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Faster autotrophic growth of anaerobic ammonium-oxidizing microorganisms in presence of nitrite, using inocula from Colombia

Crecimiento rápido autotrófico de microorganismos anaerobios oxidadores de amonio en presencia de nitrito, usando inóculos de Colombia

Short Title: Growth from Colombian inoculated anammox

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Summary

Anammox is a nitrite dependent process, catalyzed by bacteria of the order *Brocadiales*. Anammox bacteria oxidize ammonia under anoxic conditions, with nitrite as electron acceptor producing dinitrogen gas. Here, we demonstrated the presence of anammox bacteria by enriched them in a SBR reactor, with anaerobic samples taken from the bottom of a pond used in primary wastewater treatment. The enrichment reached nitrogen (N) removal rates of nearly 1.92kg N/m³/day. (The stoichiometry of the reaction matched previous anammox studies). The enriched bacterial communities were analyzed by Fluorescence In situ Hybridization (FISH), and showed a nearly 90% enrichment at the end of the experiment (day 90). As far as we know this is the first time that the anammox bacteria were enriched using Colombian inocula. The enrichment was achieved in relatively short time with high yields and have an excellent potential for application in wastewater treatment opening the opportunity to treat nitrogen-rich effluents by partial nitrification and anammox, thereby decreasing operational costs with respect to aeration (nitrification) and addition of organic electron donor (heterotrophic denitrification). This more sustainable treatment is a good alternative to control nutrient pollution in water bodies in tropical countries.

Key words : nitrogen cycle, advanced treatment, anammox, nitritation, nitrification, denitrification.

Resumen

La oxidación anaerobia del amonio (anammox), es un proceso nitrito dependiente, catalizado por bacterias del filo *planctomicetes*. Estas bacterias oxidan el amonio en ausencia de oxígeno, con nitrito como aceptor de electrones produciendo nitrógeno molecular. En Colombia, demostramos la presencia de estas bacterias mediante el enriquecimiento de cultivos en reactores por lotes, con inóculos nativos, provenientes de muestras anaeróbicas tomadas del fondo de una laguna para el tratamiento primario de aguas residuales. El enriquecimiento logrado alcanzó remociones de nitrógeno (N), en el orden de 1.92kg - N /m³/día (la estequiometría de la reacción estuvo acorde con estudios previos de anammox). La comunidad bacteriana enriquecida, se analizó mediante hibridación in situ con fluorescencia (FISH), y mostró que el enriquecimiento

contenía aprox. 90 % de bacterias anammox al final del experimento (Día 90). Esta es la primera vez que en Colombia se logra el enriquecimiento de estas bacterias con inóculos locales, hasta nuestro conocimiento. El enriquecimiento fue alcanzado en relativamente corto tiempo con altos rendimientos y tiene un excelente potencial de aplicación en el tratamiento de aguas residuales, abriendo oportunidades para el tratamiento de efluentes ricos en nitrógeno mediante nitrificación parcial y anammox, disminuyendo los costos en los procesos de aireación (nitrificación) y en la de adición de donadores orgánicos (denitrificación heterótrofa). El uso de estos tratamientos más sostenibles es una buena alternativa para el control de contaminación por nutrientes en los cuerpos de agua, en países tropicales.

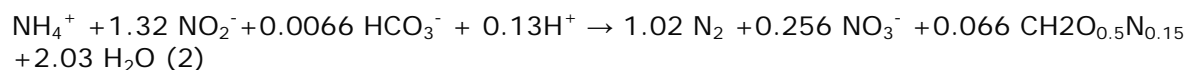
Palabras clave : Ciclo del Nitrógeno, Tratamiento avanzado, anammox, nitrificación, nitratación, denitrificación

Introduction

The anaerobic ammonia oxidation (anammox) is a catabolic process expressed by the following reaction:



The full stoichiometry of the reaction was determined experimentally (Strous *et al.*, 1999a) as shown below:



The key factor for the enrichment of anammox bacteria was the use of mineral media for autotrophic growth with carbon dioxide, ammonia and nitrite, and an efficient system for retention of biomass (Strous *et al.*, 1998). Different types of anammox bacteria have been enriched from wastewater sludge (Kartal *et al.*, 2007b; Strous *et al.*, 1999a). The enriched anammox cultures allowed the development of molecular methods to detect anammox bacteria based on both 16S rRNA and functional genes. The cultures also allowed the determination of ecophysiological parameters (Kartal *et al.*, 2007a; Strous *et al.*, 1999b) as well as the identification of the unique ladderane lipids (Sinninghe-Damsté *et al.*, 2005). Furthermore the important enzymes for anammox catabolism were located in a special prokaryotic organelle (Van Niftrik and Jetten, 2012). Recently the molecular mechanism and key enzymes (Kartal *et al.*, 2011; Shimamura *et al.*, 2007) were identified involving hydrazine synthase and hydrazine dehydrogenase. Together with the more applied studies this allowed the industrial implementation of the anammox process for the treatment of high strength waste water (Jetten *et al.*, 1997). The doubling time for anammox bacteria is estimated at 11 days under optimal conditions, although shorter times of doubling have been recently reported in the literature (Isaka *et al.*, 2006; Tsushima *et al.*, 2007). The growth yield of these bacteria has been estimated at 0.07 mol C fixed / mol NH_4^+ oxidized. This indicated that the slow growth is not caused by inefficient energy conservation but by the slow conversion of the substrate $V_{\text{max}} = 55 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$ (Jetten *et al.*, 1997; Khin and Annchatre, 2004). The temperature maxima for most anammox bacteria are around 35 degree (Kartal *et al.*, 2007a), making them very suitable for application under tropical conditions.

The application of anammox to nitrogen removing systems reduces operating costs by 60% and the required space by 50% compared to traditional technologies, as well as CO_2 emissions by 90% (Abma *et al.*, 2010; Kartal *et al.*, 2010). Other advantages are: no need for aeration, reduced sludge production given its low growth rate and it can remove over 5 to 6 times more nitrogen per cubic meter than the nitrification-denitrification

process by stages (Gao and Tao, 2011; Innerebner *et al.*, 2007; Star *et al.* 2007). The whole partial nitrification by Ammonia-oxidizing Bacteria (AOB) combined with anammox process is of great interest in effluents of anaerobic digestion, which contain high amounts of nitrogen as ammonia.

Despite the extensive knowledge of these microorganisms, few studies have been performed, studying the ability of inocula in the tropics zone, which is particularly important given the potential of the diversity of this region.

In this paper, it was investigated the selection and finding of an appropriate local anammox inoculums as a precursor to the possible application for tertiary treatment of anaerobic effluents. The results showed the potential of using anammox in treating effluent from local anaerobic digesters.

Methodology

Conditions for enrichment and reactor operation

The experiments were performed using a Sequential Batch Reactor (SBR), continuously fed (0.2 - 2 l/day) with one discharge point at the end of each cycle (see [figure 1](#)). The working volume was 5.2 l and minimum working volume was 3.2 l. The SBR was operated in cycles of 12 and 24 hours depending on the reload volume. To dispense the media a peristaltic pump was used with a capacity of 200 to 3000 ml/day, the speed of the pump (flow, ml/day) was adjusted depending on the consumption of nitrite in the reactor and the concentration of nitrite in the media. The reactor was home built, with clear acrylic material and silicone joints. Anoxic conditions were achieved by continuous addition of 20 ml/min of a gas mixture, composed by 95% Argon and 5% CO₂. The CO₂ was also the carbon source for autotrophic growth, while contributing simultaneously to pH control.

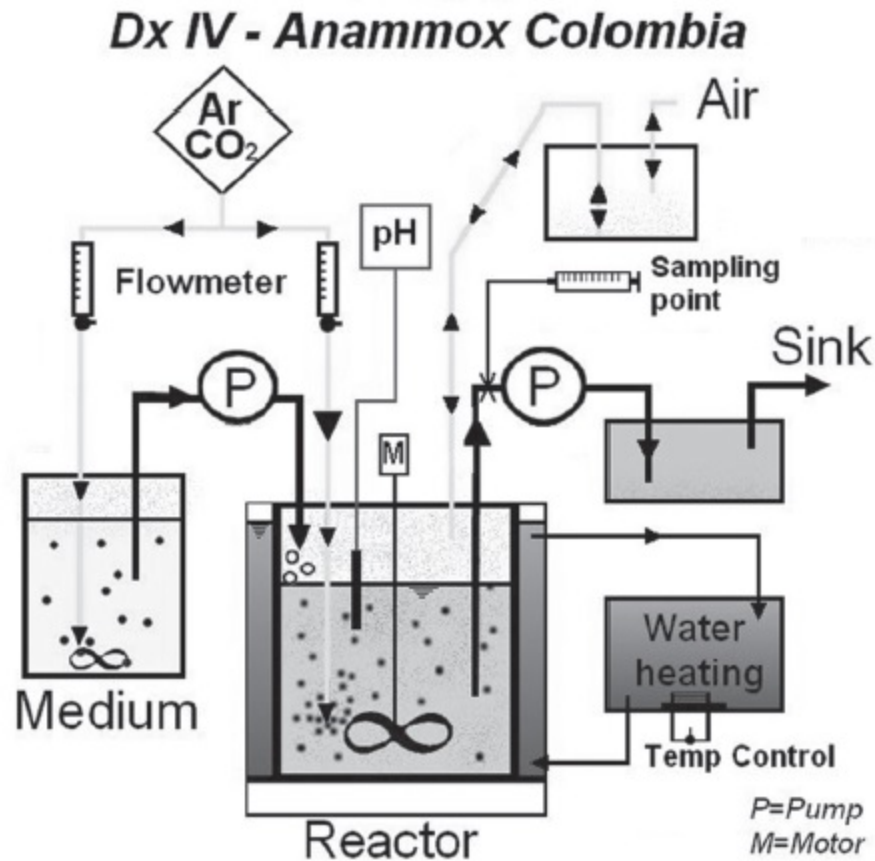


Figure 1. Configuration of SBR reactor, continuous charge and punctual discharge, full mixing, heating system, gas feed, sampling point, continuous pH meter.

The discharge took place after 10 minutes settling, allowing the efficient retention of biomass. The discharge volume corresponded to the amount of added medium, maintaining a minimum volume of 3.2 l. All the rate calculations were made based on the minimum volume. During each cycle a sample was taken for subsequent measurement of nitrite, nitrate and ammonia, by colorimetric methods while checking in situ concentration of nitrite with Merck® strips (Merckoquant®, Nitrite test, Merck-Germany). In case that no nitrite accumulated inside the reactor, the speed of the pump would be increased with a consequent augment in the medium supply, up to the maximum capacity. Once the maximum pump speed was reached, was redesigned the basal media while increasing ammonia and nitrite concentrations, and resetting medium supply to the minimum levels of the pump. Temperature was kept constant at 32o C, using a water jacket. The pH was continuously monitored with a glass membrane electrode with digital reader for pH and temperature and the pH was kept within the range of 7.2 to 7.9 by adjusting the bicarbonate content in the media and the flow of the gas mixture.

Medium composition

The media consisted of macro and micro nutrients, nitrite in the form of NaNO_2 and ammonia in the form of $\text{SO}_4(\text{NH}_4)_2$ (2-60 mM), the concentration of nitrite and ammonia in the media was increased according to the microbial activity and the capacity of the

feed pump. The media contained the following components per liter of distilled water: KHCO_3 (0.5-1) g; KH_2PO_4 10 mg; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.24 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.16 g; 1 ml of trace elements. The trace elements were added as described in Van de Graaf *et al.* (1996). The medium was kept anaerobic by adding Ar/CO₂ 95/5 % 10 ml/min continuously to the container.

Inocula

Sample used as inoculum was taken from a sedimentation pond of a sugar mill, located in "Pradera" municipality, Valle del Cauca (Colombia). The water that feeds this lake originates from sugar cane washing liquid waste and industrial processing of sugar, after settling solids, water is recycled back to washing cane. The inoculum was collected from the bottom of the pond and they contained approximately 1 g/l of TVS (Total Volatile Solids). 1.2 liters of this inoculum was added, and diluted with 2 liters of medium.

Nitrite, Nitrate and Ammonia

Nitrite was daily monitored by using colorimetric stripes (Merckoquant®, Nitrite test, Germany), with a range from 0 to 80 mg/l, in order to adjust the volume of substrate to provide, and to avoid accumulations higher to 20 mg/l. Ammonia and nitrite were weekly measured by colorimetry as described in Kartal *et al.* (2006). Nitrite was measured using spectrophotometers (UV-1800 Shimadzu®) as described in the standard methods 4500-NO₃-B (Greenberd *et al.*, 1994). Daily samples were kept in storage at -4°C, defrosted and centrifuged for 30 min at 13.000 rpm for the corresponding measures.

Molecular Studies

Samples of biomass (2 ml) were taken for DNA extraction and purification, according to the methodology described in Greenberd *et al.* (1994). Extracted DNA is used as template for polymerase chain reaction (PCR) as described previously in Schmid *et al.* (2005) with specific primers for the detection of anammox bacteria Pla46F (Neef *et al.*, 1998) and AMX368R (Schmid *et al.*, 2005) as well as for identification of general bacteria. Additionally, 2 ml of biomass are fixed in paraformaldehyde and hybridized as described in Schmidt *et al.* (2002), the probes used for hybridization were labeled with Cy3 and FAM (Fluorescein) purchased at Microsynth® (Switzerland). DNA was stained with 4',6-diamidino-2-phenylindole (DAPI). The probes for identification of anammox bacteria were: SP-Planc-0046-aA-18 (Pla46) for detecting planctomycetes, S^{-*}-Amx-0368-aA-18 (Amx368) for detecting most anammox and S^{-*}-Amx-0820-aA-22 (Amx820), and specific Kuenenia and Brocadia (Schmid *et al.*, 2000). EUBmix (mixed equimolar solution of EUB338, EUB338II and EUB338III) was used for the detection of most Bacteria, Ntspa and NIT3 (Egli *et al.*, 2003) for identifying nitrite oxidizing bacteria in equimolar solution we call NITRI mix. The samples were examined using a fluorescence microscope (Nikon®, Eclipse 90i), and photographs were taken with a digital camera (Nikon Digital Sight DS 2MBWc) and processed with specialized software (NIS-Elements). Extracted DNA is used as template for polymerase chain reaction (PCR). Pla46F and AMX368R specific primers for the detection of anammox bacteria were used.

Results and discussion

The anammox bacteria enrichment is dependent on anoxic conditions and effective biomass retention. Therefore we took great care to use strictly anoxic mineral media, continuously bubbling the bioreactor with Argon/CO₂ mixture, and applied a strict feed regime in the SBR system (see [figure 2](#)). Furthermore the pH value was kept between 7 and 7.8 and the temperature was 32 °C. The start concentrations of ammonia and nitrite were 2 mM to prevent toxicity at the start of the enrichment. The influent had a constant flow rate of 600 ml/day and cycles of 24 hours until nitrite was no longer detected in the effluent.

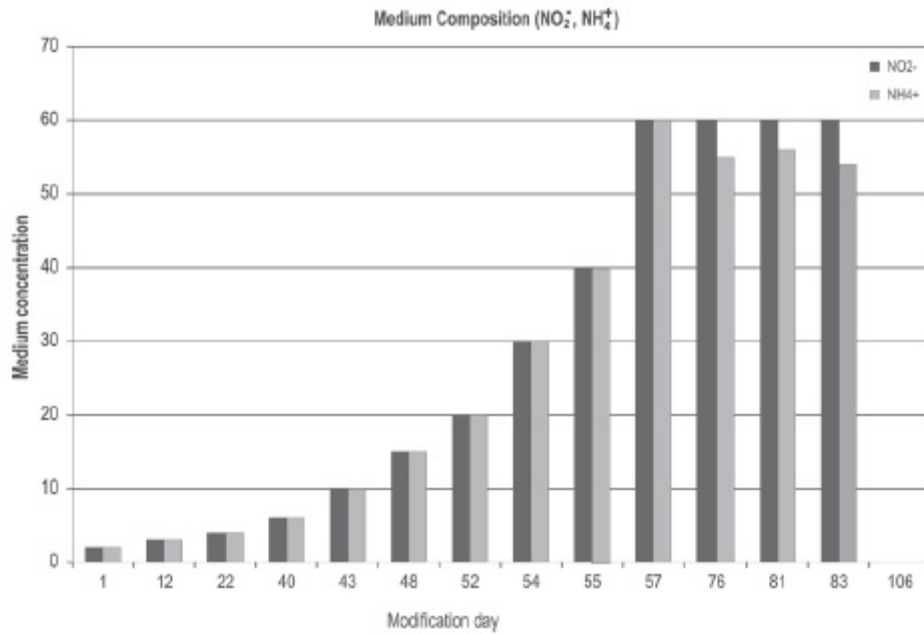


Figure 2. Ammonia and nitrite concentration changes during experimentation. Concentration gains on each component were calculated according to the growth in consumption or transformation thereof.

The conversion rates of ammonia and nitrite are shown in [figures 3](#) and [4](#), respectively, and indicated an exponential removal from day 50 onwards.

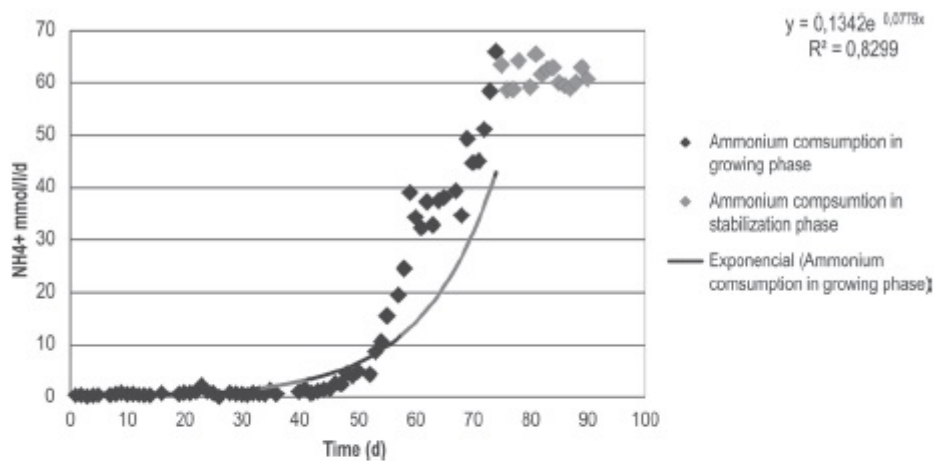


Figure 3. Transformation of ammonium throughout the experiment. Dark diamonds: exponential phase. Lighter diamonds: stabilization phase. Line: Exponential curve $Y = 0.1342 e^{0.0779x}$. Each point represents the ammonia transformed every corresponding day, in moles per liter of reactor. Due to the graphic scale and broad ranges of consumption throughout the experiment, the consumption during the first 40 days ranging from 0.3 to 3mmol/l*day is not accurately depicted. After day 70 the supplied ammonia was reduced and limited (avoiding accumulations in the reactor), while consumption remained relatively constant.

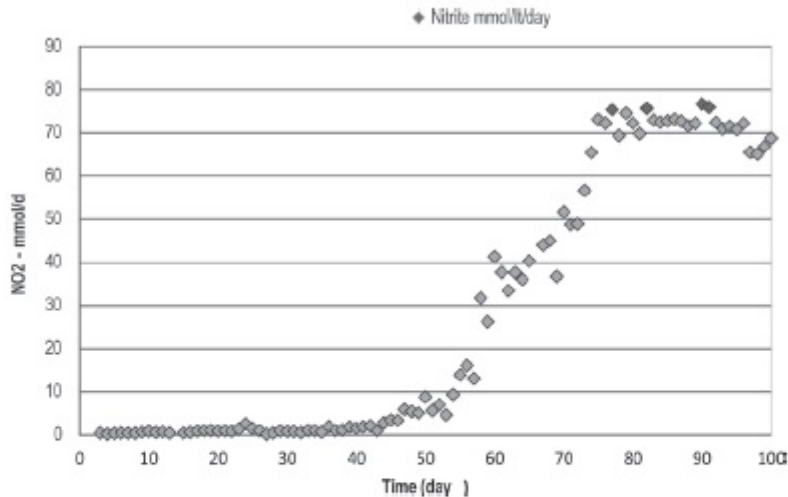


Figure 4. Nitrite conversion during the experiment. Through 0 to 40 day period consumption fluctuates from 0.6 to 4 mM (not displayed in the graph), after 40 days the growth of anammox is exponential and by day 80 consumption stabilizes.

The experiment was divided into 4 stages for its analysis: 0-15, 15-35, 35-60 and 60-90 days, according to: changes in the transformation of nitrite and ammonia, the ratio of removed nitrite/ammonia each day, the exponential variation of conversion rates and concentrations measured in the effluent.

During the first phase (days 0-15), the inoculum immediately started the consumption of substrates (nitrite and ammonia), so by day 15 of experimentation, up to 900 ml/day of media could be added. Concentrations of 3 mM of ammonia and nitrite respectively were in the medium at this point. To further increase the nitrogen load, we increased the concentration in the media whenever possible. Ammonia and nitrite accumulations in reactor were always 0.01 to 0.02 mM. The factors that could be attributed to this consumption are: the possibility of O_2 incoming for nitrification during discharge time, and the presence of organic substrate from the inoculum for heterotrophic denitrification. Anammox bacteria were not detectable with FISH, meaning they could be there but in very low concentration ($\sim 10,000$ cells/ml) (Amann *et al.*, 1995).

In the second period (day 15 to 35), we observed the nitrite/ammonia consumption ratio increased from 1 to 1.20, (see [figure 4](#)), implying a higher accumulation of ammonia inside the reactor, also absence of partial nitrification, associated with the presence of small amounts of oxygen. The O_2 could be introduced during the discharge phase, therefore low concentration of nitrate (0.05 mM) in the effluent indicated that part of the nitrite was oxidized to nitrate, to supply the anammox bacteria with sufficient electrons for CO_2 fixation (Strous *et al.*, 2006).

For the third period of the experiment (days 35 and 60), we can see a fluctuation in the nitrite/ammonia ratio from 1.20 to 1.50 returning to 1.15. The presence of a small amount of nitrifying bacteria was confirmed by FISH ($\pm 3\%$), which shows that some oxygen may still enter to the reactor during the discharge period. An increase in the consumption of nitrite in relation to ammonia was also detected ([figure 4](#)): conversion at a representative midpoint of the period (e.g. 54 days), were 33.9 mmol/day of ammonia and 44.4 mmol/day of nitrite. Thus the nitrite/ammonia ratio was 1.31 close to the

expected value (Strous *et al.*, 1998). During this period, Planctomycetes (pla46 positive cells in FISH) were becoming, dominant in the community.

Finally, during the period between days 60 and 90, we observed an increasing production of nitrate (data not shown) in good accordance with the expected anammox stoichiometry of 0.26 mmol of nitrate per 1 mmol of ammonia (equation (2)) produced anaerobically.

During this period the anammox rate increased exponentially, indicating that the bacteria population doubled every 9 days (calculated from the exponential ammonium consumption growing). The nitrite/ammonia ratio stabilized in this period (60-90) at approximately 1.16.

In summary, the nitrite/ammonia ratio of anammox process is expected to be around 1.32 (equation (2)), meanings, for every mmol of ammonia 1.32 mmol of nitrite are required. Nitrite/ammonia ratios in our enrichment were always somewhat below 1.32 which could indicate that part of the ammonia was oxidized to nitrite and nitrate under limited oxygen by AOB and nitrite-oxidizing bacteria (NOB). Indeed about 3% of the cells in the community reacted with AOB and NOB specific probes.

Results of ammonia removal rates (figure 3), show that after 50 days a substantial amount of ammonia and nitrite (figure 4) is removed by anammox bacteria. At 56 days we observed by FISH a 40 % of anammox presence (figure 6 and 7). At 90 days of enrichment, the community contained about 90% anammox bacteria, which is in good agreement with the ammonia and nitrite removal activity (245 mmol/day NO_2^- and 201 mmol/d NH_4^+). Nitrite is assumed to be the limiting substrate in this experiment as ammonium amounts were provided in excess.

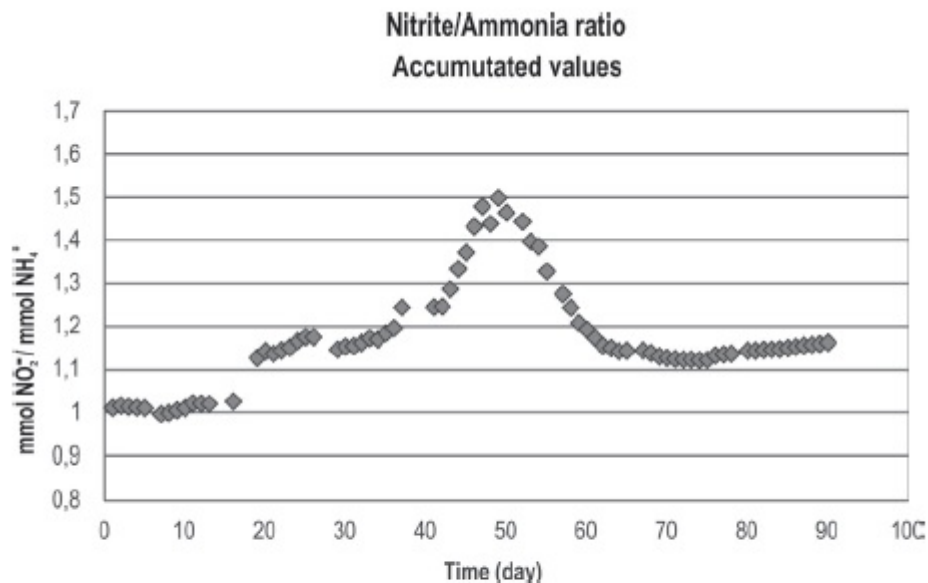


Figure 5. Nitrite transformation relation with respect to ammonium. Total values from day "0" representing changes in biomass conformation.

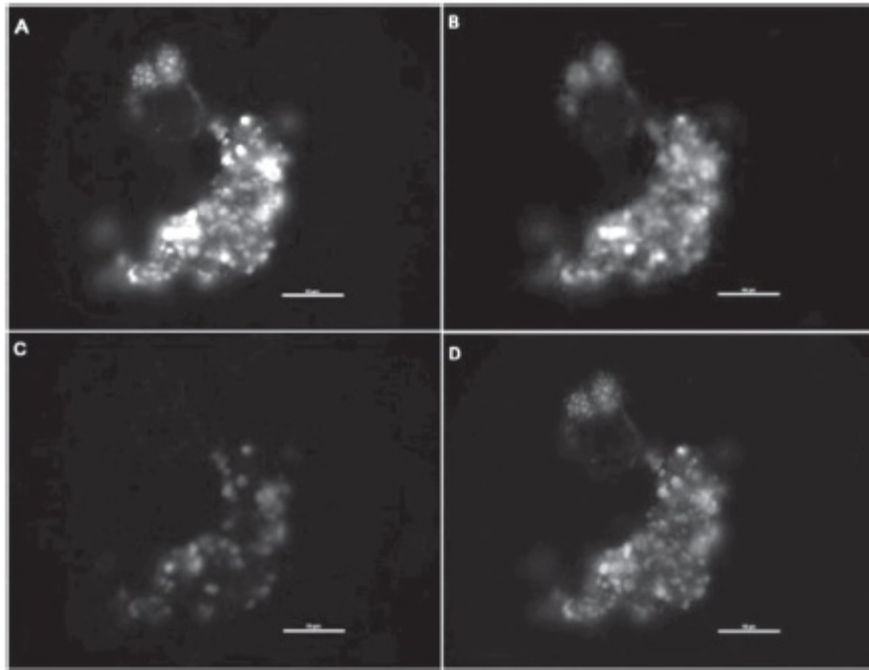


Figure 6. Detection of Anammox and bacteria in general, using FISH: A) Superposition of all three hybridizations. In lighter color, anammox flocks interacting with the rest of the mixed population. The sample was taken and fixed on day 56, by this time the community was able to transform 101 mM of nitrite and 63 mM of ammonia per day. We estimate that 40% of microbial community corresponded to anammox observed in all wells. Scale bars 10 microns. B) DAPI, C) AMX 368 specific for all known Anammox bacteria (CY3). D) EUB-mix equimolar specific solution for most bacteria (FAN).



Figure 7. Day 52, A: CY3 hybridized with an equimolar solution, Nitri-mix represents nitrating groups, which require oxygen for the conversion of nitrite to nitrate. B: CY3 + DAPI, overlapping of the two emissions. Scale bars 10 micras.

According to the anammox probes used for hybridization, a high proportion of anammox bacteria belong to the *Brocadia* and *Kuenenia* groups.

The PCR amplified fragments, for the detection of anammox, were tested by agarose gel (2%) electrophoresis and the bands size were between 300 and 400 bps as expected (322 bp, [fig. 8](#)) according with the 16s rRNA genes of anammox bacteria.

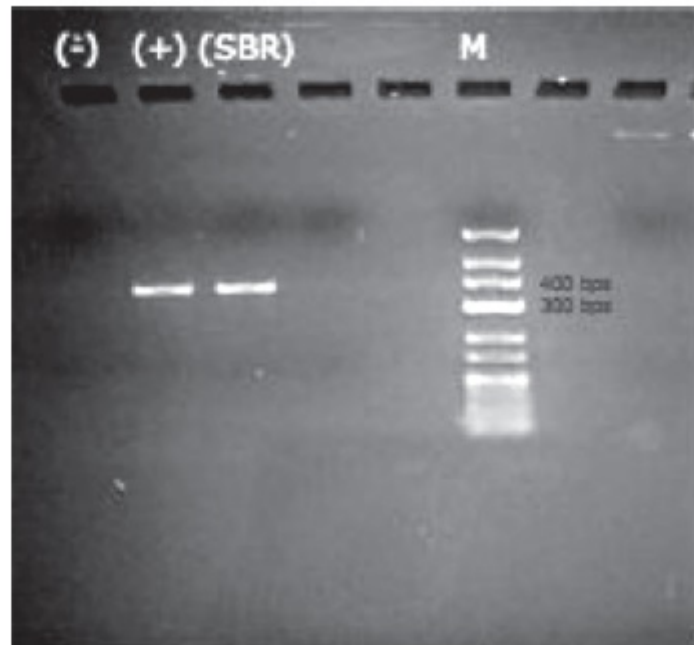


Figure 8. Detection of Anammox using PCR from SBR reactor at day 80 of operation with specific primers for the detection of anammox bacteria Pla46F and AMX368R

Conclusions

The application of anammox processes, to achieve ammonia removal, requires a preceding partial nitrification step, in which approximately 55% of ammonia will oxidize to nitrite. Once this conversion is obtained, the anammox bacteria will convert the nitrite and ammonium into nitrogen gas. Complete autotrophic removal would greatly reduce aeration costs, the need to add electron donors and recirculation during conventional nitrification and denitrification. The present experiments show the enrichment of ammonia-oxidizing anaerobic bacteria in a remarkably short period of time, with a doubling time of about 9 days during exponential growth. The presence of a local source of anammox bacteria opens the possibility of application of this novel process in Colombia and the surrounding tropics. During the experiment, we observed the presence of nitrifying bacteria inside the reactor at about 3% abundance, which would facilitate the processes of partial nitrification under oxygen limitation. The oxygen was most probably introduced during the discharge period, but was apparently rapidly consumed and resulted in some additional nitrite and nitrate production. This implies the possibility of developing co culture of anammox and AOB inside the same reactor under limited oxygen conditions similar to one step anammox or CANON systems (Sliekers *et al.*, 2003; Yan *et al.*, 2010).

The results presented above, are consistent with the presence of anammox in both terrestrial and aquatic systems. The successful enrichment of anammox from local

Colombian sources has potential for the development of local treatment systems for nitrogen. The conversion rates reached over 2.1 kg N/m³ * day, high enough for an application in the tertiary treatment of industrial effluents, anaerobic digesters or secondary recirculation lines. Average temperatures in Valle del Cauca (Colombia) match the average temperature of the experiment, suggesting there is no need for heating nitrogen removing systems. Further research on the anammox process in Colombia is necessary and requires pilot scale experiments for final implementation in local treatment plants, thereby preventing the discharge of nitrogen compounds onto our natural water bodies.

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