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Batch enrichment of anammox bacteria and study of the underlying microbial community dynamics

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HIGHLIGHTS

- Effect of nitrite supply during batch enrichment of anammox sludge was investigated.
- Anammox activity was only developed when starting at low nitrite concentration.
- Final abundance of the *hzo* gene and anammox activity were positively correlated.
- Inoculum and enrichment conditions determined final microbial community structure.
- Enrichment conditions prompted selection of anammox species belonging to Ca. *Brocadia*.

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ABSTRACT

The anaerobic ammonium oxidation (anammox) consists on the biological conversion of ammonium (NH_4^+) into dinitrogen gas under absence of oxygen. Nitrite (NO_2^-) is a substrate of the anammox reaction, but also an inhibitor at high concentrations. This study investigates the effect of nitrite on the microbial community during the batch enrichment of anammox sludge. Six inoculums collected from different environments were enriched after a conditioning pretreatment and under controlled conditions during 4 months. Concerning the mineral medium used, two different nitrite supply strategies were applied; i.e., (i) initially low concentration at 25 mg NO_2^-–N/L and progressive increase to 150 mg NO_2^-–N/L, and (ii) constant high concentration at 150 mg NO_2^-–N/L. All tested inoculums developed anammox activity but only when the enrichment was started at low nitrite concentration. In such case, the specific ammonium conversion rates finally obtained ranged from 21 ± 1 to 118 ± 1 mg NH_4^+–N/g VS/d (VS, volatile solids). Abundance of the functional gene encoding for the enzyme hydrazine oxidoreductase (*hzo*) was assessed using the real-time quantitative polymerase chain reaction (q-PCR) showing positive correlation with the anammox activity finally reported. In addition, high-throughput DNA sequencing helped to elucidate the underlying microbial community dynamics. The raw inoculum source, the conditioning pretreatment, and the cultivation conditions applied were jointly determinants of the final microbial community structure of the enrichments despite a clear convergence at the end of the experimental period. On the other hand, the cultivation conditions alone determined the selection of anammox species belonging to the genus Candidatus *Brocadia*.

1. Introduction

The anaerobic ammonium oxidation (anammox) consists on the biological conversion of ammonium (NH_4^+) into dinitrogen gas (N_2) under absence of oxygen. This is a chemolithoautotrophic microbial process where nitrite (NO_2^-) acts as the electron acceptor. The anammox reaction also involves the production of a minor fraction of nitrate (NO_3^-). According to Strous et al. [1], the corresponding molar ratios for NH_4^+ consumption, NO_2^- consumption, N_2 production, and NO_3^- production are 1.00:1.32:1.02:0.26, respectively. Metagenomic studies also suggest that nitric oxide (NO) and hydrazine (N_2H_4) are intermediates in the anammox reaction [2]. The anammox process was discovered in the early 1990s in a denitrifying fluidized bed reactor [3] and the interest in this bioprocess has ever since been rising in fundamental and

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applied research fields, such as marine ecology and environmental biotechnology [4,5].

So far, six "Candidatus" anammox bacterial genera have been enriched [6,7] from wastewater treatment facilities and freshwater environments (*Brocadia*, *Kuenenia*, *Jettenia*, *Anammoxoglobus* and *Anammoxomicrobium*), as well as from marine environments (*Scalindua*). All these genera make a monophyletic branch within the phylum *Planctomycetes*. In physiological terms, they feature a specific cytoplasmatic membrane-bound organelle known as anammoxosome, which is the locus of the anammox catabolism. They are also characterized by a low growth rate, with doubling times of 2.1–11 days (at ~30 °C) equivalent to a maximum specific growth rate of 0.065–0.334 d^{-1} [1,8]. Because of this slow biomass development, and the specialized metabolism, anammox bacteria may be difficult to culture. Yet, phylotypes related to the anammox genera have increasingly been observed by molecular means in diverse environments, such as activated sludge from wastewater treatment plants (WWTP) [9,10], marine sediments [11,12], freshwater environments [13], and terrestrial ecosystems [14].

Anammox bacteria have not been isolated in pure culture yet, thus pointing to the fact that they may coexist with other microbial species, even in bioreactors fed exclusively with mineral substrates [8,15,16]. The most frequent enrichment strategies have been based on different types of continuously operated bioreactors [17]; e.g., sequencing batch reactor (SBR), rotating biological contactor, up-flow biofilm reactor, or membrane bioreactor [1,18–20]. Alternatively, enrichments have also been developed in batch cultures [9,21–23]. Many studies have shown that successful cultivation of anammox bacteria from conventional sludge takes long time; i.e., generally from 4 months to 1 year [24]. Such time will be influenced by factors like (i) the ecological characteristics of the seeding sludge including initial concentration and relative abundance of anammox bacteria [24], (ii) effective biomass retention inside the reactor [25], and (iii) the environmental conditions applied: temperature, pH, and concentration of NH_4^+, NO_2^-, dissolved oxygen (DO), organic carbon, sulphide and other inhibitors like metals and antibiotics [26–28].

Monitoring the anammox activity usually involves the chemical analysis of relevant nitrogen (N) compounds (i.e., NH_4^+, NO_2^-, and NO_3^-) in the liquid phase. However, this strategy might be unsuccessful during the initial stages of the enrichment process, when the number of anammox cells is too low and their activity can still not be detected macroscopically. The use of culture-independent molecular methods has been proposed as a suitable method in such cases and various protocols for the DNA amplification by polymerase chain reaction (PCR) of target genes have been described in the literature, as summarized by Li et al. [12]. Anammox specific primers have been developed for amplifying ribosomal genes (16S rRNA) or functional genes such as that encoding for the enzyme hydrazine oxidoreductase (*hzo*) that dehydrogenates hydrazine to N_2. These primers have been used to assess the abundance of anammox bacteria genes within environmental samples by real-time quantitative polymerase chain reaction (q-PCR), and to analyze the microbial community diversity by molecular typing and sequencing methods [9,29–32]. Emerging next-generation sequencing (NGS) have also been applied for providing an in-depth characterization of the microbial biodiversity in anammox systems [33–36]. Yet, not so much information is available in the literature concerning the microbial community structure and dynamics during enrichment of the anammox biomass as determined by quantitative and qualitative culture-independent molecular methods.

Autotrophic nitrogen removal (ANR) applications based on anammox are promising for N-removal from municipal side-/mainstreams, industrial and agricultural wastewaters [37–40], particularly after anaerobic digestion once biodegradable organic carbon is depleted. However, anammox enriched sludge is not always available, and biomass enrichment can become the critical point for the start-up of the process. An appropriate selection of the environmental conditions applied is decisive for a successful enrichment. In this regard, NO_2^- is a substrate of the anammox reaction but may also become an inhibitor at high concentrations. Such inhibition has been reported as highly case-specific; i.e., concentrations as low as 5 and 30 mg NO_2^--N/L were found as inhibitory in some studies [41,42] whereas much higher inhibitory boundaries of 210–274 mg NO_2^--N/L were determined in other cases [43–45]. Concerning this variability, Kimura et al. [44] suggested that differences in NO_2^- concentration tolerance may be caused by the cultivation conditions used. The aim of this study is to investigate the presence of anammox populations in different inoculum sources and to assess the feasibility of enrichment in batch under two different strategies concerning NO_2^- supply. Thus, final concentrations of 150 mg NO_2^--N/L were targeted in the mineral medium used as feeding solution but testing two different supply strategies (i.e., initially low vs. high concentration) in order to evaluate the effect of the NO_2^- concentration when starting anammox batch enrichments. Use of molecular techniques will help to detect anammox bacteria and to establish correlations between macroscopically observed process parameters and the underlying microbial community dynamics. Microbial monitoring will be conducted using q-PCR and 16S rRNA gene targeted NGS.

2. Materials and methods

2.1. Inoculum sources

Six different biomass sources collected in conventional N-removal facilities were considered as inoculum (I) for batch enrichment; i.e., (I1) activated sludge collected in a municipal WWTP that combine the use of a Modified Ludzack-Ettinger (MLE) bioreactor unit and a membrane filtration loop (Betton, Brittany, France), (I2) mixture of activated and settled sludge collected in a pig slurry treatment plant with intermittent aeration and gravity settling (Meslin, Brittany, France), (I3) activated sludge collected in a pig slurry treatment plant with MLE configuration (Calldetenes, Catalonia, Spain), (I4) settled sludge -sediments- collected in a receiving lagoon treating municipal wastewater (Amanlis, Brittany, France), (I5) settled sludge -sediments- collected in polishing lagoons treating municipal wastewater (Amanlis, Brittany, France), and (I6) settled sludge collected in an intermittently aerated lagoon treating pig slurry (Almacelles, Catalonia, Spain). The volatile solids (VS) content of the samples was 0.44%, 1.82%, 0.62%, 0.82%, 0.48%, and 1.92% of the wet weight, respectively; whereas, the corresponding VS/TS ratio (TS, total solids) was 0.52, 0.64, 0.54, 0.09, 0.09, and 0.59, respectively. In order to favor biodegradation of available organic carbon before incubation for anammox biomass enrichment, a conditioning pretreatment based on promoting denitrification was carried out at room temperature during the first days after sampling by adding a NO_3^- source such as KNO_3 in pulses of 722 mg/L (100 mg N/L) and controlling the pH within the range 7.0–8.0 (HCl 2 M). Batch enrichment was started once denitrification declined (after 2–4 weeks).

2.2. Mineral medium

The synthetic nutritive solution was prepared using tap water according to a modification of the mineral medium described by Magrí et al. [45]; i.e., NH_4Cl (variable: 95–573 mg/L), $NaNO_2$ (variable: 123–739 mg/L), KNO_3 (361 mg/L), $KHCO_3$ (1000 mg/L), $FeSO_4{\cdot}7H_2O$ (9 mg/L), EDTA (5 mg/L), $MgSO_4{\cdot}7H_2O$ (240 mg/L),