Anammox bacteria enrichment and characterization from municipal activated sludge

J. C. Araujo, A. C. Campos, M. M. Correa, E. C. Silva, M. H. Matté, G. R. Matté, M. Von Sperling and C. A. L. Chernicharo

ABSTRACT

A sustainable option for nitrogen removal is the anaerobic ammonium-oxidizing (anammox) process in which ammonium is oxidized to nitrogen gas with nitrite as electron acceptor. Application of this process, however, is limited by the availability of anammox biomass. In this study, two *Brocadia*-like anammox phylotypes were successfully enriched, detected and identified from an activated sludge taken from a domestic wastewater treatment plant (Minas Gerais, Brazil) employing a Sequencing Batch Reactor (SBR). The dominant phylotype was closely related to '*Candidatus* Brocadia sinica', but one clone seemed to represent a novel species for which we propose the name '*Candidatus* Brocadia brasiliensis'. Based on Fluorescence *in situ* hybridization (FISH) analysis, this enrichment led to a relative population size of 52.7% (±15.6) anammox bacteria after 6 months of cultivation. The cultivation process can be divided into three phases: phase 1 (approximately 25 days) was characterized by heterotrophic denitrification metabolism, phase 2 was the propagation phase and phase 3 (from the 87th day onwards), in which significant anammox activity was detected. A long-term performance of the SBR showed a near perfect removal of nitrite based on the influent NO_2^--N concentration of 61–95 mg L^{-1}. The average ammonia removal efficiency was 90% with the influent NH_4^+-N concentration of 55–82 mg L^{-1}. Therefore, anammox cultivation and enrichment from activated sludge was possible under a controlled environment within 3 months.

Key words | anammox, '*Candidatus* Brocadia', cultivation and enrichment, FISH, sequencing batch reactor

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INTRODUCTION

Nitrogen is one of the major nutrients that wastewater treatments attempt to eliminate in order to avoid water pollution. Biological nitrogen removal is traditionally achieved by autotrophic nitrification and heterotrophic denitrification processes (Egli *et al.* 2001). A very promising new and more sustainable process for nitrogen removal is the anaerobic ammonium oxidation (anammox) process (Strous *et al.* 1997). The application of anammox to nitrogen removal would lead to a significant reduction of costs for aeration and exogenous electron donor as compared to the conventional nitrification–denitrification process (van Dongen *et al.* 2001).

The anammox process is the biological conversion of ammonium and nitrite to dinitrogen gas (Van de Graaf *et al.* 1996) and is performed by slow-growing deep-branching Planctomycetes (Strous *et al.* 1999). Anammox bacteria are autotrophic and have a notoriously low growth rate with minimum doubling times of several days (Strous *et al.* 1998; Tsushima *et al.* 2007). The anammox group has been split into five *Candidatus* genera: 'Brocadia', 'Kuenenia', 'Scalindua', 'Anammoxoglobus' and 'Jettenia'. Phylotypes of these genera have been identified around the world, in diverse environments, such as wastewater treatment plant sludges (Egli *et al.* 2001; Toh *et al.* 2002; Dapena-Mora *et al.* 2004), marine sediments (Tal *et al.* 2005), a few freshwater environments (Jetten *et al.* 2003), and a brackish environment (Sànchez-Melsió *et al.* 2009).

Application of the anammox process is limited by the availability of anammox biomass and the difficulty associated with growing large quantities of anammox bacteria. The isolation and enrichment of anammox biomass from a mixture

doi: 10.2166/wst.2011.632

of bacterial populations requires the optimization of conditions favoring the anammox process, while limiting the growth of any other kind of microbial population. The objectives of this study were therefore (i) to enrich and produce anammox biomass from activated sludge in a Sequencing Batch Reactor (SBR) and to gain a better understanding of the anammox enrichment process, (ii) to characterize and identify the anammox biomass enriched. The anammox biomass obtained can be used further for starting-up an anammox bioreactor. Two techniques were used to detect and confirm the successful anammox enrichment: Fluorescent in situ hybridization (FISH) and Polymerase Chain Reaction (PCR) with primers specific to anammox organisms coupled to cloning and sequencing the amplified fragments.

MATERIALS AND METHODS

Inoculum and mineral medium

The enrichment culture was started with 0.65 L of activated sludge taken from the sludge return line (from the secondary sedimentation tank) of a full-scale conventional activated sludge system treating domestic wastewater of Belo Horizonte (Minas Gerais, Brazil). The sludge was centrifuged previously and 2.6 g of TVS (total volatile solids) was inoculated in 1 L of mineral medium. The composition of autotrophic mineral medium used for the enrichment was based on previous studies (Van de Graaf *et al.* 1996; Dapena-Mora *et al.* 2004). In the beginning of the experiment, ammonium and nitrite final concentration was 30 mg L^{-1}. During the third phase of the experiment the concentrations of these nitrogen compounds were further changed as specified in the results section.

Enrichment procedure and sampling

A glass 1.3 L (BioFlo110, New Brunswick, NJ, USA) sequencing batch reactor (SBR) was used for enrichment of anammox. The reactor was fitted with a fermentor lid containing a feed inflow tube, a dissolved oxygen (DO) probe, pH probe, acid and base inflow tubes for pH control, a gas line, a sampling line, and an effluent withdrawn line. Temperature was controlled and maintained constant at 34–35 °C via a heat blanket, and the pH was controlled to 7.5. Anaerobiosis was maintained by bubbling an Ar/CO_2 (95/5%) gas mixture through the liquid. This gas mixture was also flushed in the mineral medium (feed vessel) in order to maintain anaerobic conditions in this synthetic wastewater.

The SBR was operated as a continuously fed SBR with two cycles, one of 7 h and the other of 17 h. Each cycle had three phases: in the first phase (6 h), the reactor was fed continuously (resulting in the addition of 0.5 L feed over 6 h). In the second phase (0.5 h), the stirrer and the influent supply were stopped and the biomass was allowed to settle. Finally, in the third phase (0.5 h), the supernatant (0.5 L of the liquid) was pumped out of the reactor. The minimum volume in the reactor was 0.5 L, and at the end of the cycle the final volume was 1 L. In the longest cycle (which lasted 17 h), after the filling period (6 h), biomass was allowed to react with the media for 10 h. The resulting hydraulic retention time (HRT) was 24 h. Samplings were performed every 2 days for monitoring the treatment conditions and effluent quality. Ammonium and nitrite were measured colorimetrically by the phenol-hypochlorite method (measured at 660 nm) and sulfanilic acid (measured at 520 nm), respectively, according to the Standard Methods (APHA 1998).

Fluorescence *in situ* hybridization

FISH was used to investigate abundance and presence of anammox bacteria in the enriched sludge using the general anammox probe Amx820 and the Amx1240 probe specific for '*Candidatus* Brocadia anammoxidans' (Egli *et al.* 2001). Hybridizations were performed with 40% formamide (in the hybridization buffer) and 56 mmol/L NaCl (in the washing buffer) at 46 °C for 90 min according to Egli *et al.* (2003). Probes Nso1225 for ammonia-oxidizing *β-Proteobacteria*, Nit3 for *Nitrobacter* and Ntspa662 for *Nitrospira* were also used (with 35% formamide according to Egli *et al.* 2003) in order to investigate the presence of aerobic ammonium- and nitrite-oxiding bacteria, respectively, in the enriched biomass. Counterstaining was done by using 4,6-diamidino-2-phenylindole (DAPI). FISH images were collected and recorded with an acquisition system (camera: QcolorR5C and software: Q Capture and Image–Pro Express 6.0) coupled to an Olympus BX-50 epifluorescence microscope.

DNA extraction, PCR, cloning, sequencing and phylogenetic analyses

DNA was extracted from 10 to 20 mL of the enriched biomass from SBR (after 184 days of cultivation) according to Egli *et al.* (2003). 16SrDNA fragments from isolated DNA were amplified with planctomycete-specific primer, Pla46F and anammox primer Amx820R, and Pla46F-1392R in the same conditions as described by Egli *et al.* (2001). PCR products were cloned in *Escherichia coli* JM 109 in vector

pGEM-T-easy (Promega, Madison, WI, USA) according to the manufacture's instructions. Sequencing of the clones was carried out by the Genomic Service (Genomic Molecular Engineering Ltd, São Paulo, Brazil, www.genomic.com.br). Analyses of 862 positions of the 16S rRNA gene sequences of primers Pla46F-Amx820R and 1300 bp related to primers Pla46F-1392R of the clones were compared with their closest relatives in the GenBank database by BLASTN searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al*. 1990). Further phylogenetic and molecular evolutionary analyses were performed with the MEGA 4.1 program (Tamura *et al*. 2007). The dendrogram was constructed by the neighbor-joining method (Saitou & Nei 1987). The evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al*. 2004) and are in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). Bootstrap resampling analysis for 1000 replicates was performed to estimate the confidence of the tree topologies. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of the 30 clone sequences obtained by enrichment and used for construction of the Evolutionary distance dendrogram are GQ891677-GQ891680 and GQ896513-GQ896538.

RESULTS AND DISCUSSION

Performance of the SBR used for anammox enrichment from activated sludge

The concentrations of nitrogenous compounds in the influent and effluent of the SBR as well as the nitrogen removal efficiency during the enrichment period are shown in Figure 1. As ammonium and nitrite were consumed, their concentrations in the feed were increased stepwise. Based on the obtained results, and for a better understanding of the enrichment process, the ammonium and nitrite concentration profiles were divided into three phases. Phase 1 (approximately 25 days) corresponds to the beginning of the operation period. In this phase, denitrifying activity was the favored process (anoxic environment and presence of nitrite), eliminating the organic matter present in the medium from the lysis of any aerobic bacteria. This view was supported by an initial consumption of nitrite together with an increase in the effluent ammonia concentrations compared to the influent concentrations (Figure 1). During this time, the change in environment of seed sludge might have caused the turnover of bacteria. Thus the former dormant bacteria might have been killed, causing cell lysis and breakdown of organic nitrogen to ammonia. As a result, ammonium concentrations increased. After lysis of the aerobic bacteria, lysis of the denitrifying bacteria began from lack of organic substrates. At that time (on day 25) denitrifying activity ceased and consumption of nitrite was no longer observed to about day 87th (Figure 1). The reduction of indigenous organisms which are mostly non-anammox culture and the favorable environment for the growth of anammox culture led to the selection of microbial population in the system favoring anammox bacteria. This initial phase has also been described in enrichment experiments carried out by Toh *et al*. (2002) and Dapena-Mora *et al*. (2004).

Phase 2 (from day 25th to about day 87th) corresponds to the propagation phase. In this phase the effluent nitrite concentrations were close to the influent concentrations. This suggests that the exhaustion of organic substrate from

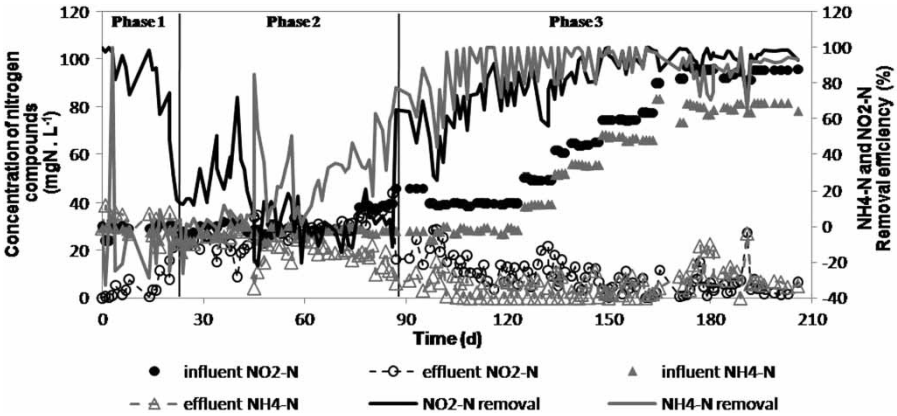

Figure 1 | Concentration of nitrogen compounds in the influent and effluent [influent NO_2^--N (filled circles), effluent NO_2^--N (empty circles), influent NH_4^+-N (filled triangles), effluent NH_4^+-N (empty triangles)], and nitrogen removal efficiency (gray solid line represents NH_4^+-N removal efficiency; black solid line, NO_2^--N removal efficiency) during 210 days of operation for the SBR.

cell lysis occurred, resulting in the increase of anammox population in favoring of the provided substrate. Anammox reaction was not observed during phase 2 (lasting approximately 65 days), but only at the end of this phase. The effluent ammonium concentrations decreased from day 62 on. Ammonium removal efficiency during this time (between 62 to 86 day) was in the range of 10% to 55% (Figure 1). A longer propagation phase clearly corresponded to a slow growing of anammox bacteria.

Phase 3 corresponds to the last phase of cultivation (at day 87 on). In this phase a decrease in the nitrogen forms, i.e. the nitrite and ammonium concentrations in the effluent were observed (Figure 1), indicating that anammox activity had occurred in the reactor. Based on the provided conditions, approximately from 70% to 95% ammonium and nitrite removal efficiencies were achieved in this phase (Figure 1). Previous studies indicated ammonium removal efficiencies of 84%, 88%, and 80% for the fluidized bed reactor (Strous *et al*. 1997), fixed bed (Strous *et al*. 1997) and SBR (Chamchoi & Nitisoravut 2007) fed on synthetic wastewater, respectively. The deviation of the system performances might be dependent on a different concentration of ammonium contained in the influent and the characteristic uniqueness of each presumably cultivated anammox culture, which also provided different ammonium consumption in each reactor.

In the present enrichment, a significant removal of ammonium and nitrite was initially observed after 3 months of operation and a near complete removal of nitrite was obtained within 4.5 months. In comparison to other studies, the time required for enrichment was relatively short. Van de Graaf *et al*. (1996) obtained anammox biomass after approximately 7 months of cultivation using an attached growth system. Egli *et al*. (2001) required 6 months to enrich an anammox culture of 88% purity from a rotating biological contactor, which had also shown significant anammox activity before the start of the enrichment period. Toh *et al*. (2002) reported biofilm growth after 1 year in a fixed-bed reactor that demonstrated anammox activity. Chamchoi & Nitisoravut (2007) detected anammox activity from different conventional sludges after 4 months of enrichment in a SBR.

FISH analysis of enriched biomass in SBR

Positive hybridization of the biomass with specific Amx820 and Amx1240 probes, confirmed the presence of anammox bacteria in SBR after 184 days of cultivation (Figure 2). By counting FISH-stained cells with an Amx1240 probe and DAPI-stained cells, it was estimated that the abundance of anammox bacteria in the SBR was 8.9 ($\pm$2.9) $\times 10^6$ ($\pm$SD) cell mL^{-1}, accounting for 52.7% ($\pm$15.6) of total DAPI-stained cells. FISH-stained cells were approximately 1.2 μm diameter doughnut-shaped cells (Egli *et al*. 2001) and typically had an inner area with very low fluorescence intensity (Figure 2(a) and 2(b), respectively). This central area, presumably a protein-rich compartment, might be similar to the 'anammoxosome' described for 'Ca. B. anammoxidans'. No positively stained cells were observed with the probes Nso1225 for ammonia-oxidizing *β-Proteobacteria*, Nit3 for *Nitrobacter* and Ntspa662 for *Nitrospira*. Although some authors reported the coexistence of ammonia- and nitrite-oxidizing bacteria and anammox in a SBR treating landfill leachate (Xiao *et al*. 2009) and in an anammox reactor (Park *et al*. 2010), FISH results obtained in the present work strongly suggest that no classical ammonium- and nitrite-oxidizing bacteria were present at significant population sizes in the enriched biomass.

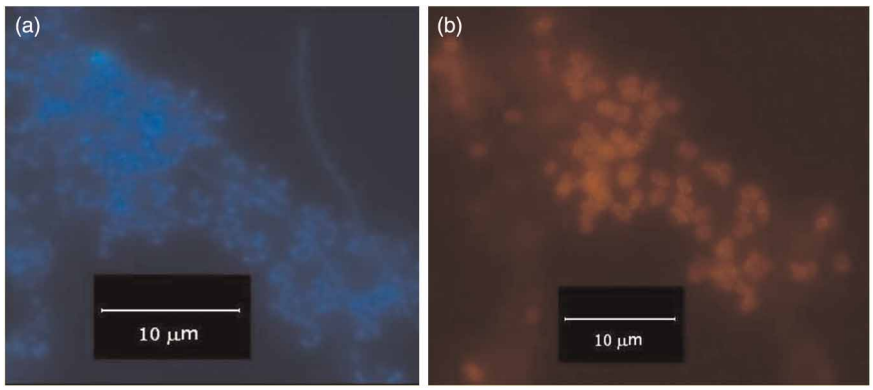

Figure 2 | FISH microphotographies of anammox enriched biomass. Cells were stained by DAPI in (a) and hybridized with Cy3-labelled probe Amx1240 for detection of *Ca*. 'Brocadia anammoxidans' cells in (b).

Phylogenetic analysis of enriched biomass

Two 16S rRNA gene clone libraries were constructed from the enriched biomass taken from the SBR after 184 day of cultivation, using primers Pla46F-Amx820R and Pla46F-1392R. Thirty clones were randomly selected and sequenced. Twenty six clones (JCA4-GQ891680; and from JCA7-GQ896514 to JCA30-GQ896538) were grouped into the same phylotype on the basis of more than 99% sequence similarity. These clones were closely related to the novel anammox species provisionally classified as '*Candidatus* Brocadia sinica' (GQ 175277) (Hu *et al*. 2010) belonging to the order *Planctomycetales* with more than 99% sequence similarity (Figure 3). One out of 30 clones (JCA11) could be assigned to a second group of anammox bacteria, suggesting that there could be microniches present in the anammox biomass that might possibly support the growth of a secondary anammox population, albeit at very low amounts. This secondary population, represented by JCA11 sequence, although related to '*Ca.* Brocadia fulgida' (with 97% sequence similarity) and more closely related to '*Candidatus* Brocadia sp. 40', seemed to constitute a new species on the '*Candidatus* Brocadia' genus based on sequence similarities commonly used to define different species (Rosello-Mora & Amann 2001) (Figure 4). We propose to provisionally classify the novel anammox species '*Candidatus* Brocadia brasiliensis' according to the taxonomic guidelines. Clones, JCA1, JCA2, and JCA3, although related to *Planctomycetales* group, were placed separately (not shown).

'*Candidatus* Brocadia' and '*Candidatus* Kuenenia' species are the most commonly found organisms in the enrichments from wastewater-treatment plants and large-scale anammox reactors (Kuenen 2008). Apparently the anammox diversity of the wastewater treatment plant sludges is large, and which organism is enriched might be determined not by inoculation but by (largely unknown) niche differentiation or other aspects, such as composition of the feed and extant substrate concentration (as observed by Park *et al*. 2010). In the present study, two 'Brocadia'-like anammox phylotypes were successfully enriched and identified from activated sludge employing a SBR, indicating that this sludge might contain several different anammox species and the cultivation conditions applied likely selected for those two.

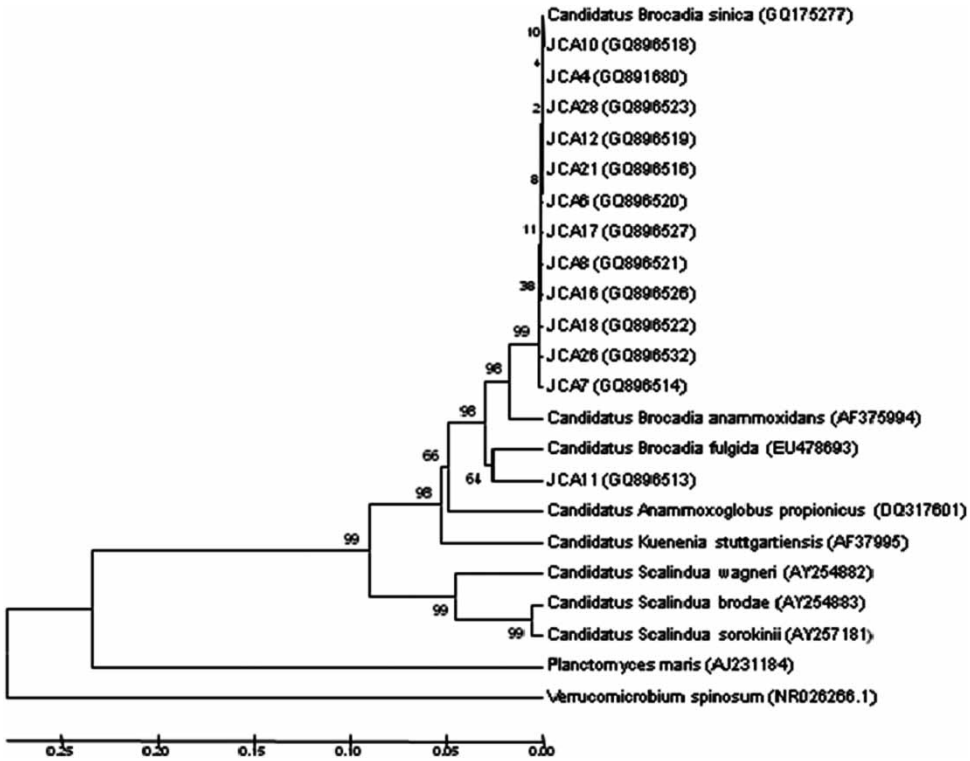

Figure 3 | Evolutionary distance dendrogram showing the affiliation of the 862 bp fragment of the 16S rRNA sequences recovered from SBR (after 184-day operation) to representative members of the divisions in the bacterial domain. Numbers at the nodes indicate the percentage of recovery of relevant branch points in 1,000 bootstrap resampling analysis. EMBL, GenBank accession numbers are indicated. The scale bar represents the estimated difference in nucleotide sequence positions.

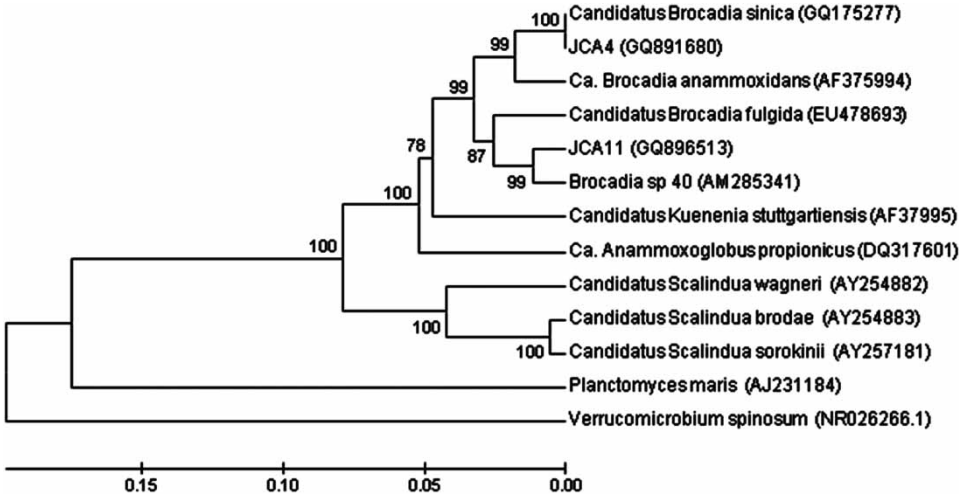

Figure 4 | Phylogenetic tree of anammox bacteria showing the positions of the clones JCA 4 and JCA11, obtained from the SBR after 184-day operation. The tree was generated by using 862 bp fragment of the 16S rRNA gene. Numbers at the nodes indicate the percentage of recovery of relevant branch points in 1,000 bootstrap resampling analyses. EMBL, GenBank accession numbers are indicated. The scale bar represents the estimated difference in nucleotide sequence positions.

CONCLUSIONS

This study has shown that an active anammox culture could be enriched from a local source of activated sludge within 3 months in a SBR. Significant anammox activity was found in the last phase of cultivation. Based on the provided conditions, approximately from 70% to 95% ammonium and nitrite removal efficiencies were achieved. Anammox-related cells labelled with Amx1240 probe accounted for 52.7% of the total bacterial population (after 6 months of enrichment). Two different anammox phylotypes could be enriched simultaneously from the same seeding source. The dominant anammox phylotype was related to a novel anammox species enriched from nitrogen removal reactors from China (provisionally named '*Candidatus* Brocadia sinica') and the secondary (minor) population seemed to constitute a new anammox species tentatively named '*Candidatus* Brocadia brasiliensis' enriched from activated sludge sample. This report demonstrates that anammox bacteria exist not only in Europe, Australia, Japan, USA and China, but could also be enriched from wastewater plants elsewhere in the world, in this case from Belo Horizonte, Minas Gerais, Brazil.

ACKNOWLEDGEMENTS

This work was supported by FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais), grant TEC 946/06; CNPq/Brazil (Conselho Nacional de Desenvolvimento Científico e Tecnológico) grant N. 471830/2006-2; FINEP (Financiadora de Estudos e Projetos), and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), grant AEX 3527/10-7.

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First received 9 February 2011; accepted in revised form 29 March 2011