

Vanotti et al. (p. 99-106)

First International Anammox Symposium IANAS 2011

May 19-21, Kumamoto Univ., Japan



Experiences with Anammox in the USA: Isolation, Preservation and Treatment Performance of *Brocadia caroliniensis*

Matias Vanotti^{1*}, Takao Fujii², Ariel Szögi¹, Michael Rothrock¹, Maria Cruz Garcia³, Airton Kunz⁴,
Albert Magri⁵, Kenji Furukawa⁶

¹ USDA-ARS, United States Department of Agriculture - Agricultural Research Service, Coastal Plains Soil, Water, and Plant Research Center, 2611 W. Lucas St., Florence, South Carolina 29501, USA;

² Sojo University, Department of Applied Life Sciences, Kumamoto, Japan;

³ ITACyL, Agriculture Technological Institute of Castilla and Leon, Valladolid, Spain;

⁴ EMBRAPA, National Research Center on Swine and Poultry, Concordia, Santa Catarina, Brazil;

⁵ GIRO Technological Centre, Mollet del Vallès, Barcelona, Spain;

⁶ Kumamoto University, Graduate School of Science and Technology, Kumamoto, Japan

Abstract

A novel anammox bacteria *Candidatus Brocadia caroliniensis*, having Accession Deposit Number NRRL B-50286 and the characteristics of oxidizing ammonia and releasing di-nitrogen under anaerobic conditions, has been discovered. It was isolated from livestock manure sludge at the USDA-ARS laboratory in Florence, South Carolina. Compared to conventional biological nitrogen removal methods, the anammox process can save more than 50% of the oxygen supply. This leads to a significant decrease in operational costs. We describe development work done to isolate, enrich, characterize and preserve the novel anammox bacteria *Candidatus Brocadia caroliniensis*. It can be used for effective treatment of wastewater having undesirable levels of ammonia, including agricultural, industrial, or municipal wastewaters. It is capable of long-term storage and reactivation after lyophilization; this protocol was used for its successful deposit with an internationally depositary authority under the provisions of the Budapest Treaty of the UN.

Key words: Anammox; *Brocadia caroliniensis*; long-term preservation; nitrogen removal; animal manure.

INTRODUCTION

The anaerobic ammonium oxidation (anammox) is a biologically mediated reaction in which ammonia is oxidized to nitrogen gas using nitrite as the electron acceptor under anaerobic conditions (Strous et al., 1998) (Eq. 1).



Compared to conventional nitrification/denitrification, a partial nitrification/anammox mode to eliminate the ammonia from wastewater reduces 58% of the oxygen requirement, 100% of the carbon requirement, and 83% of the biosolids production (Daigger et al., 2011). This leads to a significant decrease in operational costs. In addition, by-products do not include greenhouse gases. Therefore, anammox is a key technology for development of more economical and energy efficient ammonia treatment systems in the future.

In this paper we summarize development work done to isolate, enrich, characterize, and preserve a novel anammox bacteria *Candidatus Brocadia caroliniensis* (Vanotti et al., 2011) that oxidizes ammonia and releases di-nitrogen under anaerobic conditions.

MATERIALS AND METHODS

Isolation and enrichment of anammox bacteria

Anammox cultures were successfully established in three consecutive experiments with bioreactors using continuous-flow unit processes and biomass carriers seeded with sludges of manure origin from swine farms located in North Carolina, USA. Laboratory bioreactors were seeded with the manure sludge after acclimation with potassium nitrate solution (100 mg $\text{NO}_3\text{-N/L}$) to remove endogenous carbon through biological denitrification using glass vessels. The solution was renovated weekly until nitrate analyses revealed denitrification inhibition due to lack of carbon in the sludge. In a first phase of research, two sequential trials were conducted over a 5-year period to investigate conditions to isolate anammox bacteria from the swine sludges (3 years) and to optimize the anammox treatment in bioreactors (2 years). The bioreactors were operated in continuous flow and contained polyvinyl alcohol (PVA) hydrogel biomass carrier beads for immobilization and enrichment of the slow growth microorganisms (Fig. 1A). The PVA beads used in the reactor averaged 4.0 mm in diameter, a specific gravity of 1.025, and hydrous ratio (void %) of 90% (Kuraray Co., Osaka, Japan). The reactors had a volume of 1.0 L (6.5-cm-diameter glass cylinder). The reactors received a continuous flow of synthetic enrichment medium containing equal amounts of $\text{NH}_4\text{-N}$ and $\text{NO}_2\text{-N}$. It contained NH_4Cl (variable: 20-300 mg N L^{-1}); NaNO_2 , (variable: 20-300 mg N L^{-1}); KHCO_3 , 125 mg L^{-1} ; KH_2PO_4 , 27 mg L^{-1} ; $\text{FeSO}_4 \times 7\text{H}_2\text{O}$, 9.0 mg L^{-1} ; EDTA, 5.0 mg L^{-1} ; $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 240 mg L^{-1} ; $\text{CaCl}_2 \times 2\text{H}_2\text{O}$, 143 mg L^{-1} ; and trace element solution, 0.3 mL L^{-1} . The trace element solution contained $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$, 1247 mg L^{-1} ; $\text{MnSO}_4 \times \text{H}_2\text{O}$, 1119 mg L^{-1} ; $\text{CuSO}_4 \times 5\text{H}_2\text{O}$, 44 mg L^{-1} ; $\text{Al}_2(\text{SO}_4)_3 \times 14\text{H}_2\text{O}$, 201.5 mg L^{-1} ; $\text{Na}_2\text{MoO}_4 \times 2\text{H}_2\text{O}$, 129 mg L^{-1} ; $\text{CoCl}_2 \times 6\text{H}_2\text{O}$, 30 mg L^{-1} ; KCl , 100 mg L^{-1} ; EDTA, 975 mg L^{-1} . In experiment 1, the bioreactor was operated at constant hydraulic retention time (HRT) of 28 h and a variable influent total N concentration (20 – 250 mg N/L). In experiment 2, the HRT was variable (24 to 12 h), and influent concentration was constant (300 mg N/L). The process was optimized when the alkalinity in the influent was adjusted 1:1 with the $\text{NH}_4\text{-N}$ concentration in the influent; KHCO_3 added varied with the amount of ammonia in the influent at a rate 2 mg KHCO_3 per 1 mg of $\text{NH}_4\text{-N}$ (Vanotti et al., 2011). High amounts of Mg and Ca salts in the synthetic media favored isolation of anammox from manure sludge. The anammox sludge produced was used to inoculate the pilot reactor.

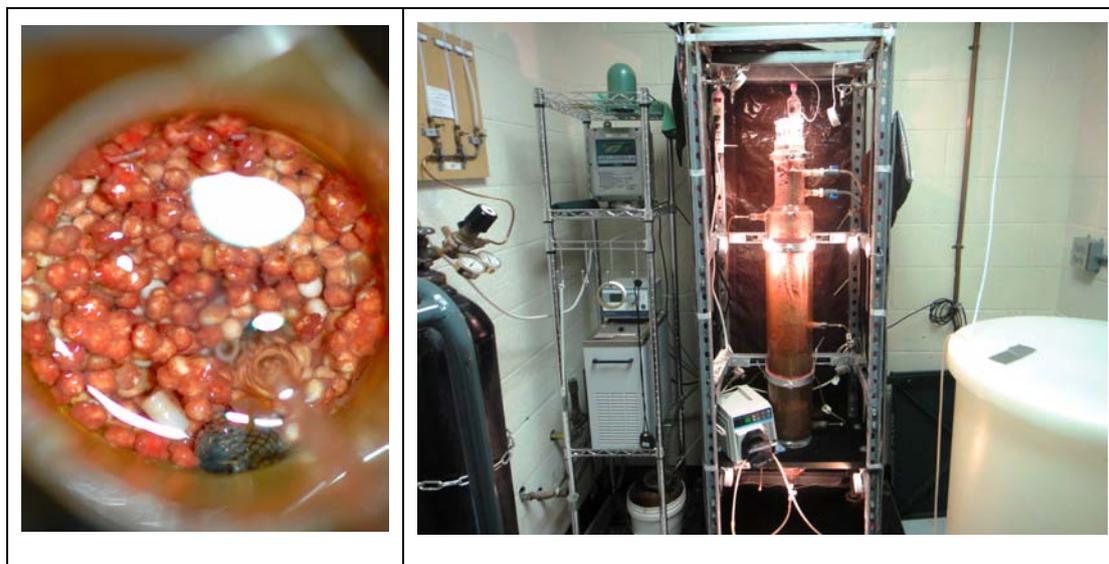


Fig. 1 (A) PVA beads with attached anammox biofilm; (B) Pilot up-flow anammox reactor using non-woven carrier.

Pilot reactor: In a second phase, a 10-L pilot reactor (9.8 L effective liquid volume, 1.20 m height) was seeded directly with the anammox red sludge biomass. A total of 111.8 g of wet anammox sludge (2.93 g total solids dry basis) was used. The reactor was configured so that influent synthetic wastewater was injected at the bottom-end of the reactor and treated water was discharged near the top of the reactor after passing through a matrix of immobilized anammox (Fig. 1B). The glass pilot reactor was jacketed on the outside to control process temperature. Process temperature was maintained at 30°C with temperature probe inside the reactor and a water heater, controller and circulator. As a biomass carrier, the reactor used a polyester non-woven material coated with pyridinium type polymer (Japan Vilene, Tokyo, Japan) designed to enhance retention of microorganisms (Furukawa et al., 2003). The reactor was kept in dark conditions. It was operated over a 3.8-yr period with various hydraulic retention times (HRT 24 to 4 h) and influent total N concentration (40 to 560 mg N/L; 50% as ammonia-N and 50% as nitrite-N). Water analyses of influent and effluent samples were performed according to Standard Methods for the Examination of Water and Wastewater.

Specific activity of anammox sludge

The specific activity of *Candidatus Brocadia caroliniensis* was determined using a 3-liter anaerobic jacketed glass reactor (Chemglass Life Sciences, Vineland, NJ, USA), which was operated in batch mode. The 3-liter reactor used 600 mL of the red anammox biomass which was developed and collected from inside the 10-L pilot reactor described *supra*. The anammox sludge had a granular structure with granules measuring 2-4 mm. After harvesting of the sludge, it was transferred immediately into the 3-L reactor and mixed with wastewater. The mixed liquor was bubbled for 5 minutes with N₂ gas (1000 ml/min) to reduce dissolved oxygen concentration to < 1 ppm. The reactor was then sealed and tested under anaerobic conditions. The concentration of suspended biomass inside the 3-liter reactor was 2,645 mg volatile suspended solids (VSS) per liter and 3,254 mg total suspended solids (TSS) per liter. Initial concentrations of ammonia and nitrite in the synthetic wastewater at the beginning of the batch test were 58 and 72 mg N/L, respectively. During the test, the reactor was mixed using a stirrer (Opti-Chem) operated at 55 rpm. The process temperature was 32.5°C. The specific activity test of the sludge, conducted in duplicate, lasted 1.5 hours. Liquid samples were extracted through a sampling port every 20 minutes to determine the changes in NH₄-N, NO₂-N, and NO₃-N concentration as a function of time. The slope of the regression lines relating N concentration and time indicated the rates of N transformations in the reactor in mg N /L-reactor/hour. The specific activity of the anammox sludge, expressed as mg N removal/mg VSS (biomass)/day, was calculated from the rates of N removal obtained (mg of N removal/L-reactor/hour) and the concentration of anammox biomass measured in the reactor (mg VSS/L-reactor).

Anammox bacteria characterization

Sludge samples from the pilot reactor were subject to chromosomal DNA extraction, PCR amplification of chromosomal DNA, cloning of amplified DNA, grouping of cloned PCR products by restriction fragment length polymorphism (RFLP), and sequencing of cloned PCR products, all according to Fujii et al. (2003).

Long-term preservation of anammox

Deposit of useful microorganisms in culture collections requires long-term preservation and successful reactivation techniques. The goal of this study was to develop a simple preservation protocol for the long-term storage and reactivation of the anammox biomass. To achieve this, anammox biomass sampled from the pilot reactor was frozen or lyophilized at two different freezing temperatures (-60°C and in liquid nitrogen (-200°C)) in skim milk media (with and without glycerol), and the reactivation of anammox activity was monitored after a 4-month storage period. Two control treatments were used: 1) freshly harvested biomass, and 2) anammox preserved in KNO₃ media (80 mg N/L) refrigerated at 4°C, with media replaced monthly. Detailed procedures for long-term preservation are provided by Rothrock et al. (2011).

RESULTS AND DISCUSSION

Isolation and treatment performance of the bio-reactors

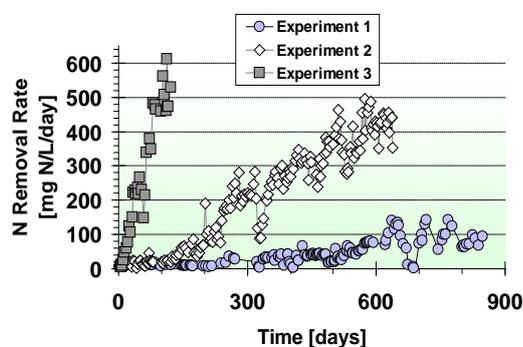


Fig. 2 compares anammox activity using manure sludge seed during isolation (start at 490 days in exp. 1 and 100 days in exp. 2), and quick start-up of pilot reactor using the isolated anammox sludge as a seed (exp. 3).

In experiment 1, the anammox reaction (with simultaneous removal of NO₂⁻ and NH₄⁺) was first noticeable at day 490 after inoculation of the reactor with manure sludge (Fig. 2). At the same time, a distinct red biomass growth, typical of the anammox planctomycetes bacteria, colonized the PVA hydrogel biomass carrier beads (Fig. 1A). These developments occurred only when high N (>200 mg/L) and adequate salt strength were used in the synthetic media. Under the conditions and protocol determined in experiment 1, it took only about 100 days in experiment 2 to develop the anammox reaction after inoculation with manure sludge (Fig. 2). Nitrogen removal rates obtained during the first-phase development were 0.4 to 0.5 kg N/m³/day. Removal of NO₂⁻ and NH₄⁺ was simultaneous at the stoichiometry: NH₄⁺ + 1.24 NO₂⁻ → 1.00 N₂ + 0.24 NO₃⁻.

Pilot reactor. The pilot reactor was seeded with the anammox-enriched sludge generated in the previous experiments. Removal of NO₂⁻ and NH₄⁺ at stoichiometric ratios of the anammox reaction occurred from day 1 of operation (Fig. 2). Nitrogen removal rate increased linearly from 0 to 600 mg N/L-reactor/day in the first 4 months of operation. After stabilization, total N removal rates up to about 1830 mg N/L-reactor/day (1.8 kg N/m³/day) were obtained. These rates were higher, about 3.6 kg/m³/day, in the bottom 10% (1 L) of the reactor where a concentrated, granular anammox developed without attachment media. Removal of NO₂⁻ and NH₄⁺ was simultaneous at the stoichiometric ratios summarized as NH₄⁺ + 1.30 NO₂⁻ → 1.06 N₂ + 0.18 NO₃⁻. These ratios

were obtained during a 3.8-year operation of the pilot reactor, from the slopes of the linear relationships shown in Fig. 3 indicating total N removal, NO₂-N removal, and NO₃-N production rates per unit of NH₄-N removal (Vanotti et al., 2011).

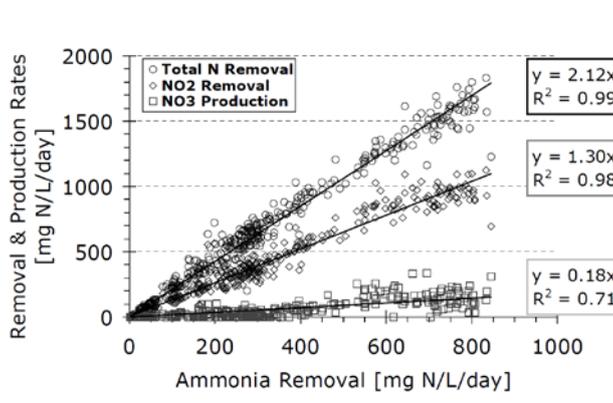


Fig. 3 is a graph depicting the stoichiometric ratio of the anammox reaction in the pilot reactor of anammox bacteria *Candidatus Brocadia caroliniensis* using various HRTs and influent total N concentrations.

Table 1 Removal of wastewater nitrogen using *Brocadia caroliniensis* (NRRL B-50286) in upflow continuous 10-L pilot reactor

	Mass Nitrogen			Removal Efficiency
	Influent	Effluent	Removal	
	-----mg N L ⁻¹ d ⁻¹ -----			%
Total N	1,735 ± 137	260 ± 122	1,477 ± 168	85 ± 7
Ammonia	804 ± 80	92 ± 71	709 ± 88	89 ± 9
Nitrite	930 ± 80	22 ± 47	915 ± 90	98 ± 5
Ammonia + Nitrite	1,735 ± 138	114 ± 101	1,621 ± 155	94 ± 6

Note: The reactor was operated continuously at hydraulic retention time of 3.9 hours. Influent wastewater containing 283.4 ± 22.4 mg-N L⁻¹ (131.4 ± 13.0 mg NH₄-N L⁻¹, 152.0 ± 13.0 mg NO₂-N L⁻¹, and 0.1 ± 0.6 mg NO₃-N L⁻¹). Data are average ± s.d. of 61 samples collected over a 350-day continuous period. Total N = NH₄-N + NO₂-N + NO₃-N.

Data in Table 1 summarize stabilized performance obtained with *Brocadia caroliniensis* in the pilot reactor during 350 days (Vanotti et al., 2011). The HRT was 3.9 hours. The average total N loading rate was 1.74 kg N/m³/day and the total N removal rate was 1.48 kg N/m³/day. The average removal efficiency obtained was 85%. Similarly, the ammonia and nitrite were removed at efficiencies of 89 and 98%, respectively (Table 1).

DNA analysis

Analysis of the RFLP fingerprint patterns revealed that all but one recovered clone exhibited the same fingerprint pattern for all three restriction endonucleases, indicating that the anammox sludge was dominated by a single bacterium. Due to the relatively low DNA sequence homology (≤96%) (Table 2) and phylogenetic relationships (Fig. 4A), it was proposed that the dominant bacteria in the Florence, SC, anammox bioreactor is a novel species of the *Candidatus* “*Brocadia*” genera. It was named *Candidatus* “*Brocadia caroliniensis*.” A strain of *Candidatus* *Brocadia*

caroliniensis was deposited on May 12, 2009, under the provisions of the Budapest Treaty (WIPO, 1980) in the Agricultural Research Culture Collection (NRRL) in Peoria, Ill., and has been assigned Accession No. NRRL B-50286.

Table 2. 16S rDNA sequence homology between *Candidatus* “Brocadia caroliniensis” (NRRL B-50286, GenBank JF487828) and anammox sequences from the GenBank Database.

Matching Sequence	GenBank Accession Number	Percent Similarity to <i>Candidatus</i> “Brocadia caroliniensis” JF487828
Uncultured bacterium clone Asahi BRW	AB456583	96%
<i>Candidatus</i> “Brocadia” sp.	AM285341	96%
<i>Candidatus</i> “Brocadia fulgida”	EU478693	96%
<i>Candidatus</i> “Brocadia anammoxidans”	AF375994	94%
<i>Candidatus</i> “Jettenia asiatica”	DQ301513	92%
<i>Candidatus</i> “Kuenia stuttgartiensis”	AF375995	90%
<i>Candidatus</i> “Anammoxoglobus propionicus”	EU478694	90%
<i>Candidatus</i> “Scalindua sorokinii”	AY257181	86%

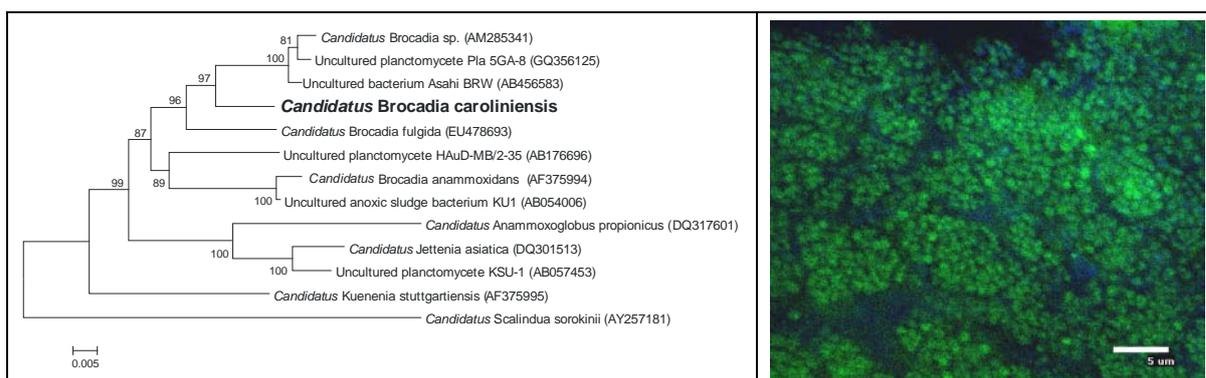


Fig. 4 (A) is a bootstrapped (n=1000) Neighbor Joining phylogenetic tree of *Candidatus* Brocadia caroliniensis in relation to major genera of anammox bacteria found in the GenBank Database; (B) is a confocal microscopy image of Brocadia caroliniensis using 16S rDNA oligonucleotides probe FLO-1

Fluorescence In-situ Hybridization (FISH) analysis was performed using a 16S rDNA oligonucleotides probe FLO-1 developed specifically for *Candidatus* Brocadia caroliniensis (Rothrock et al., 2011) and a Nikon C1 confocal microscope. The imaging obtained revealed a high density of cells growing in clusters, which is characteristic of anammox bacteria (Fig. 4B).

Specific activity of anammox sludge

Data in Fig. 5 shows the results of batch experiments used to determine the specific activity of *Candidatus* Brocadia caroliniensis. The test was done in a 3-L glass reactor containing wastewater and 600 mL of suspended anammox sludge (final sludge concentration = 2,645 mg VSS/L). The volumetric rates of N transformations in the reactor (slope of the regression lines) were NH₄-N removal= 23.32 mg N/L-reactor/hour; NO₂-N removal= 32.42 mg N /L-reactor/hour;

and $\text{NO}_3\text{-N}$ production = 7.84 mg N /L-reactor/hour. Therefore, the rate of $\text{NH}_4^+ + \text{NO}_2^-$ removal in the suspended batch reactor was 55.74 mg N/L-reactor/hour (1,337.8 mg N/L-reactor/day), and the rate of total N removal was 47.90 mg N/L-reactor/hour (1,149.6 mg N/L-reactor/day). The specific activity of *Candidatus Brocadia caroliniensis* obtained was 0.506 mg N/mg VSS/day for $\text{NH}_4^+ + \text{NO}_2^-$ removal and 0.435 mg N/mg VSS/day for total N removal.

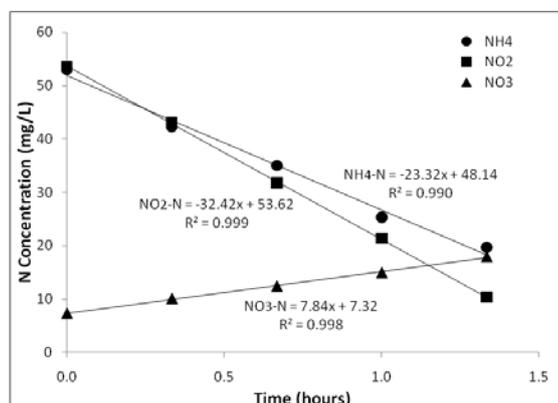


Fig. 5 Changes in NH_4^+ , NO_2^- , and NO_3^- concentrations in a batch reactor using suspended anammox.

Long-term preservation of anammox

Table 3. Stoichiometric ratios of the anammox reaction for fresh and reactivated anammox after long-term preservation compared to the ratios characteristic of *Candidatus "B. caroliniensis"* (parent bioreactor).

Treatment	Stoichiometric Ratios		
	TN Remo./ NH ₄ -N Remo.	NO ₂ -N Remo./ NH ₄ -N Remo.	NO ₃ -N Prod./ NH ₄ -N Remo.
Control 1: Fresh biomass	2.0104 NS	1.1850 NS	0.1746 NS
Control 2: Refrigeration (4°C), KNO ₃ media	2.1170 NS	1.3435 NS	0.2235 NS
Freezing (-60°C)	2.0189 NS	1.7028*	0.6840*
Freezing (-60°C)	2.0144 NS	1.6603*	0.6459*
Lyophilization, prefrozen (-60°C), Skim Milk + Glycerol	1.5650*	0.5874*	0.0222*
Lyophilization, prefrozen (-60°C), Skim Milk	2.1471 NS	1.9412*	0.7941*
Lyophilization, prefrozen (-200°C), Skim Milk + Glycerol	1.6547*	0.8417*	0.1871 NS
Lyophilization, prefrozen (-200°C), Skim Milk	2.1055 NS	1.3242 NS	0.2188 NS
Parent Bioreactor	2.12	1.30	0.18

Note: Means followed by NS are not significantly different by LSD ($P \leq 0.05$) from the stoichiometric ratios characteristic of *Brocadia caroliniensis* (parent bioreactor) obtained from 10-L continuous up-flow parent bioreactor during 3.8-year operational period (Fig. 3), noted on the 9th row; while means followed by * are significantly different ($P > 0.05$).

Candidatus "B. caroliniensis" biomass was considered reactivated when the stoichiometric ratios during two consecutive sampling periods were not significantly different from those

obtained in the parent bioreactor (Fig. 3). Of the different preservation treatments tested, only anammox biomass preserved via freezing in liquid nitrogen (-200°C) followed by lyophilization in skim milk media without glycerol achieved stoichiometric ratios for the anammox reaction similar to the biomass in both the parent bioreactor and in the freshly harvested control treatment (Table 3). To our knowledge, this is the first reported sub-zero preservation protocol for anammox bacteria. As a result of the protocol developed (Rothrock et al., 2011), the anammox bacteria *Candidatus* “*Brocadia caroliniensis*” was successfully deposited with an internationally depositary authority under the provisions of the Budapest Treaty (WIPO 1980).

CONCLUSIONS

Anammox bacteria were successfully isolated from animal waste sludge using immobilizing techniques and continuous flow reactors. Anammox reaction start-up from manure sludge seed was favored by high nitrogen influent concentrations. The obtained anammox sludge was then used for quick start-up of bio-reactors. Average nitrogen removal rates obtained were 1.5 kg N/m³-reactor/day with loading rates of 1.7 kg N/m³-reactor/day and HRT of 3.9 hours. The stoichiometric ratios were $\text{NH}_4^+ + 1.30 \text{NO}_2^- \rightarrow 1.06 \text{N}_2 + 0.18 \text{NO}_3^-$. Due to the relatively low DNA sequence homology ($\leq 96\%$) and phylogenetic relationships, the dominant bacteria isolated at USDA-ARS in Florence, South Carolina, is a novel species of the *Candidatus* *Brocadia* genera named *Candidatus* *Brocadia caroliniensis*. A specific FISH probe FLO-1 was developed. The specific N removal activity obtained was 0.506 mg N/mg VSS/day for ammonia + nitrite removal. The bacterial isolate can be preserved long term via freezing in liquid nitrogen (-200°C) followed by lyophilization in skim milk media. Using this new protocol, the anammox bacteria *Candidatus* “*Brocadia caroliniensis*” was successfully deposited with an internationally depositary authority under the provisions of the Budapest Treaty of the United Nations World Intellectual Property Organization.

REFERENCES

- Daigger, G.T., Sanjines, P., Pallansch, K., Sizemore, J., Wett, B.:** Implementation of a full-scale anammox-based facility to treat an anaerobic digestion sidestream at the Alexandria Sanitation Authority Water Resource Facility. *In Proc. WEF-IWA Nutrient Recovery and Management Conf.*, 2386-2401 (2011).
- Fujii, T., Sugino, H., Rouse, J., and Furukawa, K.:** Characterization of the microbial community in an anaerobic ammonium-oxidizing biofilm cultured on a nonwoven biomass carrier. *J. Biosc. Bioeng.* **94**, 412-418 (2003).
- Furukawa, K., Rouse, J., Yoshida, N., and Hatanaka, H.:** Mass cultivation of anaerobic ammonium-oxidizing sludge using novel biomass carrier. *J. Chem. Eng. (Japan)* **36**, 1136-1169 (2003).
- Strous, M., Heijnen, J.J., Kuenen, J.G., and Jetten, M.S.M.:** The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. *Appl. Microbiol. Biotechnol.* **50**, 589-596 (1998).
- Rothrock, M.J., Vanotti, M.B., Szogi, A.A., Garcia, M.C., and Fujii, T.:** Long-term preservation of anammox bacteria. *Appl. Microbiol. Biotechnol.* In-Press (2011).
- Vanotti, M.B., Szogi, A.A., and Rothrock, M.J.:** Novel anammox bacterium isolate. US Patent Application No. 13/013,874. US Patent & Trademark Office, Washington, DC. (2011).
- WIPO:** Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. United Nations World Intellectual Property Organization (WIPO). (Accessed 03 February 2011) <http://www.wipo.int/treaties/en/registration/budapest>. (1980)