCR reactions.

Chemical analysis

The NO_2^- and NO_3^- were determined by ionic chromatography (861 Advanced Compact IC, column Metrosep A Supp 4-250, CO_2 suppressor, Metrohm, Switzerland). Similarly, the NH_4^+ was determined by ionic chromatography (790 Personal IC, column Metrosep C2, Metrohm). The pH, electrical conductivity (EC), and DO were measured using a pHmeter (meter GLP22, probe 5203, Crison, Spain), a conductimeter (meter MM44, probe 5398, Crison), and an oximeter (meter Inolab 740, probe CellOx 325, WTW, Germany), respectively. Total inorganic carbon (TIC) was measured through sequential 5-point acid titration method to pH 4.3 (Moosbrugger *et al.*, 1993). Total suspended solids (TSS) were analyzed by weight after filtering samples through glass-fiber filters and drying at 105ºC (APHA et al., 2005). The N_2 reaction ratio was calculated through mass balance.

Results and Discussion

Un-drained reactors. High NO_2^- + NO_3^- uptake rates were measured in both reactors, R1 and R2, during the first days of the experiment due to heterotrophic denitrification (Fig. 1). Also, NH_4^+ concentrations increased at this time due to the hydrolysis of remaining organic matter. PCR amplification of *hzo* genes demonstrated the presence of anammox bacteria in AS2 at time 0 but this gene was not detected in AS1. In agreement with these results, subsequent reactor experiments evidenced the simultaneous uptake of NH_4^+ and NO_2^- only in R2. Nevertheless, a very low N-conversion rate (NCR) was still measured at the end of the 8-months (0.42 mg N g^{-1} TSS d^{-1}). In this reactor, the pH of the liquid bulk was, in average, 7.79 (±0.34), EC was 4.60 (±0.60) dS m^{-1}, and TIC ranged from 130 to 383 mg C L^{-1}. The presence of anammox bacteria in R2 was confirmed by PCR throughout the whole experimental phase (Fig. 2), whereas PCR reactions remained negative for R1 up to the end of the experimental phase.

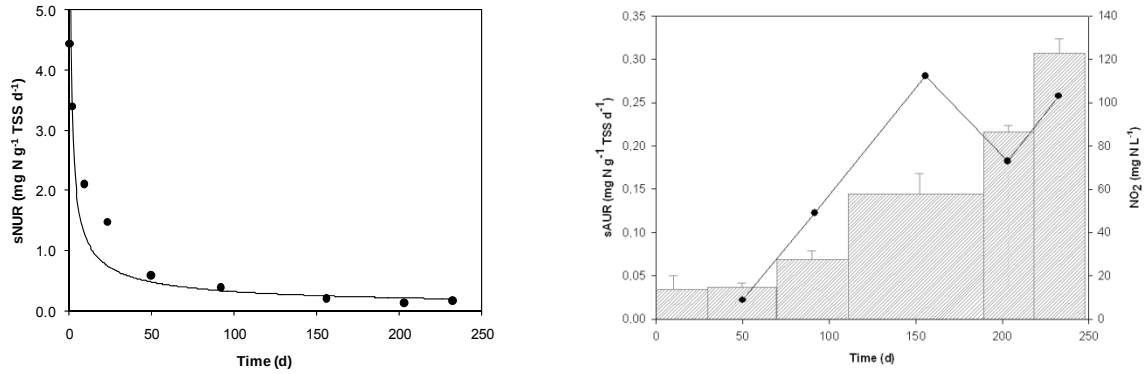

Fig. 1. Evolution of the nitrogen uptakes in R2. *Left:* specific nitrite + nitrate uptake rate (sNUR). *Right:* specific ammonium uptake rate (sAUR).

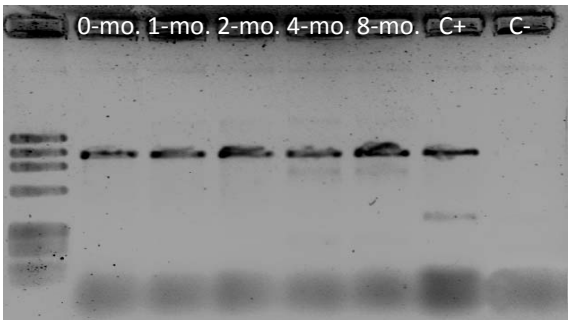

Fig. 2. Detection of the anammox-specific *hzo* gene by PCR in R2 throughout the 8-months period.

In order to assess the effect of renewing the liquid supernatant during the enrichment and its influence on the achieved conversion rate, a mixed liquor aliquot from R2 was used in a parallel anammox activity test. A positive effect was clearly evidenced upon regeneration of the synthetic wastewater (Fig. 3). On the basis of this observation, it was decided to start the operation of continuous reactors.

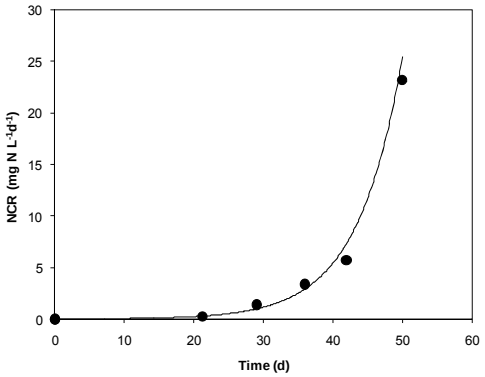

Fig. 3. Exponential increase of the anammox activity under high substrate-to-biomass ratio (0.33 g TSS L^{-1}) after sprinkling mixed liquor from R2 in synthetic wastewater SWW. NCR, nitrogen conversion rate.

Continuous reactors. Concomitant removal of NH_4^+ and NO_2^-, and production of NO_3^-, was observed already from day 0, when both bioreactors were inoculated (Fig. 4). In the case of the up-flow reactor an initial decrease in the expected activity was followed by a progressive recovery, with the NCR rising up exponentially from 4.6 to 17.3 mg N L^{-1} d^{-1} (last rate available) in a 36 day-period. Average reaction molar ratios (NH_4^+:NO_2^-:N_2:NO_3^-) measured during the same period were 1.00:1.31:1.13:0.13, comparable to those of Eq. 1. Similar results were obtained for the reactor packed with PVA gel beads. It is expected that maintaining the same operational conditions for a longer period will result in the further enrichment of anammox bacteria, and that the NCR will progressively increase to higher values, similar to those reported in the literature for high-rate conversion bioreactors (Van Hulle *et al.*, 2010). The implementation of supplementary molecular techniques such as quantitative PCR (qPCR) will be helpful to establish correlations between macroscopically observed increases in anammox activity and corresponding changes in the microbial population structure. Also, phylogenetic analysis on the anammox biomass would allow the study of the microbial community structure and dynamics in relation to operational aspects. Finally, once available the sludge enriched in anammox organisms, it could be tested in specific wastewater treatments.

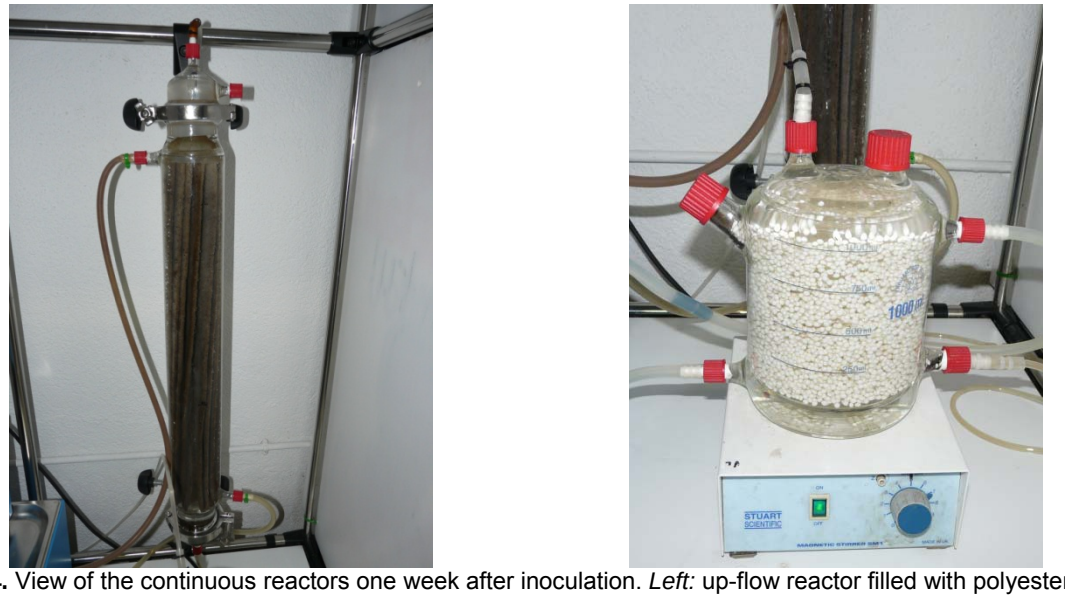

Fig. 4. View of the continuous reactors one week after inoculation. *Left:* up-flow reactor filled with polyester non-woven material. *Right:* perfectly stirred reactor packed with PVA gel beads.

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