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ANAMMOX MICROBIAL ACTIVITY IN SODIUM SALINE SOIL OF FORMER LAKE TEXCOCO, MEXICO

Actividad microbiana tipo Anammox en suelo salino-sódico del antiguo Lago de Texcoco, México

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Key words: alkaline, Anammox activity, extremophile, hzoA gene, microcosm, saline-sodic soil

ABSTRACT

The Anammox process, involving transformation of ammonium to dinitrogen, is well known in aquatic ecosystems. However, this anaerobic process in soil has been little explored, even less in extremophile soils. The saline-sodic soil of the former Lake Texcoco exposed to desertification with irrigation projects using wastewater of Mexico City, is a unique environment, yet little is known about its microbial ecology. The objective of this study was to examine the presence of Anammox microorganisms and their microcosm activity after a period of Anammox enrichment (229 days). Microcosm kinetics monitored after the 229 days enrichment experiment, showed a significant removal of ammonium and nitrite, with a significant production of dinitrogen. The stoichiometric conversion of NO_2^- and NH_4^+ to N_2 gas is complete in the studied soils, based on an Anammox process where 1 mole of NH_4^+ resulted in 1.02 moles of N_2. The presence of the Anammox functional gene *hzoA* was observed in saline-sodic soils of the former Lake Texcoco. The Anamx1 and Anamx2 operational taxonomic units (OTU) are phylogenetically close to a bacterium of the phylum Planctomycetes from a wastewater treatment reactor and marine environment (AB257585.1 and HE654780.1, respectively). Those two OTU represent indigenous or exogenous Anammox microorganisms phylotypes. The existence of microorganisms with Anammox activity in a saline-sodic alkaline soil is of great interest for the understanding of the nitrogen cycle in extremophile soils.

Palabras clave: actividad de Anammox, alcalino, extremófilo, gen hzoA, microcosmos, suelo salino sódico

RESUMEN

El proceso Anammox, que implica la transformación de amonio en dinitrógeno, es bien conocido en los ecosistemas acuáticos; sin embargo, este proceso anaeróbico en el suelo ha sido poco explorado, y menos aún en suelos extremófilos. El suelo salino sódico del antiguo lago de Texcoco, expuesto a la desertización, con proyectos de riego que utilizan aguas residuales de la Ciudad de México, es un entorno único, pero poco se sabe de su ecología microbiana. El objetivo de este estudio fue examinar la presencia de microor-

ganismos Anammox y su actividad de microcosmos tras un periodo de enriquecimiento (229 días). La cinética de microcosmos, la cual fue monitorizada después del experimento de enriquecimiento de 229 días, mostró eliminación significativa de amonio y nitrito con producción significativa de dinitrógeno. La conversión estequiométrica de NO_2^- y NH_4^+ a gas N_2 fue completa en los suelos estudiados, con base en el proceso Anammox donde 1 mole de NH_4^+ resultó en 1.02 moles de N_2. Se observó la presencia del gen funcional Anammox *hzoA* en suelos del antiguo lago salino sódico de Texcoco. Las unidades taxonómicas operativas (OTU, por su sigla en inglés) Anamx1 y Anamx 2 son filogenéticamente cercanos a cepas de una bacteria del filo Planctomycetes procedentes de un reactor de tratamiento de aguas residuales y de un medio ambiente marino (AB257585.1 y HE654780.1, respectivamente). Esas dos OTU representan los filotipos autóctonos o exógenos de microorganismos Anammox. La existencia de microorganismos con actividad Anammox en un suelo alcalino salino sódico es de gran interés para entender el ciclo del nitrógeno en suelos extremófilos.

INTRODUCTION

The anaerobic ammonium-oxidizing (Anammox) is a bacterial metabolic pathway of the nitrogen cycle in which ammonium is directly oxidized to dinitrogen (N_2) in an anoxic environment with nitrite as a final electron acceptor. The overall catabolic reaction is (Jetten et al. 1999):

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (\Delta G^{0'} = -358 \text{ kJ/mol } NH_4^+) \qquad (1)$$

Anammox microorganisms constitute a distinct phylogenetic group of bacteria, predominantly within the order of the Planctomycetales (Kartal et al. 2013). They are especially characterized by their Anammoxosome, a cell compartment where Anammox metabolism takes place, and by their very slow rate of division (Fuers et al. 2006, Fernández et al. 2014).

Despite this, in the oxygen-limited zones, the role of Anammox bacteria in the worldwide production of N_2 is today known to be important. Recent estimates suggest that this metabolism may be responsible for 30-50 % of N_2 production in oceans (Devol 2003). The past decade has seen growing interest in those bacteria due to their capacity to eliminate ammonium with a very low consumption of biomass. Moreover, as part of a process to treat nitrogen pollution, a strategy based on Anammox bacteria would allow shortening the process from two steps to one.

Anammox bacteria were first observed in wastewater treatment plants (Ren et al. 2020) but were later spotted in a wide range of natural environments (Nie et al. 2019). They were found in oceanic regions and anoxic basin (Kuypers et al. 2003), as well as deep-sea hydrothermal vents (Byrne et al. 2008). The Anammox process was also observed in freshwater zones, in hot springs (Jaeschke et al. 2009), estuaries and continental sediments (Trimmer et al. 2003, Jaeschke et al. 2010). More recently, some studies stated presence of Anammox bacteria in different types of terrestrial ecosystems, in lakeshores, wetlands and agricultural soils (Humbert et al. 2010, 2012, Wang and Gu 2013, Shen et al. 2013, Pajares and Bohannan 2016). Other studies aimed to determine if Anammox bacteria were able to adapt to extreme environments. The inhibitory effects of several culture conditions in reactors were demonstrated (Jin et al. 2012, Yu and Jin 2012) and it was particularly stated that high pH (> 9) induced an important decrease of Anammox activity. However, Anammox bacteria were also discovered in very high-temperature water containing metal sulfides (Russ et al. 2013), marine hypersaline basins (Borin et al. 2013), high-temperature petroleum reservoirs (Li et al. 2010), and acidic agricultural soils (pH < 4) (Shen et al. 2013).

The former Lake Texcoco is an ancient salty lake located in the Valley of Mexico, more than 2000 masl. This closed lake without current water outfall was drained to counter major problems of chronic flooding in nearby Mexico City. Its soils are saline and sodic, with a pH between 9.8 and 10.4, electrical conductivities in saturation extracts between 22 and 150 dS/m and a large percentage of exchangeable sodium (60-80) (Luna-Guido et al. 2000, Rojas-Oropeza et al. 2010). This site is also characterized by the presence of a network of canals of wastewater and a deficit of sewage treatment (Gómez and Díaz 2011). Ammonium was also brought into the soil during irrigation projects which used wastewater to drain salts and fertilization attempts that used sewage sludge (Beltrán-Hernández et al. 1999). Due to the high pH of Texcoco soil, an important proportion of ammonia volatilizes in form of ammonia (du Plessis

and Kroontje 1964, Vlek and Craswell 1981, Rojas-Oropeza et al. 2010). In addition, Rojas-Oropeza (2012) showed the presence of the Anammox functional gene *hzoA* in soils extracted from the former Lake Texcoco.

In this context, a better understanding of nitrogen cycles is of high interest. Therefore, in this study, our objective was to study the presence of the Anammox functional gene *hzoA* with its potential activity in extremophile soils of saline-sodic soils of the former Lake Texcoco. The hypothetic presence of Anammox microorganisms would be in relation to the anaerobic oxidation of ammonium. This will be done by analyzing the conversion rates of the different forms of nitrogen in soil samples of the former Lake Texcoco subjected to a helium atmosphere in a microcosm.

MATERIALS AND METHODS

Sampling sites

The study area (the former Lake Texcoco) is located in the Valley of Mexico at an altitude of 2210 masl, along the northeast part of Mexico City (Beltrán-Hernández et al. 1999). The superficial soil appears dry, brittle to the touch, and often contains cracks and salt crusts. The dominant vegetation on the site is saline grass (*Distichlis spicata*), which in the sampling area was < 15 cm high and sparsely distributed or absent due to the high salinity and sodicity. Six distinct sites were selected for sampling. In three of them, the salinity was considered as high (conductivity: 119.93 ± 13.9 dS/m, pH 10.0 ± 0.1), while in the three other sites it was lower (conductivity: 34.2 ± 2.8 dS/m, pH 9.1 ± 0.1). The 0-10 cm layer after removing the superficial salt crust (1 cm depth) was sampled 18 times and the soil of six plots was pooled so that three soil samples were obtained in each sampling site (n = 9 for each salinity) (February 2014). The soil samples were 2-mm sieved and characterized by triplicate. The physical and chemical properties of these soils are shown in **table I**: pH in a 1:2.5 soil-H_2O suspension using a glass electrode; electrolytic conductivity in a saturated extract; water-holding capacity of soil samples saturated with water in a funnel overnight (Rhoades et al. 1989, Thomas 1996).

Direct DNA extraction of soil

Extraction of DNA from soil was done as described by Martin-Laurent et al. (2001). One milliliter of a solution containing 100 mM Tris (pH 8.0), 100 mM EDTA, 100 mM NaCl, 1 % (wt/vol) polyvinylpyrrolidone, and 2 % (wt/vol) sodium dodecyl sulphate was added to 250 mg soil in a 2 mL mini-bead-beater tube containing 0.5 and 0.1 g of 106 mm- and 2 mm-diameter glass beads, respectively. Samples were homogenized for 30 s at 1600 rpm in a mini-bead-beater cell disruptor (Mikro-Dismembrator S; B. Braun Biotech International). Samples were centrifuged at 14 000 × *g* at 4 ºC for 1 min. The collected supernatants were incubated with 5 M sodium acetate (1:10) on ice for 10 min and centrifuged at 14 000 × *g* for 5 min. After precipitation with one volume of ice-cold isopropanol, the nucleic acids were washed with 70 % ethanol and purified using a Sepharose 4B spin column. The quality and size of the soil DNAs were verified by electrophoresis on 1 % agarose gels. DNA was quantified using a BioPhotometer at 260 nm (Eppendorf, Hamburg, Germany). Three replicates were used for DNA evaluation of each soil sample.

hzoA gene amplification

The Anammox functional gene *hzoA* was amplified from 50 ng of DNA extracted from soil with a

TABLE I. PHYSICAL AND CHEMICAL CHARACTERISTICS OF SOILS SAMPLED AT THE FORMER LAKE TEXCOCO (FEBRUARY 2014).

Soil sampled	pHH_2O	EC_s[a] DS/m	WHC[b] g/kg dry soil	Humidity %	Vegetation Description
Salinity 1	9.1 ± 0.1	34.2 ± 2.8	580	18.0 ± 1.6	Abundant *Distichlis spicata* (grass height: 30 cm), little *Eragrostis obtusiflora*, little *Chenopodium mexicana*
Salinity 2	10.0 ± 0.1	119.93 ± 13.9	540	17.4 ± 0.2	Little *Distichlis spicata* (grass height: 10 cm)

[a]ECs: electrolytic conductivity in the saturated extract; [b]WHC: water-holding capacity.

total volume of 25 µL by using 0.7 mL of the specific primers *hzoA* (9118-TATGGG TATGTC GATGGC TGA-9138 and 9583-CAACCA ACGATC CATACC GAA-9604) (designed in this work, based on *Planctomycete* KSU-1), and 2.5 U of Taq DNA polymerase (Appligene) under the following conditions: 5 min at 94 ºC, 1 min at 80 ºC (TaqPol); 1 cycle of 1 min at 94 ºC, 1 min at 55 ºC, and 1 min at 72 ºC; 10 cycles of 30 s at 94 ºC, 30 s at 55 ºC (with a decrease of 0.5 ºC per cycle), and 30 s at 72 ºC; and 30 cycles of 30 s at 94 ºC, 30 s at 52.5 ºC, and 30 s at 72 ºC, plus an additional cycle of 10 min at 72 ºC.

With the same conditions and after obtaining amplifications, these same reactions were used as template and amplifications were made with a reverse primer having at the 5' end a GC clamp (9583-CGCCCGC CGCGCG CGGCGG GCGGGG CGGGGG CACGGG GGGTAT GGGTAT GTCGAT GGCTGA-9604), allowing to hold together the double DNA chain during the following temperature gradient electrophoresis (TGGE) (Rojas-Oropeza et al. 2012).

For TGGE analysis, stapled amplifications of each gene were applied on acrylamide gels. A bi-sacrylamide (37.5:1) gradient from 56 to 66 ºC with 6 % acrylamide was used for 13 h at 60 V. After the electrophoresis, gels were revealed in a bath of SYBR-Gold with a concentration of 1:1000 (v/v) for 1 h. Digital images were analyzed in the Quantity One software from Bio-Rad Laboratories.

Sequencing and computer analysis

For DNA sequencing, visible bands were excised, resuspended in 20 µl of distilled water, and incubated for 24 h at 4 ºC. Selected DNA-bands were purified with Qiaex II Gel Extraction Kit (Quiagen). The nucleotide sequences of the DNA-band were determined by automated DNA sequencing by using the dideoxy chain-termination method and the ABI model 373A sequencer stretch (Applied Biosystems, Perkin-Elmer) of the Instituto de Fisiología Celular, UNAM. Each sequence was compared with sequences available in databases (GenBank, Blast, NCBI). A selection of Anammox bacteria from soil, wastewater treatment and marine sediment was included in the tree analysis (Nacional Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/). Derived nucleotide sequences of *hzoA* were aligned with nucleotide sequences of equivalent length using the ClustalW Multiple alignment software of Mega 7.0.21. The tree analysis was performed with Mega 7.0.21 (Kumar et al. 2016). The tree was reconstructed using the neighbor-joining method by Saitou and Nei (1987) and tree topology was evaluated by bootstrap analysis using 100 replicates.

Anammox kinetics

Forty grams of soil were placed in 125 mL glass flasks and fed with an Anammox metabolism specific medium: KH_2PO_4 3.0 g/L, K_2HPO_4 4.5 g/L, NH_4Cl 2.96 g/L, $NaNO_2$ 1.5 g/L and 1.5 mL/L of culture medium of trace elements (EDTA 5 g/L, $CaCl_2{\cdot}2H_2O$ 5.54 g/L, $CuSO_4{\cdot}5H_2O$ 1.57 g/L, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ 1.1 g/L, $CoCl_2{\cdot}6H_2O$ 1.6 g/L, $FeCl_3$ 5.0 g/L, $MnCl_2$ 5.0 g/L, $MgCl_2$ 5.0 g/L). The different soils were then complemented with distilled water up to saturation (anaerobic conditions) according to the measured water-holding capacity (**Table I**). Finally, each bottle was hermetically sealed with rubber top and aluminum ring and air was purged with helium gas for 5 min. The kinetics occurred then in an incubator at 23 ºC (average temperature of the studied sites) for a total period of 229 days.

During the whole experiment, a total of three 16-day kinetics were carried out (kinetic 1, 2 and 3). Between each kinetic, microcosms were regularly fed with 2 mL of an Anammox metabolism specific culture medium supplemented with a carbon source: KH_2PO_4 3.0 g/L, K_2HPO_4 4.5 g/L, NH_4Cl 0.82 g/L, NH_4HCO_3 0.085 g/L, $NaNO_2$ 1.5 g/L and 1.5 mL/L of culture medium of trace elements (EDTA 5 g/L, $CaCl_2 \cdot 2H_2O$ 5.54 g/L, $CuSO_4 \cdot 5H_2O$ 1.57 g/L, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 1.1 g/L, $CoCl_2 \cdot 6H_2O$ 1.6 g/L, $FeCl_3$ 5.0 g/L, $MnCl_2$ 5.0 g/L, $MgCl_2$ 5.0 g/L).

At each point of the kinetics, 20 mL of gas were extracted from the soil flasks and injected in 25 mL helium purged glass bottles for gas analysis. Nitrates, nitrites and ammonium were extracted from 10 g soil using a 0.5 M $NaSO_4$ solution.

Quantification of different nitrogen species

Measurement of concentrations of dinitrogen in gas samples was performed using a GOW-MAC gas chromatograph equipped with a thermal conductivity detector.

Nitrate, nitrite and ammonium concentrations were determined with a Skalar segmented flow analyzer based on the cadmium reduction method. Determinations of nitrite concentration alone were done through a diazo-coupling reaction producing a red-purple coloration absorbing light at 540 nm. Determinations of ammonium concentration were based on the modified Berthelot reaction. Quantification of each nitrogen species was characterized by triplicate.

Statistical analyses

The physicochemical results obtained were analyzed and compared statistically using a one-way analysis of variance (ANOVA) with PROC GLM with a level of significance of 95 % ($p < 0.05$), followed by a Tukey HSD test (SAS/STAT[R] software [2017], sas.com/stat).

RESULTS AND DISCUSSION

Presence of the Anammox functional gene *hzoA* in alkaline saline-sodic soils of the former Lake Texcoco

Following specific amplification of the studied gene, two *hzoA* partial gene sequences were obtained in the salinity 1. These sequences were individually analyzed using BLAST, to obtain sequences with the highest levels of similarity and allow construction of the dendrogram (**Fig 1**).

The diversity of the *hzoA* gene is low, as it presents a range-weighted richness (R_r) of 0.12:

$$R_r = N^2 \times (T_g/100\ ^\circ C) \quad (2)$$

where N is the total number of bands and T_g the gradient of temperature (Marzorati et al. 2008). The fragments Anamx1 and Anamx2 are phylogenetically close to the bacterium Candidatus *Jettenia caeni* of the phylum Planctomycetes, from an anaerobic ammonium-oxidizing enrichment culture (AB257585.1), followed by an uncultured Planctomycetes from the Baltic Sea (HE654780.1) (Shimamura et al. 2007, Klawonn et al. 2012). Irrigation with wastewater from Mexico City, for more than a century, could explain the exogenous origin of these operational taxonomic units (OTU). However, considering that the phylum Planctomycetes was aggregated with a very wide range of natural environments (Klawonn et al. 2012, Pajares and Bohannan 2016, Wiegand et al. 2018), it is not possible to determine whether these two OTU represent indigenous or exogenous microorganism phylotypes.

The *hzoA* gene is observed only in the lower salinity (salinity 1). Wang et al. (2020) observed that the dominance of bacterial groups is related to salinity: an increase in salinity changes the dominance of Planctomycetes, Proteobacteria and Bacteroidetes to Proteobacteria and Firmicutes.

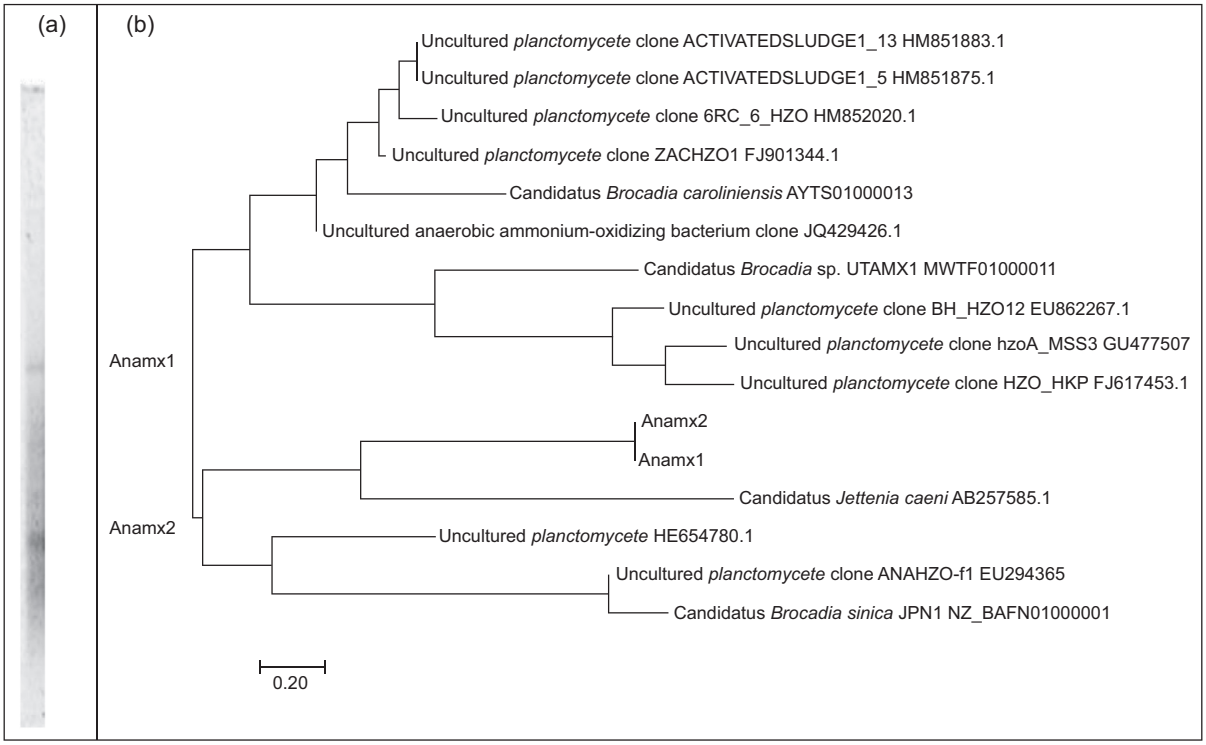

Fig 1. (a) TGGE analysis of the gene amplified with the *hzoA* specific primers, and (b) dendrogram of the *hzoA* gene fragments, both present in soils sampled at the former Lake Texcoco. Comparison of temperature gradient gel electrophoresis sequences and sequences from GenBank. Phylogenetic distances were determined analyzing neighbor-joining.

Activity of the Anammox function in the alkaline saline-sodic soil of former Lake Texcoco

The Anammox kinetics was performed in three steps: the first step at the initial time, the second step after 40 days of enrichment and the third after 229 days of enrichment. The presence of Anammox microorganisms presents a low diversity. An experiment of enrichment culture was initiated in microcosms. Of the three 16-day kinetics monitored during the 229 days enrichment experiment, only the third one allowed to observe significant consumption of ammonium and nitrite, with a significant production of dinitrogen. It is possible to compare the consumption and production rates of the different nitrogen species during the exponential phase of the third kinetics, with the average rates of the three kinetics (**Table II**).

The production of N_2 gas and the concentrations of ammonium (NH_4^+) and nitrite (NO_2^-) in microcosm soils of the studied salinities are presented in **figures 2** and **3**. The production of N_2 gas is observed in microcosm (**Fig 2**). Kinetics carried out in sterilized soils (not shown) do not follow this trend, implying that this gas production is indeed induced by anaerobic microbial activity. It can also be noticed that the production of gas in the sites of salinity 2 is at least as significant as in the soil of salinity 1.

As expected in the case of Anammox activity, NH_4^+ and NO_2^- concentrations followed the opposite trend, a nitrogen removal. While, in each case, NO_2^- practically disappeared completely in less than two days, NH_4^+ showed an average decrease of 54 % during the kinetics, both in the case of each salinity soils (**Fig 3**). After the Anammox enrichment period

TABLE II. AVERAGE CONSUMPTION AND PRODUCTION RATES OF THE DIFFERENT NITROGEN SPECIES DURING THE THREE KINETICS AND DURING THE EXPONENTIAL PHASE OF THE THIRD KINETICS.

	Salinity 1			Salinity 2		
	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3
Average of kinetics 1 and 2 exponential phase slope (mg/kg dry soil/d)						
NH_4^+ consumption	1.98	2.02	2.13	3.32	3.04	1.92
NO_2^- consumption	1.26	1.35	1.19	1.09	1.21	1.23
N_2 production	6.39	3.82	5.54	2.29	2.71	2.85
Kinetics 3 exponential phase slope (mg/kg dry soil/d)						
NH_4^+ consumption	16.70	15.07	13.37	64.81	37.93	15.27
NO_2^- consumption	1.99	2.39	1.35	1.13	3.24	4.99
N_2 production	57.55	98.80	27.17	24.72	36.27	32.09
p-values (N_2 production between kinetics 1-2 and 3)	0.00001	0.04033	0.00759	0.00267	0.00189	0.00025

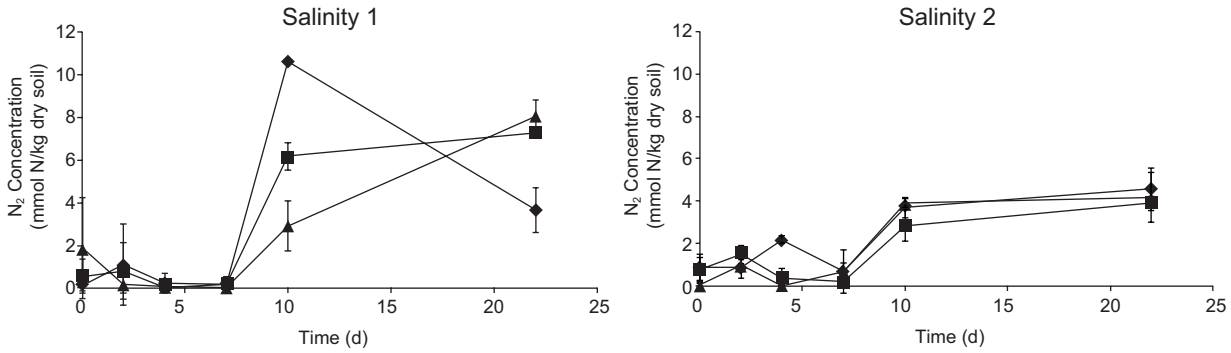

Fig 2. N_2 production in a microcosm environment during the third Anammox kinetics for the three sites with each salinity (after 229 days of Anammox enrichment): ■ site 1; ♦ site 2; ▲ site 3. Salinity 1, low conductivity soils; salinity 2, high conductivity soils. Determination by triplicate. ANOVA with $p < 0.05$.

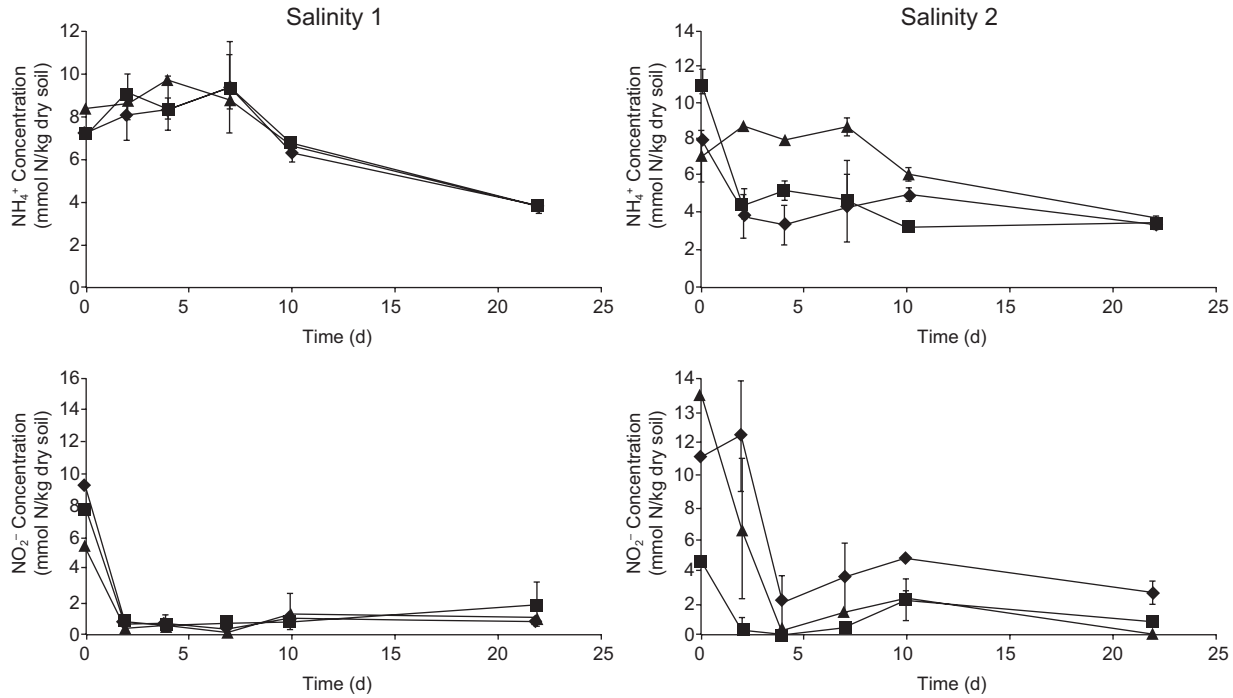

Fig 3. NH_4^+ and NO_2^- concentrations in a microcosm environment during the third Anammox kinetics for the three sites with each salinity (after 229 days of Anammox enrichment): ■ site 1; ♦ site 2; ▲ site 3. Salinity 1, low conductivity soils; salinity 2, high conductivity soils. Determination by triplicate. ANOVA with $p < 0.05$.

of 229 days, the stoichiometric conversion of NO_2^- and NH_4^+ to N_2 gas is complete in the studied soils, based on the Anammox process where 1 mole of NH_4^+ resulted in 1.02 moles of N_2 (Saricheewin et al. 2010). These measurements highlight the association of NO_2^- and NH_4^+ consumption with the production of N_2 gas. Each time, the limiting element is NO_2^-, as the whole nutrient is practically consumed since the fourth day of kinetics for each site of both salinities. It might be possible to consider that Anammox speed is slightly higher in the case of salinity 1 soils.

Therefore, in this study, the presence of a functional gene *hzoA* confirmed the Anammox process in saline-sodic extremophile soil of the former Lake Texcoco. Our hypothesis was also confirmed by stoichiometric analysis: Anammox activity was present in the two salinities, which is related to the presence of Anammox microorganisms. We are facing a test that strengthens the theory of the influence of the physical and chemical conditions of a saline-sodic soil on the assembly and co-occurrence of the microbial community (Guan et al. 2021). The presence and diversity of microorganisms can occur in the same process, in different times under similar conditions: hydraulic pulse, anaerobiosis, redox potential and nutrient availability.

CONCLUSIONS

The sodium saline soil of the former Lake Texcoco contains the Anammox gene with low diversity, phylogenetically close to the phylum Planctomycetes. Furthermore, Anammox activity was observed after an enrichment period with a specific culture medium for the Anammox process. The existence of microorganisms with Anammox activity in a saline-sodic alkaline soil is of great interest to understand the nitrogen cycle in extremophile soils.

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