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# Bacterial Biodiversity from an Anaerobic up Flow Bioreactor with ANAMMOX Activity Inoculated with Swine Sludge

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## ABSTRACT

*The present study aimed to describe the bacterial community present at an anaerobic up flow bioreactor with ANAMMOX activity, inoculated with the sludge from the anaerobic pond of a swine slurry treatment system. The description was based on the molecular DNA techniques using primers for amplification of complete 16S rRNA gene and also new primers to amplify smaller fragments from 16S rRNA. During the bioreactor operation time, the bacterial community changed significantly, increasing the nitrogen removal efficiency, reaching after 500 days a removal rate of 94%. The complete PCR amplification of 16S rRNA gene generated 17 clones, where three presented similarity with* Candidatus Jettenia asiatica *(97%), twelve with* Janthinobacterium *(99%) and two with uncultured clones. The PCR amplification of 436 base pairs had generated 12 clones, of which eight presented 96-100% similarity with* Candidatus Anammoxoglobus propionicus, Planctomycete *KSU-1 and one with* Pseudomonas *sp. (99%) and three with uncultured clones.*

**Key words**: ANAMMOX, anaerobic pond, sequencing, swine manure

## INTRODUCTION

The biological nitrogen removal is an important biotechnological process with a high ecological and economical significance (Li et al., 2009). Discovered in the 1990s at an anoxic denitrifying pilot bioreactor of a wastewater treatment plant, Anaerobic Ammonium Oxidation (ANAMMOX) is an alternative nitrogen removal process, in which ammonium is oxidized to nitrogen gas ($N_2$) using as the electron acceptor, the nitrite, under anoxic conditions (Kartal et al., 2007).

The ANAMMOX process has advantages over the conventional processes for the combination of nitrification/denitrification such as reduction of oxygen demand and dinitrogen monoxide

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emission, as well decrease in sludge production in wastewater treatment plants (Kartal et al., 2010). The bacteria's responsible for the ANAMMOX process belong to the Planctomycete phylum which forms a large phylogenetic branch and includes genera such as Candidatus, Brocardia, Kuenenia, Scalindua, Anammoxglobus and Jettenia (Zhang et al., 2008).

Bacteria with ANAMMOX activity have been found in different bioreactors for effluent treatment, such as the completely autotrophic nitrogen removal over nitrite (CANON) bioreactor, the sequential batch bioreactor (SBR), and other wastewater treatment plants (Kuenen, 2008). They have also a significant role in nitrogen cycle for different natural ecosystems, such as freshwater, marine and sediment environments (Kartal et al., 2010).

Since ANAMMOX bacteria have low growth rate, it was not possible so far, to isolate them in pure culture. The molecular technologies are especially important for studying the ANAMMOX and the bacterial diversity in ANAMMOX related environments. In this context, the present work aimed to study the bacterial community by the phylogenic analysis of 16S rRNA,, present at an anaerobic up flow bioreactor with ANAMMOX activity, inoculated with sludge from the anaerobic pond of a treatment system for swine slurry and, also to develop a new primer set to amplify a smaller fragment from 16S rRNA, making molecular techniques easier, and improving the knowledge about the ANAMMOX bacteria.

## MATERIAL AND METHODS

### Bioreactor and nitrogen analysis

A 2-L bioreactor was inoculated with sludge (4 g/L TSS), collected from an anaerobic pond from the experimental swine slurry treatment system, at Brazilian Agriculture Research Company (Embrapa Swine and Poultry), Concórdia, SC, Brazil (Schierhold Neto et al., 2006). The bioreactor was fed with the synthetic substrate prepared according to Schierhold et al. (2006), with 100 mgL·d of $NO_2$ -N and 100 mg/L·d of $NH_4^+$-N and kept at pH 8.0. The bioreactor activity was monitored by the analysis of $NH_4^+$-N, using ammonium selective electrode, N-$NO_2^-$ and N-$NO_3^-$ determined by the Flow Injection Analysis System FIAlab 2500 (FIAlab Instruments, USA) with spectrophotometric detection system, provided with light source "LED" of tungsten-halogen (400 to 700 nm), optical fiber 200 mm and UV/VIS detector USB4000 (Ocean Optics, USA) (Schierholt et al., 2006). To avoid the interference of oxygen in the process, $N_2$ was bubbled until 0.5 mg/L of dissolved oxygen. The Hydraulic Retention Time (HRT) was adjusted between 18.5 and 24 h. After 75 days of acclimatization, the bioreactor started to develop ANAMMOX activity (Schierhold et al., 2006). The bioreactor was monitored during the 500 days of operation, analyzing the nitrogen twice a week.

### Fluorescence in situ hybridization – FISH

The samples were collected at days 20, 116, 250 and 500 from the bioreactor. The hybridization procedures were performed as previously described (Amann et al., 1995) and samples were fixed with para-formaldehyde (4%). The fixed samples were hybridized with different probes (five) as shown in Table 1, and stained with DAPI. To determine the percentage of organisms, the amount of cells hybridized with specific probe were compared with DAPI stained cells. For visualization, an epifluorescence microscope (Olympus BX41, USA) was used.

### Sample and genetic material extraction

At day 500 of operation, 0.01 g of red biomass was collected from the bioreactor. The biomass was applied to the FTA elute card (Whatman Bioscience, USA), and the genetic material was extracted from the card according to the manufacturer's instruction. The extracted solution was mixed two times with appropriate volume of phenol/chloroform/isoamylalcohol (50:48:2) and once with chloroform/ isoamylalcohol (96:4) to remove the proteins present in the solution as contaminants and dissolved phenol.

### Complete Amplification of 16S rRNA gene

The complete amplification of 16S rRNA gene was performed using conserved eubacterial primers 6F (5'-ggagagttagatcttggctcag-3') and 1492r (5'-ggttaccttgttacgact-3'). PCR parameters were 2 min initial denaturation at 94°C, followed by 25 cycles of 15 sec at 94°C, 30 sec at 58°C, and 30 sec at 68°C (Viancelli et al., 2009).

### Amplification of 436 base pair (bp) fragments from 16S rRNA gene

A forward primer 436F (5'–agcggtgaaatgcg–3') and a reverse primer 436R (5'–gggtttcgctcgtta–3')

were developed by amplifying a fragment of 436 bp between the 677-1112 bp from 16S rRNA region, having as development base a conserved region from *Candidatus Brocadia anammoxidans* nucleotide sequence deposited in Genbank (AF375994). The polymerase chain reaction was performed in a thermocycler (Eppendorf, UK), using the following parameters: 10 pmol each primer, 2.5 U of *Taq* Polimerase, 10% PCR buffer, 2 mM $MgCl_2$, 200 μM each dNTP, 3 ng DNA and ultrapure water in a final volume of 25 μL. The amplification was conducted in the following conditions: 3 min initial denaturation at 95ºC; 30 cycles of 45 sec denaturation at 95ºC, 1 min annealing at 60ºC, 1 min extension at 72ºC; and a final extension of 7 min at 72ºC. The amplified fragments were analyzed by 1% agarose gel electrophoresis, stained with Etidium Bromide (5 μg/mL), and visualized under UV light.

**Table 1-** Probe sequences and formamide concentration required for FISH.

| Probe | Specificity | Sequence | Form |
|---|---|---|---|
| EUB MIX | All bacteria | 5'-gctgcctcccgtaggagt-3' | 20% |
| | | 5'-cagccacccgtaggtgt-3' | |
| | | 5'-ctgccacccgtaggtgt-3' | |
| Nso190 | All AOB | 5'-cgatcccctgcttttctcc-3' | 55% |
| NIT3 | *Nitrobacter* sp | 5'-cctgtgctccatgctccg-3' | |
| NEU | *Nitrosomonas* sp | 5'-cccctctgctgcactcta-3' | |
| AMX820 | C.B. anammoxidans | 5'-aaaacccctctacttagtgccc -3' | 40% |
| | C.K. stuttgartiensis | | |

**Cloning, sequencing and phylogenetic analysis of amplified fragments**

The fragments amplified by PCR were purified using the GFX PCR and Gel DNA purification kit (GE Healthcare, UK). Purified fragments were inserted into pCR® 2.1-TOPO® vector (Invitrogen, USA) and transformed into DH5α *Escherichia coli* competent cells (Sambrook et al., 2001). The clones were grown in Luria-Bertani medium plates supplemented with ampicillin (50 mg/ml). Colonies were chosen and the plasmidial DNA was extracted by alkaline method (Sambrook et al., 2001). The plasmids containing the fragments were analyzed with ABI3130 analyzer and BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). Sequences were aligned using Clustal W 1.8.3 (Thompson et al., 1994) from BioEdit 7.0.5 package (Hall, 1999). After the alignment, the sequences were compared with the sequences from GenBank using BLAST tool (Altschul et al., 1990) and the phylogenetic analysis was conducted with software MEGA 3 (Kumar, et al., 2004).

**PCR detection limit**

To test the detection limit of the PCR that amplified the 436 pb, an ANAMMOX positive sample (confirmed by sequencing) containing 3 ng of DNA, was diluted ($10^{-1}$ until $10^{-5}$), submitted to PCR and 1% agarose gel electrophoresis as described above.

## RESULTS AND DISCUSSION

During the 500 days of operation, the bioreactor presented a maximum nitrogen removal rate of 1916 mg N L/d (between the days 400 - 500 of operation). The value obtained represented 94% of nitrogen removal (Table 2) and was much higher than the traditional process of nitrification/denitrification, which removed about 300 mg N L/d. It was almost two times more than the removal by the sequential batch bioreactors and gas-lift with ANAMMOX activity (1070 mg N L/d) (Dapena-Mora et al., 2004). The stoichiometric index for $NH_4^+$:$NO_2^-$:$NO_3^-$ obtained was 1:1.18:0.24 (Schierhold et al., 2006) that was similar to that found by Jetten et al., (2000) 1:1.31:0.22.

The analysis of the bacterial community showed the presence of ammonium oxidizing bacteria from *β-proteobacteria* group (detected by NSO probe), *Nitrosomonas* spp (detected by NEU probe), *Nitrobacter* sp. and ANAMMOX (detected by NIT3 and AMX820 probes, respectively). Figure 1 showed that with the amount of organisms, the ANAMMOX activity increased along the operation days and, a decrease in the other bacterial populations occurred. Ammonium

oxidizing bacteria (genus *Nitrosomonas, Nitrosospira, Nitrosococcus, Nitrosovibrio, Nitrosolobus*) were responsible for a little conversion of ammonium to nitrate or nitrite, explaining the difference found between the stoichiometric equation found in the reactor and the described in the literature.

The increase in the amount of organisms with anaerobic ammonium oxidation activity (Fig. 1) was in accordance with the nitrogen removal rate and ANAMMOX stoichiometry until day 250. However, on day 500, the percentage of organisms decreased but the nitrogen removal rate increased. The reason of reduction of organism with ANAMMOX activity probably was due to the sampling procedure since the reactor did not have a homogeneous distribution of these organisms.

**Table 2 -** Highest values of nitrogen loading, removal and % removal performed during the 500 days of operation of the reactor.

| Days | nitrogen loading rate (mg N L/d) | nitrogen removal rate(mg N L/d) | nitrogen removal (%) |
|---|---|---|---|
| 0 – 100 | 305.6 | 191.3 | 59 |
| 100 – 200 | 597.0 | 535.5 | 89 |
| 200 – 300 | 744.0 | 638.8 | 85 |
| 300 – 400 | 1404.9 | 1245.7 | 88 |
| 400 – 500 | 1653.2 | 1569.9 | 94 |

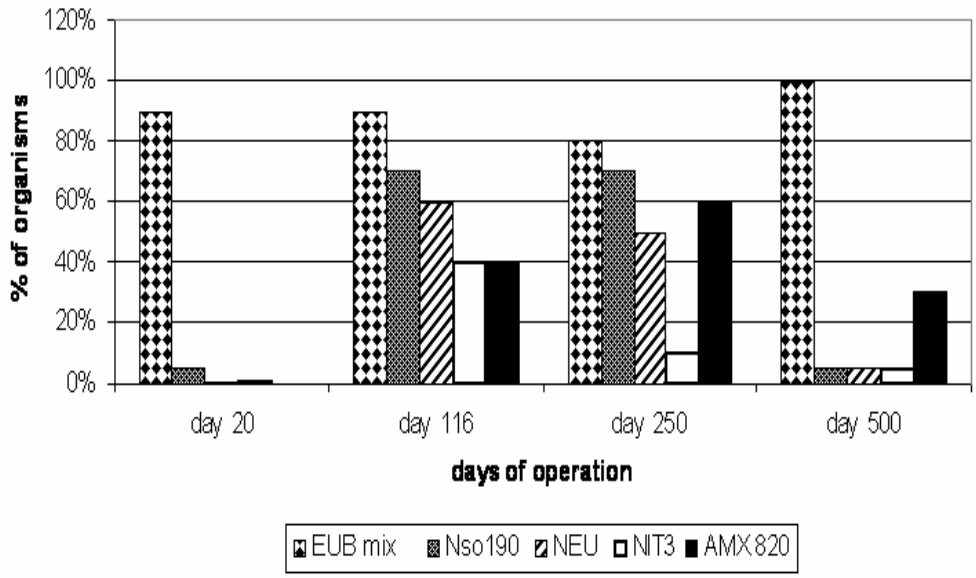


**Figure 1 -** Bacterial diversity present in the bioreactor, analyzed by FISH during the 500 days of bioreactor operation. Probes detected total eubacteria (EUB mix); *β-proteobacteria* group (NSO 190); *Nitrosomonas* spp (NEU); *Nitrobacter* sp. (NIT3) and ANAMMOX (AMX820).

Since the stoichiometric index, removal rate and FISH analysis showed the presence of organisms with ANAMMOX activity, molecular techniques of PCR and sequencing were performed to amplify and characterize the ANAMMOX species present in the bioreactor. A complete PCR targeting for 16S rRNA gene was performed and the fragments amplified were cloned and sequenced. From the 17 clones obtained from the 16S rRNA, 12 presented 99% similarity with *Janthinobacterium* sp. (sequences FJ225383, FJ225384 and FJ225385) and were aerobic facultative nitrate-reducing bacteria, typically present in the soil and water. Although these were aerobic organisms, they had been isolated in aerobic and anaerobic conditions. Organisms with 99% similarity to

*Janthinobacterium* were found in a microbial ammonium-assimilating community in animal waste treatment systems (Sasaki et al., 2005).

Three clones (sequences FJ225387 and FJ225388) presented 97% similarity with ANAMMOX *Candidatus Jettenia asiatica* and strain *Planctomycete* KSU-1 and 94%, 92% and 91% with *Candidatus Anammoxoglobus propionicus*, *Candidatus Brocadia anammoxidans* and *Candidatus Kuenenia stuttgartiensis*, respectively. From these three clones, two showed exactly the same nucleotide sequence (FJ225387), and the FJ225388sequence showed only a single base difference with that (FJ225387). These two clones were named *Brasilis concordiensis* 1 and 2 (Viancelli et al., 2009).

Parallel to the amplification of 16S rRNA gene, a PCR targeting a 436 bp region was performed. Figure 2A shows the results of PCR standardizing with a specific 436 bp amplification. The amplified fragment was sequenced and confirmed the ANAMMOX similarity. This DNA sample was used as PCR positive control. After the standardizing, the technique was performed to test the limit of detection using an initial amount of 3 ng of DNA. The DNA was serially diluted (from $10^{-1}$ to $10^{-5}$) and submitted to PCR. Figure 2B showed that the last amplification occurred with 0.3 ng of genetic material, which was less than the used in other studies: 30 to 80 ng (Penton et al., 2006). This indicated that the PCR described here could be performed with low quantities of DNA, which would be very interesting, considering the low amount of ANAMMOX bacteria found in natural environment samples.

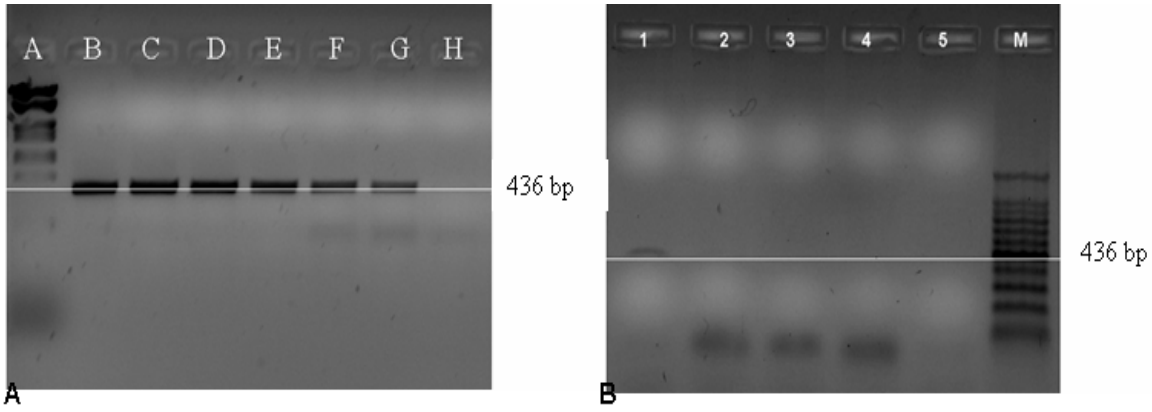


**Figure 2 A -** PCR standardizing with new primer set: A: 100 bp Latter; B-G samples with 3ng of DNA; H: negative control. **B:** PCR with serial DNA dilution to test the primers limit of detection: 1: dilution $10^{-1}$; 2: dilution $10^{-2}$; 3: dilution $10^{-3}$; 4: dilution $10^{-4}$; 5: dilution $10^{-5}$; M: DNA ladder 100 bp.

The biomass samples from the bioreactor were submitted to PCR and the amplified fragments of 436 pb were cloned and sequenced. This generated 12 clones, eight similar with ANAMMOX organisms (sequences GQ850472, GQ850476, GQ850473, GQ850479 and GQ850478), *Candidatus Anammoxoglobus propionicus* (100%), Planctomycete KSU-1 (96%), *Candidatus Jettenia asiática* (98%) (Fig. 3).

*Candidatus Anammoxoglobus propionicus* was found in a culture medium enriched with oxidizing propionate, acetate and formate with nitrate as final acceptor of electrons. *Candidatus Anammoxoglobus propionicus* was the first ANAMMOX bacterium with defined niche. The oxidation of propionate and ammonium when at a mixed culture, presented competitive behavior with ANAMMOX and other denitrifying heterotrophic bacteria by the oxidation of propionate in the presence of ammonium, nitrite and nitrate. After four months of *Candidatus Anammoxoglobus propionicus* culture, according to Kartal et al. (2007), they eliminated other ANAMMOX species (Brocadia anammoxidans) from the reactor.

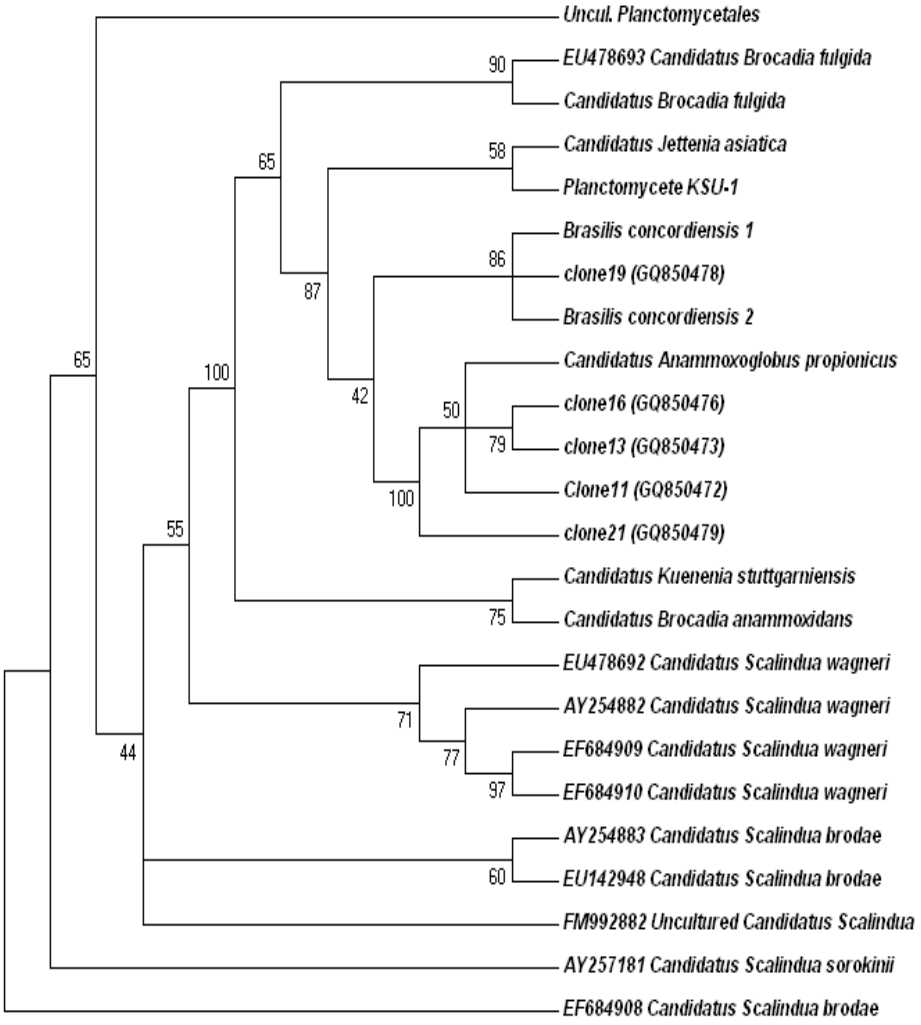


**Figure 3 -** Dendogram of sequences found in the present study (GQ850472, GQ850476, GQ850473, GQ850479, GQ850478) and sequences from other studies deposited in GenBank.

*Planctomycete* KSU-1 was identified as the dominant bacteria species in a biofilm ANAMMOX reactor with an average total-nitrogen removal rate of 984 mg N L/d (Fujii et al., 2002) and presented 92% similarity with *Candidatus Brocadia anammoxidans*. Although it was not classified as an ANAMMOX organism, *Planctomycete* KSU-1 probably was a new member of Brocadia genus. In other studies, *Candidatus Jettenia asiatica* was described as the dominant bacteria in a granular sludge ANAMMOX bioreactor, where AAOB were also found belonging to *β-Proteobacteria*. The nitrogen removal rate of the reactor was 800 mg N L/d (Quan et al., 2008), somewhat lower compared to the removal rate obtained in this study (1916 mg N L/d).

One clone (sequence GQ850474) showed 99% similarity with *Pseudomonas* sp. These are denitrifying bacteria that under anaerobic conditions use nitrate as final acceptor electrons and have been found on SBR reactors converting all $NO^-_3$ in the medium to $N_2$ (Merzouki et al., 1999). Three others had similarity with uncultured bacterium clones isolated from the swine sludge.

In the present study, two similar ANAMMOX organisms (*Candidatus Jettenia asiatica* and *Candidatus Anammoxoglobus propionicus*) were identified at the same time in the bioreactor. However, Kartal et al. (2007) when working with

enrichment culture concluded that two ANAMMOX species rarely were found in the same place, or in the same bioreactor. This could be explained because the bacterial community analyzed in the present study was not from a pure culture and the species were still competing. Probably, in due course of time, one might get eliminated by competition as reported by Kartal et al (2007). Considering the above, it was possible to conclude that the PCR developed could be easily applied in monitoring the ANAMMOX bacteria, because its protocol procedures and high capacity to detect little DNA amount, permitted that. By the high performance in nitrogen removal rate, ANAMMOX could be a powerful and cheap technology to treat the sludge rich in ammonium like swine sludge.

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