Investigation of Aerobic and Anaerobic Ammonium-oxidizing bacteria presence in a small full-scale wastewater treatment system comprised by UASB reactor and three polishing ponds

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Abstract This work applied PCR amplification method and Fluorescence *in situ* hybridization (FISH) with primers and probes specific for the aerobic ammonia-oxidizing β-*Proteobacteria* and anammox organisms in order to detect these groups in different samples from a wastewater treatment system comprised by UASB reactor and three polishing ponds. Seven primer pairs were used in order to detect Anammox bacteria, such as: the forward primer Pla46rc (specific for the *Planctomycetales*) together with either the reverse primer 1392r (E*ubacteria)*, Amx368 (specific for "*Brocadia*", "*Kuenenia*" and "*Scalindua*"); or Amx820 (specific for "*Brocadia*" and "*Kuenenia*"), or Amx1240 (specific for "*Brocadia anammoxidans*"), or Amx667R. Primer pairs Brod541f-Brod1260r (specific for *Scalindua brodae* and *Scalindua wagneri*) and An7f-An1388r (specific for all anammox organisms Genera "*Ca. Brocadia"*, "*Ca. Kuenenia*", and "*Ca. Scalindua"*) were also tested. Results with primers Pla46rc/Amx368r and Pla46rc/Amx667r suggest the presence of Anammox bacteria in the UASB reactor and also in the three polishing ponds. However, FISH results indicated the absence of Anammox and aerobic ammonia-oxidizing bacteria in these samples, suggesting that nitrification and anammox were not the major reactions responsible for total nitrogen removal in this wastewater treatment system.
Keywords Anammox; *in situ* hybridization; Nitrogen removal; PCR detection; Polishing ponds; UASB reactor

INTRODUCTION

Biological nitrogen removal is often accomplished by microbial processes such as nitrification and denitrification. Objectively, these can not be considered as a sustainable process, as they do not satisfy the requirements for low energy costs and low sludge production. A very promising new and more sustainable process for nitrogen removal is the anaerobic ammonium oxidation (Anammox) process (Strous *et al*., 1997). In this process ammonium is oxidized to nitrogen gas with nitrite as electron acceptor.

After the first discovery, nitrogen losses, which could only be explained by the anammox reaction, were reported in various wastewater treatment facilities including landfill leachate treatment plants in Germany, Switzerland, and England (Helmer *et al*., 1999; Egli *et al*., 2001, Schmid *et al*., 2003), as well as in semitechnical wastewater treatment plants in Germany (Schmid *et al*., 2000), Belgium (Pynaert *et al*., 2003), Japan (Fujii *et al*., 2002), Australia (Toh and Ashbolt, 2002), and United States (Tal *et al*., 2003).

Anammox organisms are classified in a group of *Planctomycete* bacteria. The *planctomycetes*, five of which have been named provisionally *Candidatus Brocadia anammoxidans*, *Candidatus Kuenenia stuttgartiensis*, *Candidatus Scalindua wagneri*, *Candidatus Anammoxoglobus*

propionicus (Kartal *et al*., 2007) and *Candidatus Jettenia asiatica* (Quan *et al*., 2008) are an interesting group of bacteria with many rare or unique properties. They are coccoid bacteria with a diameter of less than 1 μm (Van Niftrik *et al*., 2004). They have a doubling time of approximately 11 days and are physiologically distinct from the other known *Planctomycetes*: - they are anaerobic chemolithoautotrophs. Many anammox bacteria have not yet been isolated; therefore, molecular ecological techniques such as Fluorescence *in situ* hybridization (FISH) and specific polymerase chain reaction (PCR) amplification are essential for future research on these bacteria (Schmid *et al*., 2005)

In small wastewater treatment systems, conceptual simplicity is a very important point in order to garantee sustainability of the plant operation over the years. This is especially the case in developing countries, in which coping with excessive mechanization and energy consumption is usually an obstacle towards a smooth and reliable operation (von Sperling *et al*. 2008). Systems comprised by a UASB (Upflow Anaerobic Sludge Blanket) reactor followed by shallow polishing (maturation) ponds in series have been applied in different parts of Brazil, leading to good results in terms of organic matter removal and excellent results regarding coliform removal (Van Haandel & Lettinga 2004; von Sperling *et al.* 2005). Polishing ponds in series have proven to produce an effluent suitable for unrestricted irrigation. It is also worth of mention that the pond system is the only capable of removing the four categories of pathogenic organisms (bacteria, viruses, protozoan cysts and helminth eggs) (von Sperling *et al.* 2008).

The purpose of the present study was to investigate the Anammox and ammonia-oxidizing bacteria presence in a small full scale wastewater treatment system (comprised by UASB reactor and three polishing ponds) by using the molecular technique of PCR (Polymerase Chain Reaction) with primers specific for the amplification of the 16S rDNA of anammox organisms and fluorescence *in situ* hybridization (FISH) in order to verify if Anammox and/or aerobic ammonia oxidation process would be occurring in polishing ponds and being responsible for ammonia removal in these systems.

METHODS

Study Area and Experimental Set-up

The wastewater treatment system investigated is located in Belo Horizonte, Brazil, and involved the following units in series: one UASB reactor, three shallow polishing (maturation) ponds (Figure 1). The UASB reactor was cylindrical, with the gas-solids-liquid separator in the central part, with gas collection. The ponds were rectangular, with a length-to-width ratio around 5, and hydraulic retention times of 4 days (ponds 1 and 2) and 2 days (pond 3). The population equivalent of the system was around 200 inhabitants. The behaviour of the system was evaluated in detail by von Sperling *et al* (2008), using physical-chemical and biological parameters, based on three years of intensive monitoring. These data will not be presented here.

Five samples were collected: sludge from the UASB reactor (U4); effluent from the UASB reactor (E5); sediment from polishing pond 1 (P1), which treats the effluent from the UASB reactor; sediment from polishing pond 2 (P2), which treats the effluent from P1; sediment from polishing pond 3 (P3), which treats the effluent from P2.

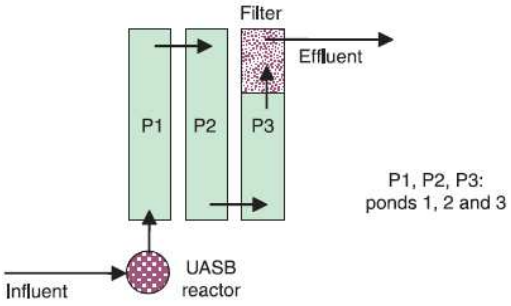

Figure 1: Flowsheet of the investigated system.

DNA extraction and PCR amplification.

DNA was extracted from 2 to 4 ml of the sludge and sediment samples according to Egli *et al*. (2003). To detect anammox bacteria, the following pairs of primers were used (Table 1): primer Pla46rc (used as foward primer) and 1392r; Pla46rc and anammox-specific primer Amx368r; Pla46rc and Amx820r; Pla46rc and Amx1240r; and Pla46rc and Amx667r. Amplification conditions used were the same for all pairs of primers, which included an initial denaturation step consisting of 4 min at 94° C, 35 cycles consisting of 45 s of denaturation at 94°C, annealing for 50 s at 56°C (or 60°C for Pla46F/1392R), and elongation for 1 min at 72 ^{0}C, and a final extension step consisting of 7 min at 72°C. Amplification conditions for the following primers Brod541f-Brod1260r and An7f-An1388r were: 95°C for 3 min, 30 cycles of 95°C for 45 s, 60°C (for Brof541F-Brod1260R) or 63°C (for An7F/An1388R) for 1 min, 72°C for 1 min, and final extension at 72°C for 7 min. All PCR amplifications were performed two times in two separate PCR events. The presence and size of amplification products were determined by agarose (1%) gel electrophoresis of 5 µl aliquots of the PCR products.

Table 1. Primers used for the detection of *Planctomycetales* and anammox organisms.

Primer	Specificity	Sequence (5´→3´)	Reference
Pla46rc	*Planctomycetales*	GGATTAGGCATGCAAGTC	Egli *et al.* (2001)
Amx368r	All anammox organisms (Genera "*Ca. Brocadia*", "*Ca. Kuenenia*", and "*Ca. Scalindua*").	CCTTTCGGGCATTGCGAA	Schmid *et al.* (2000)
Amx667r	Anammox organisms	ACCAGAAGTTCCACTCTC	Van der Star *et al.* (2007)
Amx820r	Genera "*Ca. Brocadia*" and " *Ca. Kuenenia*"	AAAACCCCTCTACTTAGTGCCC	Schmid *et al.* (2000)
1392r	Universal bacterial primer	ACGGGCGGTGTGTAC	Ferris *et al.* (1996)
Amx1240r	"*Ca. Brocadia anammoxidans*"	TTTAGCATCCCTTTGTACCAACC	Schmid *et al.* (2000)
An7f	All anammox organisms (Genera "*Ca. Brocadia*", "*Ca. Kuenenia*", and "*Ca. Scalindua*").	GGCATGCAAGTCGAACGAGG	Penton *et al.* (2006)
An1388r		GCTTGACGGGCGGTGTG	Penton *et al.* (2006)
Brod541f	" Ca. *Scalindua brodae* and *Scalindua wagneri*"	GAGCACGTAGGTGGGTTTGT	Penton *et al.* (2006)
Brod1260r		GGATTCGCTTCACCTCTCGG	Penton *et al.* (2006)

Fluorescence *in situ* hybridization (FISH) analysis

The FISH method involves application of oligonucleotide probes to permeabilized whole microbial cells and specifically hybridize the cells to their complementary target sequence in the ribosomes.In this study, about 500 ml of sediment samples from the three polishing ponds, sludge and effluent samples from the UASB reactor were collected to determine the presence of anammox and aerobic ammonia-oxidizing bacteria. The probes corresponding to these bacterial groups and hybridization conditions employed are described in Table 2. Samples were prepared according to Sawaittayothin & Polprasert (2007), by applying 15ml of each sample in 15 ml of 0.85% NaCl solution, centrifuged at 150 rpm for 60 min to extract the bacterial cells from the sediment, and again centrifuged at 3000 rpm for 10 min to separate the bacterial cells from other contaminants. The supernatant from the step described before was centrifuged again at 4000 rpm for 10 min to make bacterial cells to settle. The cells were fixed with paraformaldehyde 4% and hybridizations with fluorescent probes were performed as described by Egli *et al*. (2003).

Table 2. Oligonucleotide probes and hybridization conditions used for FISH analysis.

Probe name	Specificity	Sequence (5´to 3´)	Formamide (%)/NaCl(mM)[a]	Reference
Amx 820	*Candidatus "Brocadia anammoxidans", Cand. "Kuenenia stuttgartiensis"*	AAAACCCCTCTACTTAGTGCCC	40/56	Schmid *et al.* (2000)
Nso190	Many but not all ammonia-oxidizing β-*Proteobacteria*	CGATCCCCTGCTTTTCTCC	50/28	Mobarry *et al.* (1996)
Nso1225	Almost all ammonia-oxidizing β-*Proteobacteria*	CGCCATTGTATTACGTGTGA	35/80	Mobarry *et al.* (1996)

[a] Formamide (%) in the hybridization buffer and NaCl (mM) in the washing buffer, respectively, required for specific *in situ* hybridization (Egli *et al.* 2003)

RESULTS AND DISCUSSION

Detection of anammox organisms

PCR amplification results using different primer pairs for the amplification of 16S rDNA from *Planctomycetales* and anammox organisms from different polishing pond sediments and sludge samples are shown in Table 3. With the conserved primers Pla46rc and Amx368r, products of the appropriate size 0.32kb were obtained for DNA of all samples tested, as can be seen in Figure 2A. Results with the primers Pla46rc and Amx667r (specific to anammox organisms) confirmed the presence of anammox bacteria in sediment samples from the three polishing ponds (as shown in Figure 2B), but did not for UASB samples (sludge and effluent). From looking at the amount of product formed in P3 sediment sample it appeared that this sample contained the highest amount of anammox amplifiable material per amount of DNA isolated (Fig. 2B). With primers Pla46rc/Amx820r, and Pla46rc/Amx1240, specific for, respectively, *Kuenenia* and *Brocadia*, and *Brocadia anammoxidans*, none of the samples tested showed positive results (not shown).

PCR results suggest that anammox bacteria were present in the UASB reactor before entering into the polishing ponds. Since the results with primers An7f/An1388r and Brod541f/Brod1260r were negative for all samples tested, and according to Penton *et al.* (2006) these primers are more specific for the detection of anammox organisms, compared to primers Pla46rc, Amx368r, and

Amx820r, it is therefore still necessary to confirm the presence of anammox in sediment samples through cloning and sequencing the amplified fragments.

Table 3. PCR amplification results with different primer pairs for the amplification of 16S rDNA from *Planctomycetales* and anammox organisms (for the specificity of the primers see Table 1).

Pair of primers	Pond 1 sludge	Pond 2 sludge	Pond 3 sludge	UASB sludge	UASB effluent
Pla46rc/1392r	+	+	+	+	+
Pla46rc/Amx368r	+	+	+	+	+
Pla46rc/Amx667r	+	+	+	-	-
P46rc/Amx820r	-	-	-	-	-
P46rc/Amx1240r	-	-	-	-	-
An7f/An1388r	-	-	-	-	-
Brod541f/Brod1260r	-	-	-	-	-

(+) positive result, amplification product was visualized on agarose gel
(-) negative result, no amplification product was formed

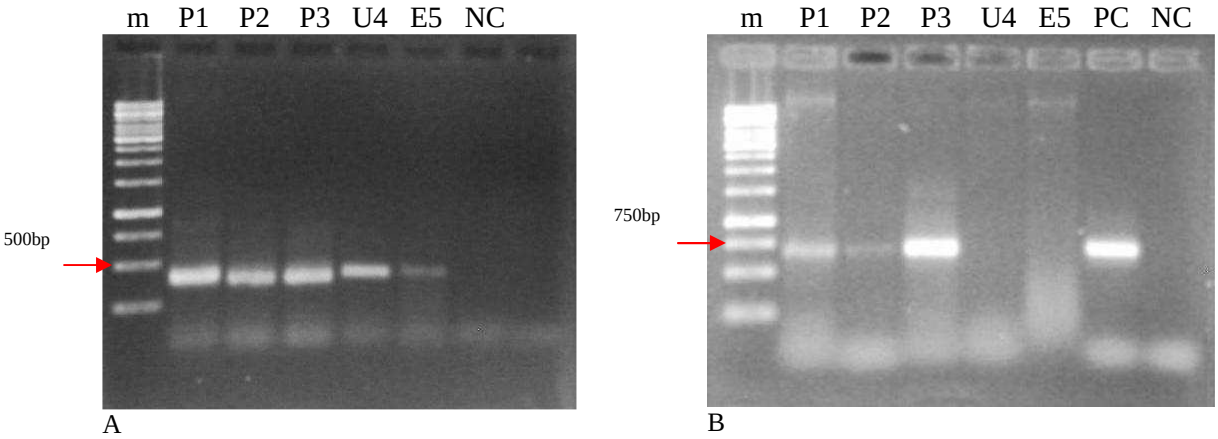

Figure 2. A-PCR detection of anammox-amplifiable 16S rDNA with primers Pla46rc and Amx368r specific for *Brocadia*, *Kuenenia* and *Scalindua*. B-PCR detection with primers Pla46rc and Amx667r. Lanes: m, 1 Kb ladder (Fermentas); P1, sediment from polishing pond 1; P2, sediment from polishing pond 2; P3, sediment from polishing pond 3; U4 sludge from UASB reactor; E5, effluent from the UASB; NC is the negative control (without DNA); PC is a positive control from an anammox enrichment reactor.

Ammonia removal in the system

Table 4 presents the mean removal efficiencies (and mean concentration of N-compounds) of the parameters investigated in the system in the previous study, conducted over a three-year period (von Sperling *et al.* 2008). It can be noticed that the highest ammonia removal values occurred in polishing ponds 2 and 3.

Table 4. Summary table of mean removal efficiencies and mean concentration of the nitrogen compounds and other parameters (modified from von Sperling *et al*., 2008).

Parameter	Mean removal efficiencies (%)				
	UASB	Pond 1	Pond 2	Pond 3	Overall
BOD	73	9	12	-13	81
COD	62	-14	4	-31	71
TSS	68	-36	1	-25	78
Total N		21	28	26	56
Ammonia-N		24	32	34	57
Parameter	Mean concentration (mg. l^{-1})				
Ammonia -N	31	24	16	13	
NO_2^- - N	0.10	0.28	1.02	1.63	
NO_3^- - N	0.15	0.15	0.20	0.20	

Average efficiencies calculated based on the average influent and effluent concentrations of each unit.

FISH Results

The FISH results shown in Table 5 indicated the absence of aerobic ammonia oxidizing bacteria probably because the DO concentration of the sediment samples was close to 0 mg $.l^{-1}$. Although ammonia removal efficiencies occurred systematically along the ponds (24, 32, and 34% for polishing pond 1, 2, and 3, respectively as shown in Table 4), nitrite and nitrate values are low, implying a reduced nitrification. This was also confirmed by Most Probable Number (MPN) analysis of *Nitrosomonas* and *Nitrobacter* from polishing ponds water samples (not shown). The Anammox bacteria require NO_2^- as electron acceptor to oxidize NH_4^+ to become gaseous N_2. Because of the absence of nitrifying bacteria (ammonia-oxidizing bacteria, as reported above) and the low NO_3^- and NO_2^- concentrations in the samples, the FISH results did not indicate the presence of Anammox bacteria in the polishing ponds sediments (and also in UASB reactor), or Anammox bacteria could be present but below FISH detection limit (around 10^3 and 10^4 cells . ml $^{-1}$). This was also the case in a WWTP, located in Pitsea (UK). Although the biomass from the UK had high anammox activity, no anammox bacteria could be detected with the general anammox probe (Amx820), indicating that known anammox species were below the FISH detection limit (Schmid *et al*., 2003). Sawaittayothin & Polprasert (2007) also applied FISH to determine the predominant species of bacteria growing in constructed wetlands treating municipal landfill leachate. They verified that nitrification/denitrification or Anammox were not the major reactions responsible for total nitrogen removal in the wetlands but cattail plant uptake was the major mechanisms.

Table 5. FISH analysis for bacteria identification in polishing pond sediments and UASB samples.

Probe Name	P. Pond 1	P. Pond 2	P. Pond 3	UASB sludge	UASB effluent
Amx820 (Anammox)	ND	ND	ND	ND	ND
Nso190 (Ammonia oxidizing-bacteria)	ND	ND	ND	ND	ND
Nso1225 (Ammonia oxidizing-bacteria)	ND	ND	ND	ND	ND

Remark: ND- non-detectable

CONCLUSIONS

The FISH analysis did not reveal the presence of nitrifying and Anammox bacteria in this wastewater system, or they could be present but below FISH detection limit. Thus, the ammonia removal observed in the system might be associated with NH_3 stripping, associated with the pH

increase resulting from the intensive photosynthetic activity in the ponds (mechanism under investigation).

In spite of FISH results, 16S rDNA genes of anammox organisms were amplified from polishing ponds sediments suggesting that anammox organisms were present and may be more common (and widespread) than previously thought, and also that anammox reaction could be a widely occurring phenomenon in wastewater treatment plants. In principle, nitrogen losses which could be only explained by the anammox process were not verified in this wastewater system, or they were too low to be noticed. While the presence of the anammox 16S rRNA genes does not equate with *in situ* anammox activity, these genes do serve to identify samples for more intensive study and serve as molecular markers for better tracking of candidate populations for anaerobic ammonium oxidation.

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