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ANAMMOX ACTIVITY OF SLUDGE COMING FROM WETLAND MONOCOTS (*Typha* sp.): KINETIC STUDY

ACTIVIDAD ANAMMOX EN LOS LODOS PROCEDENTES DE MONOCOTILEDÓNEAS DE HUMEDALES (*Typha* sp.): ESTUDIO CINÉTICO

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Abstract

The anaerobic ammonium oxidation (anammox) activity was evaluated using sludge obtained from the rhizome of *Typha* sp., found in a natural wetland localized in the Jóse Antonio Álzate dam, Toluca Valley, Mexico. Five NO_2^--N concentrations (15, 30, 50, 70 and 100 mg/L) were evaluated and the ammonium remained constant at 100 ± 10 mg N/L. All cultures presented a lag phase in the ammonium consumption. Nevertheless, after the phase lag the ammonium oxidation was linked to nitrite reduction, producing N_2. The nitrite consumption efficiencies for the concentrations evaluated from 15 to 50 mg/L of NO_2^--N were above 95%, while the ammonium consumption efficiency increased from 14 to 48%. In experiments with 70 and 100 mg/L of NO_2^--N, nitrite consumption efficiencies were 74 and 58% respectively. The higher nitrite concentrations tested inhibited the anammox process since the specific rates diminished at 0.51 and 0.44 mg NO_2^- -N/g VSS d respectively. This study provides new and useful information about the anammox activity in sludges coming from wetlands, and these can be used as potential inoculum to treat wastewaters into anammox reactors, or in constructed wetlands.

Keywords: anammox, wetlands, specific activity, nitrite, *Typha*.

Resumen

Se evaluó la actividad de oxidación anaerobia de amonio (anammox) usando un lodo obtenido del rizoma de *Typha* sp., de un humedal natural localizado en la presa José Antonio Álzate, en el valle de Toluca, México. Se evaluaron cinco concentraciones de N-NO_2^- (15, 30, 50, 70 y 100 mg / L) manteniendo constante la concentración de N-NH_4^+ en 100 ± 10 mg/L. Todos los cultivos presentaron una fase lag en el consumo de amonio, concluida esta fase, la oxidación del amonio estuvo acoplada a la reducción del nitrito, con producción de N_2. Las eficiencias de consumo de nitrito para las concentraciones de 15 a 50 mg/L de N-NO_2^- fueron mayores a 95%, mientras que las eficiencias de amonio aumentaron de 14 a 48%. En los experimentos con 70 y 100 mg/L de N-NO_2^-, el consumo de nitrito fue de 74 y 58%, respectivamente observando un fenómeno de inhibición, reflejado en la disminución de las velocidades específicas (0.51 a 0.44 mg N-NO_2^-/g SSV d). El estudio proporciona información nueva y útil sobre la actividad anammox en lodos procedentes de humedales, los cuales pueden ser utilizados como inóculo potencial para el tratamiento de aguas residuales en reactores anammox ó en humedales construidos.

Palabras clave: anammox, humedales, actividad específica, nitrito, *Typha*.

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1 Introduction

The Anaerobic Ammonia Oxidation (anammox) process was discovered in a denitrifying pilot plant (Mulder *et al*., 1995). The anammox reaction involves the oxidation of ammonium coupled to the reduction of nitrite to produce the main product, molecular nitrogen, and a minor product, nitrate (Eq. 1) (Strous *et al*., 1998) under anoxic conditions. The biological reaction is catalyzed by chemolithoautotrophic bacteria which have been identified as a distinct phylogenetic order, the Brocadiales, which is part of the phylum Planctomycetes (Kartal *et al*., 2008; Jetten *et al*., 2009). Since its discovery, it has gained attention due to its importance in the global nitrogen cycle that has been estimated accounts for over 50% of nitrogen loss in marine ecosystems (den Camp *et al*., 2006). Anammox has mainly been investigated in wastewater treatment plants, enrichment cultures, in sediments and water columns (Dalsgaard *et al*., 2003; Zhu *et al*., 2013). However, there is few information of anammox in natural and constructed freshwater wetlands (Zhu *et al*., 2010).

$$NH_4^+ + 1.3NO_2^- + 0.0425CO_2 \rightarrow 1.042N_2 + 0.22NO_3^- \\ 0.425CH_2O_{0.5}N_{0.15} + 1.87H_2O + 0.09OH^- \quad (1)$$

The José Antonio Álzate Dam (JAAD) in the Toluca Valley, Mexico, receives wastewaters of origin domestic and industrial, through the Lerma River (Barceló-Quintal *et al*., 2012a, 2012b). The uncontrolled wastewaters discharges from municipal and industrial activities, as well as from agricultural runoffs, untreated or partially treated; induce contamination of water sources, risking more and more its complete use becoming a potential source of diseases. The main pollutants contained in the dam are organic matter, metals, phosphorous and reactive nitrogen (3 at 60 mg/L) (López-Galván *et al*., 2010; Barceló-Quintal *et al*., 2012a). Nitrogen removal is necessary because of the environmental impact, such as eutrophication, acidification and toxicity to aquatic life (Téllez-Pérez *et al*., 2013). The dam have wetlands with monocots (*Typha, Juncus and Eichhornia*), the wetlands are buffer zones between land and waters that maintain good water quality for reducing nutrients and pollutants (Fisher and Acreman, 2004; Ramírez-Carrillo *et al*., 2009; Trepel 2010; Wang and Gu, 2013). It has been shown in several studies the capacity of wetlands to remove organic matter, nitrogen, phosphorus, suspended solids, pathogens and heavy metals (Fisher and Acreman, 2004; Humbert *et al*., 2012; Waki *et al*., 2015). This capability is attributed to biological diversity they possess, for instance, it has been reported that the mechanism of nitrogen removal was conducted by: 1) ammonium assimilation by plants and 2) microbial activity through nitrification followed by denitrification (Verhoeven and Meuleman, 1999; Vymazal, 2007; Knox *et al*., 2008; Lee *et al*., 2009). However, the participation of anammox microorganisms, can also be assumed to contribute to nitrogen removal, but there is relatively little literature about the anammox activity in natural and constructed wetlands (Zhu *et al*., 2010).

Most of the studies that have been conducted were focused on evaluating the diversity of anammox bacteria, abundance and population dynamics (Zhu *et al*., 2011; Humbert *et al*., 2012), distribution patterns (Waki *et al*., 2015) but knowledge about the specific activity of anammox bacteria is scarce and particularly in soils wetland. Therefore, the goal of this study was focused on the respiratory activity of anammox process using anoxic sludge as inoculum potential in the treatment of dam's water. So in this paper the anammox activity was evaluated using sludge taken from the rhizome of *Typha* sp. of this natural wetland in the dam Jose Antonio Alzate from the Lerma River.

2 Materials and methods

2.1 Collection and preservation of microbial consortium

Ten plants of *Typha* sp. were collected of five sample points of the natural wetland present in the dam. It was collected with a portion of soil at the roots (Leander, 1972). These were carried at room temperature in plastic bags. The sludge obtained was spiked in an Erlenmeyer flask with 1000 mL of sterile isotonic solution (sodium chloride 0.90%). The mixing was homogenized at 20 min., and it allowed settling the sludge in an Imhoff cone. This operation was performed three times in order to determine the amount of total suspended solids (SSV). The biomass concentration quantified was 4.7 ± 0.2 g VSS/L (APHA, 2005).

2.2 Batch cultures

Batch cultures were conducted in 125 mL serological bottles containing 60 mL of basal medium formulated with the following compounds (mg/L): $NaHCO_3$

(2500); $NaH_2PO_4{\cdot}H_2O$ (57.5); $CaCl_2{\cdot}2H_2O$ (100); $MgSO_4{\cdot}7H_2O$ (200); and 1.5 mL/L of trace element solution. Trace element solution contained (in mg/L) $FeSO_4$ (5000) and ethylenediaminetetraacetic acid (EDTA) (5000); $ZnSO_4{\cdot}7H_2O$ (430); $CoCl_2{\cdot}6H_2O$ (240); $MnCl_2$ (629); $CuSO_4{\cdot}5H_2O$ (250); $Na_2MoO_4{\cdot}2H_2O$ (220); $NiCl_2{\cdot}6H_20$ (190); $MgCl_2$ (500). The initial sludge concentration was 2 ± 0.01 g of VSS/L the liquid and headspace were flushed during 3 min, with He/CO_2 mixture (80/20, v/v) to exclude oxygen from the assays. All bottles were sealed with butyl rubber stoppers and aluminum crimp seals. Separate bottles were set up for gas phase measurements of N_2. Abiotic controls lacking inoculum were run in parallel to monitor the possible abiotic elimination of the substrates. Biotic controls lacking nitrite or ammonium were also included to correct for compound losses not associated with anammox. All assays either abiotic or biotic were carried out by duplicate in an orbital shaker 150 rpm and 35°C. The initial pH for all batch cultures was of 7.5 ± 0.2. Five concentrations of $N\text{-}NO_2^-$ (15, 30, 50, 70 and 100 mg/L) were evaluated and the concentration of electron donor remained constant at 100 ± 10 mg $N\text{-}NH_4^+$/L. Microbial activity was evaluated in terms of consumption efficiency (Eq. 2), production yield (Eq. 3) and a specific consumption rate (Eq. 4). The kinetic parameters such as the specific rates were calculated using the Gompertz model and non-linear regression program (OriginPro 8.0). Each bottle was an independent experimental unit, which was sacrificed after sampling. Samples were filtered (0.45 μm) and analyzed for ammonium, nitrite, and nitrate determination.

$$\%E_N = \frac{mg\ N\ initial/L - mg\ N\ final/L}{mg\ N\ initial/L} \times 100 \quad (2)$$

$$Y_{P/S} = \frac{mg\ of\ product/L}{mg\ substrate\ consumed/L} \quad (3)$$

$$q_{N-NO_2^-} = \frac{mg\ NO_2^- - N\ consumed}{g\ VSS\ h} \quad (4)$$

2.3 Analytical methods

Ammonium was analyzed by a selective electrode (Phoenix electrode company, USA). Nitrite and nitrate were measured by ultraviolet spectrophotometry (Thermo Scientific, GENESYS 10S) with xenon lamp provides high intensity at 300 and 350 respectively and quartz cells 1 cm optical path (Modified of APHA 2005). Standard curves of nitrate and nitrite were prepared with the mineral medium used in batch cultures to reduce interferences. Before sampling, all liquid samples were filtered through a 0.45-μm nylon membrane. Biogas production was measured using columns with NaCl solution (300 g/L, pH = 2) and the composition was determined only at the end of the kinetic by gas chromatography with thermal conductivity detector (Gow Mac model 550). Temperatures for the column, injector, and detector were 50, 100, and 110 °C, respectively. Helium was used as carrier gas at a constant flow rate of 16 mL/min. The stainless steel column (Porapak Q mesh 100-80) was of 1.20-m long and 1/8" diameter.

3 Results and discussion

3.1 Control assays

Batch assays were performed for a period of approximately 55 h. Abiotic cultures spiked with NO_2^--N and NH_4^+-N did not show any chemical reaction, because at the end of the batch experiments the initial concentrations remained without change. Regarding to inoculated treatments, NO_2^--N consumption without electron donator (NH_4^+-N) and vice versa were evaluated in order to observe contribution of a possible endogenous metabolism. At the end of the batch assays ammonium was almost completely recovered (~98 %), while for the nitrite assays it showed a consumption of 8%, this can be attributed to the endogenous metabolism or the presence of residual organics in the culture favoring the denitrification process (Peng and Zhu, 2006; Torà *et al*., 2011). Finally, these experimental data showed that consumption of both substrates were mainly associated to the anammox activity of the sludge.

3.2 Anammox activity assays

Figure 1 shows the time-course of nitrogen compounds consumption. In batch cultures spiked with 15 mg/L NO_2^--N, it presented a lag phase of 2 h; while batch cultures with 70 and 100 mg/L NO_2^--N displayed lag phases around 5 h. Nonetheless, nitrite consumption did not present lag phase. Nitrite was consumed from the beginning, due to the endogenous metabolism as was described above. In overall, in all batch cultures the ammonium oxidation was linked to nitrite reduction, being the main product, N_2. For instance, the nitrite consumption efficiencies for the concentrations evaluated from 15 to 50 mg/L of NO_2^--N were above 95% (Table 1).

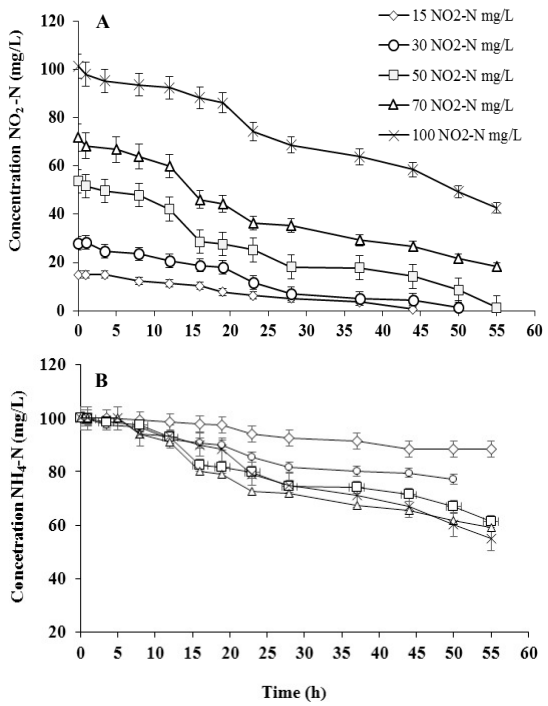

Fig. 1. Profiles of consume of an anammox enrichment culture to different concentrations of nitrite evaluated (A) NO_2^- -N; (B) NH_4^+-N.

The ammonium consumption efficiency increased from 14 to 48%, this means, that initial nitrite concentration improved the ammonium consumption. The values obtained for the N_2 production yield indicated that total nitrogen consumed was recovered mainly as molecular nitrogen. The low efficiencies for ammonium consumption might suggest that initial nitrite concentration was not enough to convert all ammonium up to N_2.

In batch assays evaluated with 70 and 100 mg/L of NO_2^--N, nitrite consumption efficiencies were 74 and 58% respectively. The ammonium consumption efficiencies were 46 and 55%, respectively. At these initial nitrite concentrations the substrate affected was the nitrite profile, due to ammonium consumption continued increasing. These low efficiencies might be associated to inhibitory troubles. For example, there are works indicating that anammox process can be inhibited by their substrates (nitrite and ammonium) and also for the nitrate produced. Nitrite is the most important inhibitor, because it has reported an IC_{50} for the nitrite specific activity ranging from 98 to 350 NO_2^--N mg/L. Rowe *et al*. (1979) and Rake and Eagon (1980) suggested that nitrite is an uncoupler of the respiratory chain.In the case of ammonium, the IC50 reported ranges from 770-980 NH_4^+-N mg/L and for nitrate of 630-980 NO_3^--N mg/L (Strous *et al*., 1999; Dapena-Mora *et al*., 2007). The level of inhibition is strongly related to several factors such as cell concentration in the cultures, history of inoculum, among others.

The diminishing in the specific consumption rates for nitrite indicated inhibitory effects, as can be seen in the Figure 2. The last initial nitrite concentration evaluated was in the range of inhibition reported by Dapena-Mora *et al*., 2007. At concentration of 100 mg/L of NO_2^--N, nitrite affected stronger the respiratory process of anammox, since nitrite consumption diminished, and also the N_2 production diminished up to 0.81; this value suggested that 19% of total nitrogen consumed followed another metabolic fate. The nitrogen missing might be in form of anammox intermediates like hydroxylamine or hydrazine (Jetten *et al*., 1999).

According to Sun *et al*. (2011), the activity of anammox process can be corroborated from the stoichiometry of the process. Strous *et al*., (1998) stablished the stoichiometry of the anammox reaction, as can be seen in Eq. (1). The stoichiometric molar ratios in the anammox assays of this study were consistent with the stoichiometry of anammox reaction regardless of initial concentration of nitrite.

Table 1. Efficiencies consumption, performance N_2 and specific consumption rates nitrite at different concentrations of NO_2^- -N evaluated.

NO_2^- -N (mg /L)	$\%E_N$ NO_2^- -N	$\%E_N$ NH_4^+-N	Y_{N_2}	q NO_2^- -N*	R^2
15.2 ± 0. 6	99.6 ± 1.1	14.5 ± 0.8	0.98 ± 0.06	0.54 ± 0.03	0.98
30.3 ± 0.7	96.7 ± 0.9	28.2 ± 0.4	0.96 ± 0.05	0.56 ± 0.02	0.98
50.1 ± 0.5	97.3 ± 1.7	48.7 ± 0.3	0.98 ± 0.04	0.59 ± 0.04	0.91
70.2 ± 0.6	74.7 ± 1.2	46.3 ± 0.6	0.94 ± 0.06	0.51 ± 0.03	0.92
100.0 ± 0.6	58.9 ± 1.2	55.7 ± 1.2	0.81 ± 0.03	0.44 ± 0.03	0.94

qs * specific substrate consumption rate obtained by adjusting Gompertz

Table 2. Nitrogen balance, stoichiometric ratio of culture to different concentrations of nitrite evaluated

NO_2^--N Initial concentration (mg/L)	NH_4^+-N Consumptions (mg/L)	NO_2^--N Consumptions (mg/L)	NO_2^- removed/ NH_4^+consumed Relations (mol/mol)
15.2 ± 0. 6	10.5 ± 0.7	15.1 ± 0.2	1.39 ± 0.3
30.3 ± 0.7	21.1 ± 0.5	29.3 ± 0.8	1.38 ± 0.2
50.1 ± 0.5	36.5 ± 0.5	48.7 ± 1.1	1.33 ± 0.4
70.2 ± 0.6	34.9 ± 1.2	52.4 ± 0.9	1.51 ± 0.3
100.0 ± 0.6	41.7 ± 0.9	58.3 ± 0.5	1.40 ± 0.3

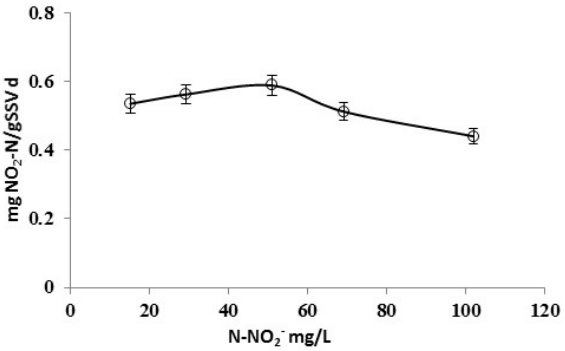

Fig. 2. NO_2^--N consumption rate evaluated in batch cultures. Batch cultures were carried out in duplicate. Error bars represent the standard deviation.

The reactant ratios of NO_2^- consumption to NH_4^+ consumption were all very near the benchmark value of anammox stoichiometry at 1.32 (Table 2). Overall, the results indicated that anammox was the dominant process in the cultures.

Finally, the sludge taken from the roots of *Typha* sp. showed the metabolic capability for carrying out the anammox process. In this work, the characterization of the bacterial microflora was not possible. Nonetheless, phylogenetic studies in natural and artificial wetlands have identified anammox bacterial like *Candidatus* and *Anammoxoglobus* genus (Zhu *et al*., 2011; Waki *et al*., 2015).

Conclusions

The sludge taken from the roots of *Typha* sp. showed the potential to carry out the anammox biological process. At initial nitrite concentrations below to 70 mg/L were obtained high consumption efficiencies for nitrite, whereas for above, the consumption efficiencies for nitrite diminished. At higher nitrite concentrations was observed an inhibitory phenomenon, however, the reaction time was relatively short. Therefore, the use of this kind of sludge might shorten the start-up process. The anaerobic ammonium oxidation is an emerging technology for nitrogen removal that provides a more environmentally sustainable and cost effective alternative compared to conventional biological treatments.

Nomenclature

Anammox	Anaerobic Ammonia Oxidation
$\%E_N$	efficiency of consume of NO_2^--N or NH_4^+-N, %
JAAD	Jóse Antonio Álzate dam
q_{NO_2-N}	specific substrate consumption rate mg of N consumed/g VSS h
VSS	volatile suspended solids, g/L
$Y_{P/S}$	anammox yield, mg of N_2 produced/mg of N consumed

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