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## Anammox sludge immobilized in polyvinyl alcohol (PVA) cryogel carriers

Albert Magrí\*, Matias B. Vanotti, Ariel A. Szögi

USDA-ARS Coastal Plains Soil, Water and Plant Research Center, 2611 W. Lucas St., Florence, SC 29501, USA

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## ABSTRACT

This study evaluated the use of PVA cryogels to encapsulate slow-growing anammox bacteria for deammonification treatment of wastewater. The cryogel pellets were prepared by freezing-thawing at  $-8\text{ }^{\circ}\text{C}$ . On average, pellets contained  $11.8\text{ mg-TSS/g-pellet}$  of enriched anammox sludge NRRL B-50286 (*Candidatus Brocadia caroliniensis*) in 4-mm cubes. They were tested with synthetic and partially nitrified swine wastewater using continuous stirred-tank reactors packed at 20% (w/v). The immobilized gel was retained inside the reactor by a screen that eliminated the need of sludge recycling. The stoichiometry of anammox reaction was maintained for more than 5 months under non-sterile conditions. The process was not limited by substrates availability unless quite low N concentration ( $<5\text{ mg/L}$ ) achieving  $>93\%$  removal efficiency. In mass balances,  $>80\%$  of the potential N conversion activity was achieved ( $2920\text{ mg-N/kg-pellet/d}$ ). In addition, the immobilized bacteria were resilient to inhibition at high nitrite concentrations ( $244\text{--}270\text{ mg-N/L}$ ).

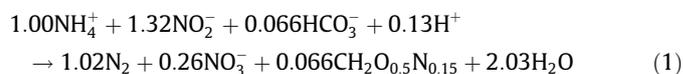
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## 1. Introduction

Discovery in the early 1990's of the anaerobic ammonium oxidation (anammox) (Mulder et al., 1995) as a new pathway to biologically convert ammonium ( $\text{NH}_4^+$ ) to dinitrogen gas ( $\text{N}_2$ ) under absence of oxygen has arisen great expectations in the field of wastewater treatment. However, due to the low growth rate of anammox bacteria (doubling time in the range of 1.8–11 days; Isaka et al., 2006; Strous et al., 1998), one of the main challenges for implementing the anammox process is to ensure bacterial cells' retention inside the reactors. For this reason, immobilization of microbial cells has received increasing interest in wastewater treatment to minimize the risk of biomass wash-out from the reactors and provide stabilized treatment (Quan et al., 2011). These immobilization techniques include self-immobilization as granular biomass (Dapena-Mora et al., 2004; López et al., 2008), attachment on the surface of a carrier forming biofilm (Ni et al., 2010; Tsushima

et al., 2007), and entrapment of the microbial biomass into gel pellets (Furukawa et al., 2009; Isaka et al., 2007).

The anammox process is especially suitable for the removal of nitrogen (N) from wastewaters containing high ammonium and low biodegradable organic carbon (Paredes et al., 2007). This process consists of a chemolithoautotrophic bioconversion mediated by Planctomycetes-like bacteria that under anoxic conditions oxidize  $\text{NH}_4^+$  using nitrite ( $\text{NO}_2^-$ ) as the electron acceptor. According to the anammox reaction proposed by Strous et al. (1998) (Eq. 1),  $\text{NH}_4^+$  and  $\text{NO}_2^-$  are converted to  $\text{N}_2$  and nitrate ( $\text{NO}_3^-$ ) under stoichiometric molar ratios of 1.00:1.32:0.26:1.02 for  $\text{NH}_4^+$  consumption,  $\text{NO}_2^-$  consumption,  $\text{NO}_3^-$  production and  $\text{N}_2$  production, respectively.



The use of synthetic polymers such as urethane, polyethylene glycol (PEG), and polyvinyl alcohol (PVA) for the entrapment of microorganisms was reported as advantageous in the field of wastewater treatment. These advantages include closely packed design of bioreactors, non-toxicity to microorganisms, mechanical strength and long life span of gels, enhanced process efficiency, and resilience to overloading rates as demonstrated in the studies of Sumino et al. (1992), Tanaka et al. (1996) and Vanotti and Hunt (2000) on immobilization of nitrifying biomass. Furukawa et al. (2009) demonstrated that both anammox and nitrifying biomass can be immobilized in PEG gel pellets and used in connected reactors for the successful deammonification treatment of anaerobic digested liquor containing high ammonium concentration ( $1400\text{--}1600\text{ mg NH}_4^+\text{-N/L}$ ).

**Abbreviations:** ACR, ammonium conversion rate; BOD, biological oxygen demand; COD, chemical oxygen demand; CSTR, continuous stirred-tank reactor; DO, dissolved oxygen; EC, electrical conductivity; HRT, hydraulic residence time; NCE, nitrogen conversion efficiency; NCR, nitrogen conversion rate; NLR, nitrogen loading rate; NRE, nitrogen removal efficiency; NRR, nitrogen removal rate; PEG, polyethylene glycol; PVA, polyvinyl alcohol; SBR, sequencing batch reactor; SNCR, specific nitrogen conversion rate; SNRR, specific nitrogen removal rate; TSS, total suspended solids; VSS, volatile suspended solids.

\* Corresponding author. Permanent address: GIRO Technological Centre, Rambla Pompeu Fabra 1, 08100 Mollet del Vallès, Barcelona, Spain. Tel.: +34 93 579 67 80; fax: +34 93 579 67 85.

E-mail addresses: [albert.magri@gmail.com](mailto:albert.magri@gmail.com) (A. Magrí), [matias.vanotti@ars.usda.gov](mailto:matias.vanotti@ars.usda.gov) (M.B. Vanotti), [ariel.szogi@ars.usda.gov](mailto:ariel.szogi@ars.usda.gov) (A.A. Szögi).

Two techniques have been proposed to entrap microorganisms using PVA; in both cases the microorganisms are first mixed with a PVA solution and then the PVA is polymerized. For the PVA cross-linking or hardening step, one technique uses chemicals and the other uses freezing. For example, microorganisms can be immobilized using PVA, alone or mixed with alginate, into spherical gel beads through dripping into a gelation solution. This chemical technique has been used with anammox (Hsia et al., 2008; Quan et al., 2011; Zhu et al., 2009). The other cell immobilization technique involves the PVA cryogels, which are prepared by physical cross-linking through the freezing/thawing method (Lozinsky and Plieva, 1998). In the field of wastewater treatment, Furukawa et al. (1994) used the PVA-freezing technique at  $-20\text{ }^{\circ}\text{C}$  to immobilize marine nitrifying sludge, whereas Vanotti and Hunt (2000) applied the PVA-freezing technique at  $-4\text{ }^{\circ}\text{C}$  to immobilize swine wastewater nitrifying sludge. Previous research showed that exposure to freezing temperatures ( $-20\text{ }^{\circ}\text{C}$  during 2 months) may cause irrevocable inactivation of anammox bacteria (Vlaeminck et al., 2007), but the effect of short time exposure to freezing temperatures (needed for PVA polymerization and cell entrapment) has not been investigated.

While nitrite is an essential substrate for the anammox reaction, there is evidence that high nitrite concentration inside an anammox reactor may also become an inhibitor of the anammox reaction and, therefore, it can be an important parameter to control. Kimura et al. (2010), in their experiments with anammox entrapped in PEG gel pellets, determined that  $274\text{ mg NO}_2^- \text{-N/L}$  was the maximum concentration before nitrite inhibition occurred. In other cases, however, much lower nitrite concentrations ( $44\text{--}100\text{ mg NO}_2^- \text{-N/L}$ ) were found as inhibitory of the anammox (Egli et al., 2001; Strous et al., 1999).

The aim of this research was to evaluate the effectiveness of entrapping anammox biomass in pellets using PVA as gel carrier and the freezing polymerization technique. In addition, we investigated the following three aspects of the immobilized anammox process: the recovery time of the anammox pellets after immobilization; the stabilized anammox reaction using both synthetic wastewater and partially nitrified swine wastewater; and the effect of high nitrite concentration on the activity of the immobilized anammox.

## 2. Methods

### 2.1. Anammox sludge

The anammox bacteria used was *Candidatus Brocadia carolinienensis* deposited under the provisions of the Budapest Treaty (WIPO, 1980) in the Agricultural Research Service Culture Collection (NRRL) at Peoria, Illinois, USA, with accession number NRRL B-50286 (Vanotti et al., 2011b). It was isolated from sludges of nitrification–denitrification systems treating liquid swine manure and cultivated in a 10-L jacketed up-flow reactor (120 cm) (hereafter called parent reactor) at the ARS laboratory in Florence, South Carolina, USA. The parent reactor contained a biomass support made of polyester non-woven material (Japan Vilene Co., Tokyo, Japan). At the time of sludge collection for this PVA immobilization study, the parent reactor was being fed with synthetic wastewater containing  $152.7\text{ mg NH}_4^+ \text{-N/L}$  and  $152.7\text{ mg NO}_2^- \text{-N/L}$  and operated with a hydraulic residence time (HRT) of 4 h, an N-loading rate (NLR) of  $1735\text{ mg N/L/d}$ , and a water temperature of  $30\text{ }^{\circ}\text{C}$ . Under these conditions, the N-conversion efficiency (NCE) obtained was 94% and the total N-removal efficiency (NRE) was 85% (Vanotti et al., 2011b). Stoichiometric molar ratios for the anammox reaction ( $\text{NH}_4^+$  consumption: $\text{NO}_2^-$  consumption: $\text{NO}_3^-$  production: $\text{N}_2$  production) obtained in the parent reactor during steady-state conditions (three

years) were  $1.00:1.30 \pm 0.009:0.18 \pm 0.006:1.06 \pm 0.005$ , respectively (Vanotti et al., 2011b). The removal of  $\text{NH}_4^+$  plus  $\text{NO}_2^-$  inside the 120-cm-long parent reactor was gradual as the wastewater passed through: 34% of the N removal occurred at the bottom, 33% at the middle, and 33% at the top. Therefore, the anammox sludge was collected from three different heights (bottom, middle, and top) of the parent reactor by means of a long probe and a peristaltic pump.

### 2.2. Immobilization procedure

The anammox sludge extracted from the parent reactor was encapsulated in polymer gel pellets of PVA according to the freezing method described by Vanotti and Hunt (2000). The anammox sludge had a granular structure with granules measuring 2–4 mm and had a reddish color (Fig. A.1). It was concentrated in a beaker to  $14.9 \pm 0.9\text{ g TSS/L}$  ( $11.3 \pm 0.7\text{ g VSS/L}$ ;  $\text{VSS/TSS} = 0.76$ ) by rapid settling (5 min) and decanting. The decanting was not straightforward because about 25% of the anammox sludge floated in the beaker. A sieve 0.25-mm pore size that covered the beaker was used and retained all the anammox sludge during decanting. The PVA polymer used for the preparation of the pellets was PVA-HC in powder form (100% saponification, 2000 polymerization, Kuraray Co., Tokyo, Japan). The PVA powder was sprinkled on warm water and mixed by hand to form a polymer suspension of 20% (w/v). The suspension was then autoclaved at  $121\text{ }^{\circ}\text{C}$  during 50 min to obtain complete PVA dissolution. After cooling to  $37\text{ }^{\circ}\text{C}$ , 400 mL of the polymer solution were mixed with 440 mL of the concentrated anammox sludge (Fig. A.1). The mixture was transferred into four non-stick aluminized steel pans ( $23 \times 23\text{ cm}$ ) to make four sheets approximately 4-mm thick. Subsequently, the pans were leveled and cooled for 2 h at  $8\text{ }^{\circ}\text{C}$ , and frozen for 17 h at  $-8\text{ }^{\circ}\text{C}$ . After fast thawing for about 15 min inside a warm water bath (Fig. A.2), immobilized anammox pellet cubes of about 4-mm length were prepared using a sushi knife. The immobilized pellets were washed for 30 min under  $\text{N}_2$  bubbling with synthetic medium (section 2.7.1) until foaming produced by unpolymerized PVA stopped. Wet weight of the pellets was determined after draining the pellets during 1 min over a 1-mm sieve. Corresponding volume was determined using water displacement in a 1-L graduated cylinder. The pellets density was  $1.035\text{ g/mL}$ . Conditioning of immobilized anammox pellets was performed in closed glass vessels during 2 days at  $33 \pm 1\text{ }^{\circ}\text{C}$  under fill-and-draw procedure using the same synthetic medium and applying a NLR of  $230\text{ mg N/L/d}$ . Thereafter, the activity of the anammox pellets was tested using continuous stirred-tank reactors (CSTR).

### 2.3. Immobilized reactors set-up and operation

The pelletized anammox biomass obtained above (557 g wet) was split into two cell culture glass flasks (Celstir, Wheaton, Millville, NJ, USA) that were operated as CSTR (Fig. 1). Each reactor had a working volume of 1.4-L and a pellet packing ratio of 20% (w/v). Process temperature was controlled at  $33 \pm 1\text{ }^{\circ}\text{C}$  using a heated water bath. Magnetic stirring speed was set to 100 rpm. The influent was supplied by an inflow peristaltic pump (model 7553-80, Cole-Parmer, Vernon Hills, IL, USA) from a 55-L tank through a feed line Tygon tubing (3.2-mm i.d.). The influent liquid was bubbled with  $\text{N}_2$  each time the influent tank was refilled and covered with a floating polystyrene foam sheet to reduce gas exchange with the atmosphere. The feed line was introduced inside the reactor through a rubber stopper using Chemflour PTFE tubing (4.8-mm i.d.) up to the bottom. The effluent line used the same Chemflour PTFE tubing that exited the reactor through a rubber stopper. To retain the pellets inside the reactor, an outlet port was assembled at the beginning of the effluent line. The port consisted of a plastic

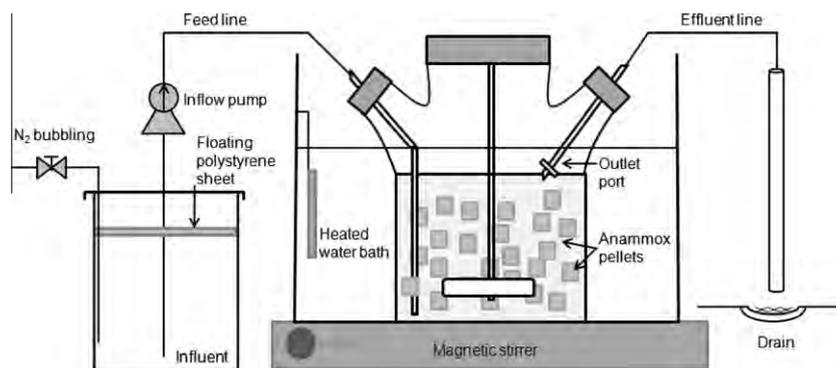


Fig. 1. Schematic diagram of continuous-flow stirred-tank reactor used for nitrogen treatment of wastewater with immobilized anammox pellets.

cylinder 1-cm diameter and 1-cm in length covered with a 1-mm fiberglass screen. The position (height) of the outlet port inside the reactor determined the liquid surface level and working volume of the CSTR. Outside the reactor, the effluent line was prolonged 20-cm with a flexible tubing 3.2-mm i.d. and 6.3-mm o.d. and fitted into a wider tube 9.5-mm i.d. that conveyed the effluent by gravity to a drain. The reactors were always closed. They were also covered with black plastic sheet to avoid light, which is known to have a negative effect (30–50% reduction) on anammox activity (van de Graaf et al., 1996).

The recovery of activity and stabilized treatment after immobilization were evaluated during 120 days. The two reactors were operated under identical NLR conditions using synthetic wastewater. The complete formulation of the synthetic wastewater is described in section 2.7.1. During the first 38 days, the synthetic wastewater contained 70 mg  $\text{NH}_4^+$ -N/L and 90 mg  $\text{NO}_2^-$ -N/L. Afterwards (day 38–120), N concentrations were increased to 150 mg  $\text{NH}_4^+$ -N/L and 150 mg  $\text{NO}_2^-$ -N/L. These influent N concentrations were selected since there was previous evidence of non-inhibition of the anammox biomass at these concentrations. The NLR was adjusted in the range of 490–2780 mg N/L/d according to the influent concentration by varying the flow rates from 2 to 9 mL/min. Corresponding HRTs were 11.7–2.6 h. Flow rates were measured daily by collecting the effluent in a graduated cylinder during 5 min and adjusted to target rates as needed. The influent tanks were refilled weekly with fresh synthetic wastewater. At that time, the tanks were bubbled for about 10 min with  $\text{N}_2$  to reduce DO concentration below 1 mg  $\text{O}_2$ /L. Liquid samples were taken daily from the influent tank and the effluent line for chemical analysis. After evaluation of recovery after immobilization and stabilized treatment, the immobilized reactors were used to evaluate anammox activity with partially nitrified swine wastewater and the inhibition by high nitrite concentrations.

#### 2.4. Pellets activity test using batch testing

The activity of pellets was also evaluated using batches at the end of the recovery period. The batch experiments were conducted under the same conditions described above (mixing, temperature, pellet packing and effective volume) using the same 1.4-L CSTR reactors (Fig. 1) without a feed line and the following five modifications: initial N concentration was 150 mg N/L ( $\text{NH}_4^+$  +  $\text{NO}_2^-$ ), the effluent line was connected to the feed line to form a closed loop that included the inflow pump and an in-line sampling port, the outlet port inside the reactor was lowered 1 cm below the liquid surface, the pump was turned on for 3 min during each sampling, and liquid samples (3.5 mL) were collected through the in-line sampling port at 0.5- to 1-h intervals for water quality analyses. The batch experiments were done in duplicate.

#### 2.5. Initial activity of the anammox biomass

The specific activity of the anammox biomass before immobilization was evaluated through batch tests. This determination was done in a 3-L anaerobic jacketed reactor (Chemglass Life Sciences, Vineland, NJ, USA) using fresh sludge collected from the parent reactor. Anammox biomass content inside the batch reactor was  $3.00 \pm 0.35$  g VSS/L ( $3.81 \pm 0.55$  g TSS/L). Water temperature was  $33 \pm 1$  °C. Substrate used for the specific activity tests (2) were dilutions of that fed in the parent reactor with initial concentrations of both ammonium and nitrite of 50 or 110 mg N/L. The two different initial conditions provided a broader range of substrate concentrations under non-limiting nitrite levels. After adding the biomass and the liquid substrate into the 3-L reactor, bubbling with  $\text{N}_2$  was provided during 10 min to decrease DO concentration to <1 mg/L. The batch tests were then started; they lasted 2 h and liquid samples were extracted every 15–20 min for chemical analyses. The tests were done in duplicate.

#### 2.6. Nitrite inhibition test

The effect of nitrite on anammox activity in the gel carrier was evaluated using continuous feeding tests. The concentration of nitrite in the influent was gradually increased every 3 days from 150 to 700 mg  $\text{NO}_2^-$ -N/L at increments of 50 mg  $\text{NO}_2^-$ -N/L using  $\text{NaNO}_2$ . The sodium added (246–1150 mg Na/L) was lower than the threshold concentration of 2300 mg Na/L considered inhibitory to anammox (Dapena-Mora et al., 2007). The  $\text{NH}_4^+$ -N concentration was kept constant at 150 mg/L under nominal flow rate of 4 mL/min (5.8 L/d), and the dosage of the rest of the chemicals in the synthetic wastewater was the same as detailed in section 2.7.1. Water temperature was  $33 \pm 1$  °C. Influent and effluent samples were collected daily during the test. Inhibition was determined by examining the relationship between NCR and the nitrite concentration inside the reactor.

#### 2.7. Substrates

##### 2.7.1. Synthetic wastewater

The chemical composition of the synthetic wastewater used throughout the experiments including the parent reactor contained  $\text{NH}_4\text{Cl}$  (variable: 267.5–583.5 mg/L),  $\text{NaNO}_2$  (variable: 443.6–3450 mg/L),  $\text{KHCO}_3$  (variable: 140–305.4 mg/L),  $\text{KH}_2\text{PO}_4$  (27 mg/L),  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (9 mg/L), EDTA (5 mg/L),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (240 mg/L),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (143 mg/L), and trace element solution 0.3 mL/L. Trace element solution contained:  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (1247 mg/L),  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  (1119 mg/L),  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (44 mg/L),  $\text{Al}_2(\text{SO}_4)_3 \cdot 14\text{H}_2\text{O}$  (201.5 mg/L),  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  (129 mg/L),  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  (30 mg/L), KCl (100 mg/L), EDTA (975 mg/L). Carbonate alkalinity was adjusted

according to the influent  $\text{NH}_4^+$ -N concentration at a ratio of 1:1 (Vanotti et al., 2011b). Therefore,  $\text{KHCO}_3$  added in the influent varied at a rate of 2 g  $\text{KHCO}_3$  per 1 g  $\text{NH}_4^+$ -N.

### 2.7.2. Partially nitrified swine wastewater

Swine wastewater was obtained from a pig farm located near Clinton (Sampson Co., NC, USA) after solid–liquid separation using polyacrylamide as flocculant agent (Vanotti et al., 2009). The liquid fraction was stored under anaerobic conditions in barrels (208 L) at ambient temperature for about 3 months before use in the experiments. Subsequently, partial nitrification with the target of 57% ammonium oxidation to nitrite was conducted using a high performance nitrifying sludge (Vanotti et al., 2011a) in a lab-scale continuously aerated SBR as described by Magrì et al. (2011). The partially nitrified swine wastewater was diluted 5.75 times with distilled water to obtain a nitrite concentration of 150 mg  $\text{NO}_2^-$ -N/L. The pH was adjusted to 7.50 using HCl 1.19 N (0.72 mL/L), and levels of DO were adjusted below 1 mg  $\text{O}_2$ /L by bubbling with  $\text{N}_2$ . Measured values for this feed were pH  $7.56 \pm 0.03$ , electrical conductivity (EC)  $3.28 \pm 0.20$  dS/m, alkalinity  $205 \pm 34$  mg  $\text{CaCO}_3$ /L, chemical oxygen demand (COD)  $193 \pm 23$  mg  $\text{O}_2$ /L, 5-day biological oxygen demand ( $\text{BOD}_5$ )  $<10$  mg  $\text{O}_2$ /L, orthophosphate phosphorus ( $\text{PO}_4$ -P)  $16 \pm 1$  mg P/L,  $\text{NH}_4^+$ -N  $103 \pm 3$  mg N/L, and  $\text{NO}_2^-$ -N  $144 \pm 3$  mg N/L.

### 2.8. Analytical methods

All treated and untreated liquid samples were analyzed according to Standard Methods for the Examination of Water and Wastewater (APHA, AWWA and WEF, 1998). Alkalinity was determined by acid titration to an endpoint of pH 4.5 and expressed as  $\text{CaCO}_3$  (Standard Method 2320 B). Total suspended solids (TSS) were determined after filtration through a 1.5- $\mu\text{m}$  glass microfiber filter and drying to constant weight at 105 °C (Standard Method 2540 D), and volatile suspended solids (VSS) were determined after later ignition in a muffle furnace at 500 °C for 15 min (Standard Method 2540 E). The COD was determined through the closed reflux colorimetric method (Standard Method 5220 D) with addition of 10 mg sulfamic acid for each mg  $\text{NO}_2^-$ -N present in the sample before digestion to avoid interference by nitrite (Standard Method 5220 A). For  $\text{BOD}_5$ , we used biological incubation tests (Standard Method 5210 B). Inorganic nitrogen forms were determined after sample filtration to 1.5  $\mu\text{m}$  using an auto-analyzer:  $\text{NH}_4^+$ -N was determined by the automated phenate method (Standard Method 4500-NH<sub>3</sub> G),  $\text{NO}_2^-$ -N +  $\text{NO}_3^-$ -N were determined by the automated cadmium reduction method (Standard Method 4500- $\text{NO}_3^-$  F), and  $\text{NO}_2^-$ -N alone was determined by applying the same colorimetric method without the cadmium reduction step.  $\text{NO}_3^-$ -N was then calculated by subtraction. The orthophosphate phosphorus ( $\text{PO}_4$ -P) was determined by the automated ascorbic acid method (Standard Method 4500-P F). The pH was measured using a pH-meter (Pinnacle 540, Corning Inc., Corning, NY, USA), EC referred to 25 °C was measured by a portable meter (model 126, Orion Research Inc., Beverly, MA, USA), and DO was measured by a portable meter (YSI 550A, YSI Inc., Yellow Springs, OH, USA).

### 2.9. Nitrogen transformation calculations

The nitrogen conversion rate (NCR) was defined as the removal of  $\text{NH}_4^+$  and  $\text{NO}_2^-$  from the liquid, and the nitrogen removal rate (NRR) was defined as the total removal of nitrogen from the liquid and considers the removal of  $\text{NH}_4^+$  and  $\text{NO}_2^-$  and the production of  $\text{NO}_3^-$  (Eq. 1). Under continuous-flow operation of reactors, the NCR was calculated from the difference in concentrations of  $\text{NH}_4^+$  and  $\text{NO}_2^-$  between the influent and the effluent, divided by the measured HRT. The NRR was calculated from the difference in

concentrations of  $\text{NH}_4^+$ ,  $\text{NO}_2^-$ , and  $\text{NO}_3^-$  between the influent and the effluent, divided by the measured HRT. Stoichiometric reaction ratios were calculated according to the difference in concentrations of  $\text{NH}_4^+$ ,  $\text{NO}_2^-$ , and  $\text{NO}_3^-$  between influent and effluent and expressed per unit of  $\text{NH}_4^+$  removal. The  $\text{N}_2$  stoichiometric reaction ratio was calculated through mass balance (1 mol  $\text{N}_2$  = 2 atoms N) considering the other three measured nitrogen species. Under batch operation, NCR and NRR were calculated from the corresponding time-dependent slopes for the evolution in the concentration of  $\text{NH}_4^+$ ,  $\text{NO}_2^-$ , and  $\text{NO}_3^-$ . Specific activities of the anammox sludge, sNCR and sNRR, were determined from the NCR and NRR plus the corresponding VSS concentration in the reactor.

### 2.10. Statistical analyses

Data were analyzed by means of standard error, linear and non-linear regression analyses, and analysis of variance. Linear regression analyses were used to describe N-conversion during batch tests. Non-linear regression analysis of data obtained in the nitrite inhibition test was carried out in SAS (SAS Institute Inc., Cary, NC, USA) by fitting a spline plateau-linear function using the NLIN procedure and the iterative resolution method of Gauss–Newton. Analysis of variance was used to determine the effect of the NLR on reactor performance under stable capacity using GLIMMIX procedure in SAS. Significant differences among treatment means were evaluated using least squares means ( $\alpha = 0.05$ ).

## 3. Results and discussion

### 3.1. Activity of the anammox sludge before immobilization

The anammox sludge NRRL B-50286 (*Candidatus Brocadia caroliniensis*) exhibited good specific N removal activity before immobilization. Average nitrogen removal rates (NCR and NRR) obtained in two batch reactor tests (Fig. 2) were  $1162 \pm 176$  mg N/L/d and  $1048 \pm 102$  mg N/L/d, respectively. Corresponding specific N removal activities (VSS = 3 g/L) were  $0.40 \pm 0.11$  g N/g VSS/d (SNCR), and  $0.36 \pm 0.08$  g N/g VSS/d (SNRR). They were above median values of 0.20 g N/g VSS/d (range: 0.06–1.60,  $n = 14$ ) obtained for a variety of anammox sludges of diverse origins as reported by Van Hulle et al. (2010).

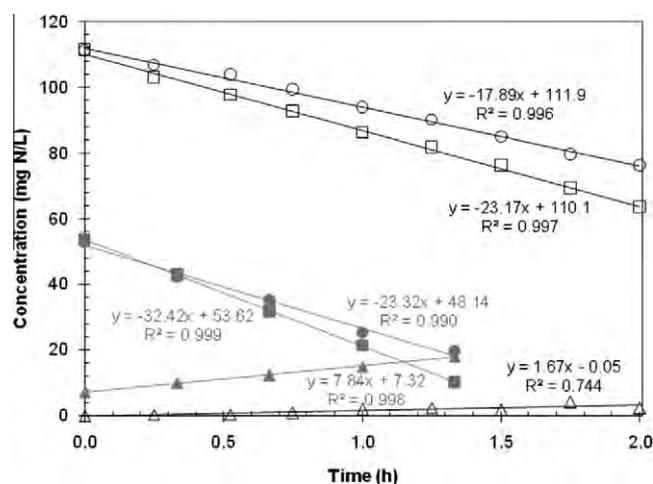


Fig. 2. Concentration profiles of ammonium (○), nitrite (□), and nitrate (△) during batch testing experiments for the anammox sludge collected from the parent reactor (before immobilization). Data show two batches (empty symbols and gray filled symbols) at different initial N concentrations.

### 3.2. Production of immobilized anammox pellets and potential activity

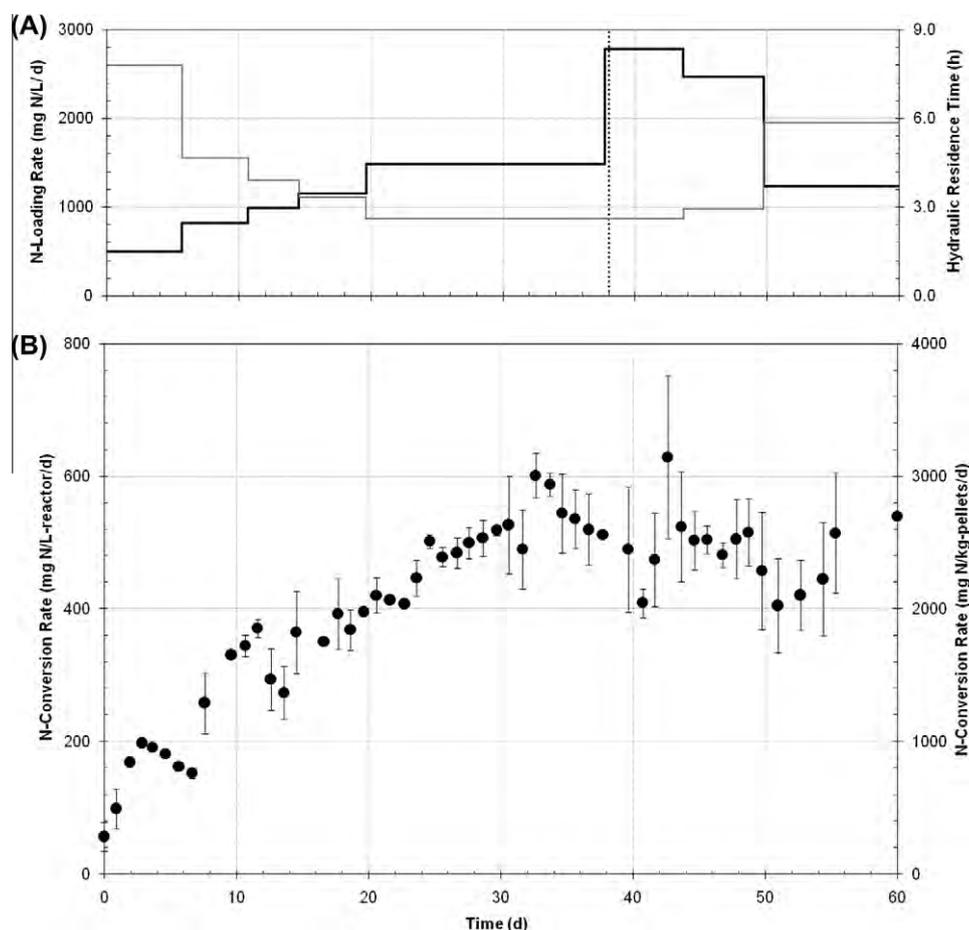
The mixture of PVA-HC aqueous solution and concentrated anammox sludge, with a final PVA concentration of 9.5%, was successfully immobilized using the PVA-freezing method (Fig. A.2). The immobilized anammox had an elastic, rubber-like appearance and exhibited good gel strength. The PVA cryogel pellets did not experience problems of adhesion and agglomeration reported for immobilization of cells using 100% PVA and chemical polymerization (Zhu et al., 2009); they remained dispersed in the stirred reactor (100 rpm) used in this study (Fig. A.3). Pellets were produced at a rate of 663 g (wet) per 1000 mL of the polymer-anammox sludge initial mixture. Even though the density of these pellets was 1.035 g/mL, without stirring they were buoyant and floated up to the surface due to the intense bubbling of  $N_2$  gas noticed throughout the experiments (Fig. A.4). The exception was during the inhibition study (section 3.6) when anammox activity was severely inhibited; the  $N_2$  bubbling stopped and the pellets sank to the bottom.

The concentration of anammox sludge contained in the PVA cryogels at the time of immobilization was 8.97 mg VSS/g-pellet (11.77 mg TSS/g-pellet). At the actual loading rate of 199 g-pellet/L-reactor (packing ratio = 20%), the initial concentration of immobilized anammox sludge in the CSTR reactors was 1.78 g VSS/L (2.34 g TSS/L). Considering the specific activity of the anammox sludge obtained before immobilization ( $SNCR = 0.40 \pm 0.11$  g N/g VSS/d; section 3.1) plus the concentration of anammox sludge in the pellets and the loading rate of pellets in the reactor, the potential N conversion capacity of the immobilized anammox was 3.58 mg N/g-pellet/

d and 712 mg N/L-reactor/d (20% packing). Since this potential assumes full recovery of anammox sludge activity after immobilization, it was used as the metric to assess recovery and performance of the immobilized anammox in this study.

### 3.3. Activity of immobilized anammox after freezing

Perhaps the largest concern in the creation of the anammox cryogels was the effect that freezing temperatures during polymerization may have on bacteria survival and reactivation. Vlaeminck et al. (2007) demonstrated that long-term storage of sludge under sub-zero freezing (2 months at  $-20$  °C) caused irrevocable inactivation of anammox bacteria. Similarly, Rothrock et al. (2011) could not reactivate anammox sludge preserved during 4 months at  $-60$  °C even with the use of skim milk media and glycerol as cryoprotectants. On the other hand, these last authors found that pre-freezing with liquid nitrogen ( $-200$  °C) was a necessary step for successful long-term preservation of anammox via lyophilization. In the present study, the PVA gel entrapment procedure via freezing included sub-zero temperatures of  $-8$  °C during 17 h. These conditions produced gels of good strength but severely hindered the anammox activity because the SNCR decreased 90% from  $0.40 \pm 0.11$  before immobilization to  $0.04 \pm 0.01$  g N/g VSS/d after. However, this decrease was not permanent and the N conversion activity (both  $NH_4^+$  and  $NO_2^-$ ) of the immobilized anammox recovered quickly; it reached levels of 73, 76 and 82% of the potential capacity before immobilization after approximately one, two



**Fig. 3.** Recovery of anammox activity after whole-cell entrapment in gel pellets using PVA-freezing polymerization. (A) Time courses of the nitrogen loading rate (—) and hydraulic residence time (---) in the continuous-flow immobilized reactors. Vertical dashed line at day 38 indicates change in substrate concentration. (B) Nitrogen conversion rates (NCR) obtained. Error bars represent  $\pm$  the standard error from duplicate reactors.

**Table 1**  
Effect of flow rate and nitrogen loading on conversion activity of immobilized anammox.

Run (No.)	Q <sup>a</sup> (L/d)	HRT (h)	Influent N conc. (mg N/L)	NLR (mg N/L/d)	NCR (mg N/L-reactor/d)	NCR (mg N/kg-pellet/d)	NCE (%)	Conversion activity relative to potential <sup>b</sup> (%)
1 <sup>c</sup>	6.0	5.6 (0.2)	301 (4)	1290 (27)	539 (19)	2709 (95)	42 (2)	76 (3)
2	3.1	10.8 (0.5)	300 (2)	671 (34)	460 (13)	2312 (65)	69 (2)	65 (2)
3	5.5	6.2 (0.2)	304 (2)	1191 (36)	581 (11)	2920 (55)	48 (1)	82 (2)
LSD <sub>0.05</sub> <sup>d</sup>		0.9	NS	97	44	221	6	6

<sup>a</sup> Q, Influent flow rate; HRT, Hydraulic residence time; Influent N conc. = 50% NH<sub>4</sub><sup>+</sup> and 50% NO<sub>2</sub><sup>-</sup>; NLR, Nitrogen loading rate; NCR, Nitrogen conversion rate; NCE, Nitrogen conversion efficiency.

<sup>b</sup> Conversion potential: NCR of the immobilized anammox relative to the specific activity of the anammox sludge before immobilization (3578 mg N/kg-pellet/d).

<sup>c</sup> Runs 1–3 were conducted at days 50–62, 92–101 and 104–123 after immobilization. Values are means of duplicate reactors (standard errors in parentheses).

<sup>d</sup> LSD: Least significant difference at  $P \leq 0.05$ ; NS: not significant.

and four months, respectively, as discussed in the following sections.

### 3.4. Immobilized pellets activity using continuous flow treatment

#### 3.4.1. Recovery of anammox activity after immobilization

Most of the activity of the immobilized anammox pellets was recovered in the first 30 days (Fig. 3B). During this period, the pellet's activity responded positively to gradual increases of NLR in the range of about 500–1400 mg N/L/d (Fig. 3A). Under these conditions, the NCR increased one order of magnitude up to achieving stabilization: from an initial value of  $57 \pm 22$  mg N/L-reactor/d obtained at day 1 ( $0.28 \pm 0.11$  mg N/g-pellet/d) to a value of  $519 \pm 8$  mg N/L-reactor/d at day 30 ( $2.61 \pm 0.04$  mg N/g-pellet/d). Thus, considering the specific activity and amount of anammox sludge used for the preparation of pellets, 73% of the potential N conversion activity was recovered after one month of continuous operation. While gradual increases of NLR increased pellet response, an excessive twofold increase (applied at days 38–49) did not increase further the pellet's anammox activity (Fig. 3). It is concluded that gradual increases of NLR are preferred for reactivation of immobilized anammox with the PVA-freezing polymerization technique.

#### 3.4.2. Effect of flow rate and NLR on pellet activity

The conversion capacity of the immobilized anammox was evaluated using two flow rates (3 and 6 L/d) and the same influent N concentration (Table 1). The high flow rate was repeated resulting in two high and one low NLR conditions (runs 1, 3 vs. 2). There was a significant effect of the applied loading rate on the conversion rates achieved. With the first high NLR condition at two months of testing (run 1; 1290 mg N/L/d), the potential activity was 76%. At the lower NLR of 671 mg N/L/d in run 2 (this is lower than the pellet potential of 712 mg N/L/d), the anammox activity (NCR) significantly decreased 15% from the previous run. However, the activity was quickly reversed and improved when high NLR conditions were reestablished in run 3. Coinciding with the higher NCR attained of 2920 mg N/kg-pellet/d ( $3.02$  kg N/m<sup>3</sup>-carrier/d) at four months of continuous testing, the immobilized anammox reached 82% of the potential conversion activity (the activity of the sludge before immobilization).

Different prepolymer composition and hardening procedures used for anammox entrapment in gel carriers affect the macroporous internal structure of the gel carrier (Isaka et al., 2007; Lozinsky and Plieva, 1998; Quan et al., 2011; Zhu et al., 2009) and its suitability to promote enhanced growth and N removal capacity of the technology. For example, the NCR attained with the PVA cryogels ( $3.02$  kg N/m<sup>3</sup>-carrier/d) is higher than that obtained by Zhu et al. (2009) (approximately  $0.62$  kg N/m<sup>3</sup>-carrier/d) that used PVA/alginate gel hardened with CaCl<sub>2</sub> to immobilize anammox. On the other hand, higher NCRs ( $11$ – $29$  kg N/m<sup>3</sup>-carrier/d) have been

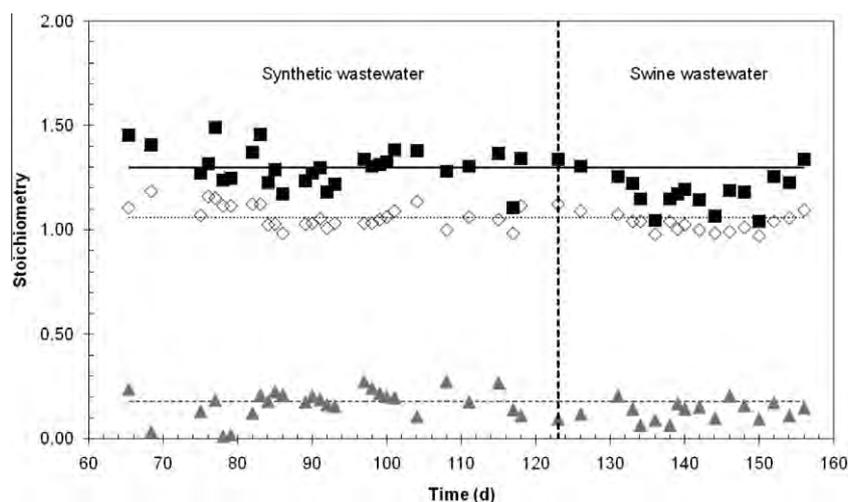
reported with immobilized anammox sludge using two gel entrapment techniques: 1) PVA/alginate gel carriers cross-linked with CaCl<sub>2</sub> and NaNO<sub>3</sub> (Quan et al., 2011), and 2) PEG gel carriers made with PEG prepolymer, N,N,N',N'-tetramethylene-diamine (a promoter) and potassium persulphate (an initiator) (Isaka et al., 2007). In these studies, a significant increase in anammox activity was also described in the subsequent days after immobilization (25–120 d). Since their initial TSS concentrations ( $0.72$ – $5.00$  g/L-reactor) were similar to the initial TSS concentration in our study ( $2.34$  g/L-reactor), the high N conversion activity obtained by Isaka et al. (2007) and Quan et al. (2011) could only be explained by significant growth of anammox bacteria in the gel carriers as a result of improved microenvironment conditions in the gel structure. Therefore, it would be important for effective industrial bio-treatment that, in addition to good physical strength and durability, the anammox gel carrier also has an internal porous structure that promotes nutrient and gas transport and sustains growth and high population of anammox bacteria.

#### 3.4.3. Stoichiometric characteristics of immobilized anammox

The removal molar ratios of NO<sub>2</sub><sup>-</sup> to NH<sub>4</sub><sup>+</sup> and production molar ratios of NO<sub>3</sub><sup>-</sup> and N<sub>2</sub> to NH<sub>4</sub><sup>+</sup> removal for the anammox reaction (Eq. 1) were reported to be 1.32, 0.26 and 1.02, respectively (Strous et al. 1998). The average ( $\pm$ s.e.) stoichiometric ratios of the immobilized anammox throughout the 120-d period using synthetic medium as influent were  $1.30 \pm 0.02$  for NO<sub>2</sub><sup>-</sup> to NH<sub>4</sub><sup>+</sup>,  $0.23 \pm 0.01$  for NO<sub>3</sub><sup>-</sup> to NH<sub>4</sub><sup>+</sup>, and  $1.04 \pm 0.01$  for N<sub>2</sub> to NH<sub>4</sub><sup>+</sup>. This indicates that the transformations are in agreement with the anammox reaction. The stoichiometric ratios of anammox obtained were quite stable, especially the ratio NO<sub>2</sub><sup>-</sup> to NH<sub>4</sub><sup>+</sup>. For example, during the first 60 days after immobilization the ratios (NH<sub>4</sub><sup>+</sup>:NO<sub>2</sub><sup>-</sup>:NO<sub>3</sub><sup>-</sup>:N<sub>2</sub>) were 1.00:1.30:0.26:1.02, and during the second 60 days of more stabilized treatment the ratios were 1.00:1.30:0.17:1.07 (Fig. 4). These ratios coincide with the stoichiometric ratios of 1.00:1.30:0.18:1.06 obtained for the anammox sludge NRRL B-50286 in the parent reactor (Vanotti et al., 2011b) that was used in the study. The results obtained in these experiments indicate that the stoichiometric anammox activity was successfully retained in the PVA cryogel carriers.

#### 3.4.4. Anammox reaction using partially nitrified swine wastewater

The anammox reaction was also achieved in one of the CSTRs packed with immobilized anammox pellets during a 30-d period (days 126–156) once synthetic wastewater was changed by partially nitrified swine wastewater. Average NCR attained during this phase was  $336 \pm 21$  mg N/L-reactor/d ( $1688 \pm 106$  mg N/kg-pellet/d) corresponding to about 60% of the NCR obtained for the synthetic wastewater. Under similar change in conditions, Yamamoto et al. (2008) observed an activity decrease from about 10–20% the activity obtained for the synthetic wastewater treatment in a fixed-bed anammox reactor. Measured stoichiometric



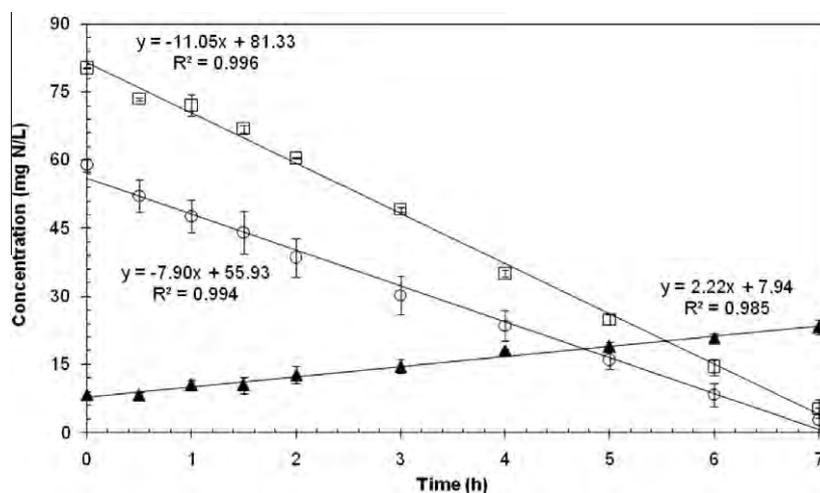
**Fig. 4.** Stoichiometric molar ratios obtained with the immobilized anammox pellets during feeding trials with synthetic wastewater and partially nitrified swine wastewater.  $\text{NO}_2^-:\text{NH}_4^+$  (■),  $\text{N}_2:\text{NH}_4^+$  (◇),  $\text{NO}_3^-:\text{NH}_4^+$  (▲). Horizontal lines indicate the corresponding stoichiometric molar ratios of the anammox sludge NRRL B-50286 (*Candidatus Brocadia caroliniensis*) used for immobilization (1.30, 1.06, and 0.18).

ratios were slightly different from those when processing synthetic wastewater, which were consistent with the ratios of the parent reactor (Fig. 4). This deviation was especially evidenced in the case of the removal ratios of  $\text{NO}_2^-$  to  $\text{NH}_4^+$  that declined from 1.30 to 1.05 in the first 10 days and recovered back up to initial value of 1.30 at day 30. Thus, average stoichiometric ratios obtained when feeding partially nitrified swine wastewater (58% conversion  $\text{NH}_4^+\text{-N}$  to  $\text{NO}_2^-\text{-N}$ ) were 1.00:1.18:0.13:1.03. This behavior was contrary to that observed by Yamamoto et al. (2008) when processing partially nitrified swine wastewater digester liquor (58% conversion  $\text{NH}_4^+\text{-N}$  to  $\text{NO}_2^-\text{-N}$ ) in a fixed-bed anammox reactor. They described that presence of biodegradable organic carbon (COD = 141 mg/L) and suspended solids in the influent inhibited the treatment performance and caused some heterotrophic denitrification to occur as evidenced by a significant increase of the  $\text{NO}_2^-$  to  $\text{NH}_4^+$  ratio. It increased from 1.26 using synthetic wastewater to 1.67 using partially nitrified swine wastewater (Yamamoto et al., 2008). Although the COD of the partially nitrified swine wastewater in our study was higher (193 mg/L), an increased  $\text{NO}_2^-$  to  $\text{NH}_4^+$  reaction ratio (indicating concurrent heterotrophic denitrification) was not detected. Thus, the COD was mainly attributable to non-biodegradable organic

carbon or to colloidal solids that were not retained in the immobilized pellet reactor. Working with anammox sludge entrapped in PEG gel carriers, Furukawa et al. (2009) reported that high influent TSS after partial nitrification of swine wastewater had weak adherence to gel pellets (PEG) and that reaction ratios obtained using the PEG gel carriers and partially nitrified swine wastewater were typical of non-inhibited anammox reaction (1.00:1.31:0.17 for removal of  $\text{NH}_4^+$ , removal of  $\text{NO}_2^-$ , and production of  $\text{NO}_3^-$ , respectively). These results suggest that the use of the gel carriers appears to provide additional protection to anammox microorganisms that could otherwise be negatively affected by colloidal solids in partially nitrified swine wastewater.

### 3.5. Immobilized pellets activity using batch treatment

Activity of the anammox pellets was also evaluated through a discontinuous batch test by modifying the CSTR set-up from continuous to batch at the end of the recovery period (day 25). Data in Fig. 5 show the obtained concentration profiles of  $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_2^-\text{-N}$ , and  $\text{NO}_3^-\text{-N}$  during the assay and the corresponding performance evaluation is presented in Table 2. The NCR obtained in the batch was  $459 \pm 10$  mg N/L-reactor/d (2307 mg N/



**Fig. 5.** Concentration profiles of ammonium (○), nitrite (□), and nitrate (▲) during batch testing experiments of immobilized anammox pellets after recovery (day 25). Error bars represent  $\pm$  the standard error from duplicate reactors. Corresponding N-removal efficiency calculations are shown in Table 2.

**Table 2**  
Performance of immobilized anammox pellets in batch treatment.

Parameter	Units	Average (±s.e.) <sup>a</sup>
Time length of the batch test	h	7
Initial concentrations: NH <sub>4</sub> <sup>+</sup> , NO <sub>2</sub> <sup>-</sup> , and NO <sub>3</sub> <sup>-</sup>	mg N/L	59 (1), 80 (0), 8 (0)
Final concentrations NH <sub>4</sub> <sup>+</sup> , NO <sub>2</sub> <sup>-</sup> , and NO <sub>3</sub> <sup>-</sup>	mg N/L	3 (3), 5 (2), 23 (2)
Ammonium removal rate <sup>b</sup>	mg N/L/d	193 (6)
Nitrite removal rate <sup>b</sup>	mg N/L/d	266 (4)
Nitrate production rate <sup>b</sup>	mg N/L/d	53 (3)
Ammonium removal efficiency <sup>c</sup>	%	95 (5)
Nitrite removal efficiency <sup>c</sup>	%	93 (2)
Nitrogen conversion rate (NCR)	mg N/L/d	459 (10)
	mg N/kg-pellet/d	2307 (50)
Nitrogen removal rate (NRR)	mg N/L/d	406 (7)
	mg N/kg-pellet/d	2040 (35)
Nitrogen conversion efficiency (NCE)	%	94 (1)
Nitrogen removal efficiency (NRE)	%	79 (2)

<sup>a</sup> Data are average of two reactors. Values in parenthesis are standard errors.  
<sup>b</sup> Removal and production rates calculated from the slopes of concentration profiles of ammonium, nitrite and nitrate shown in Fig. 5.  
<sup>c</sup> Removal efficiencies calculated from the initial and final concentrations.

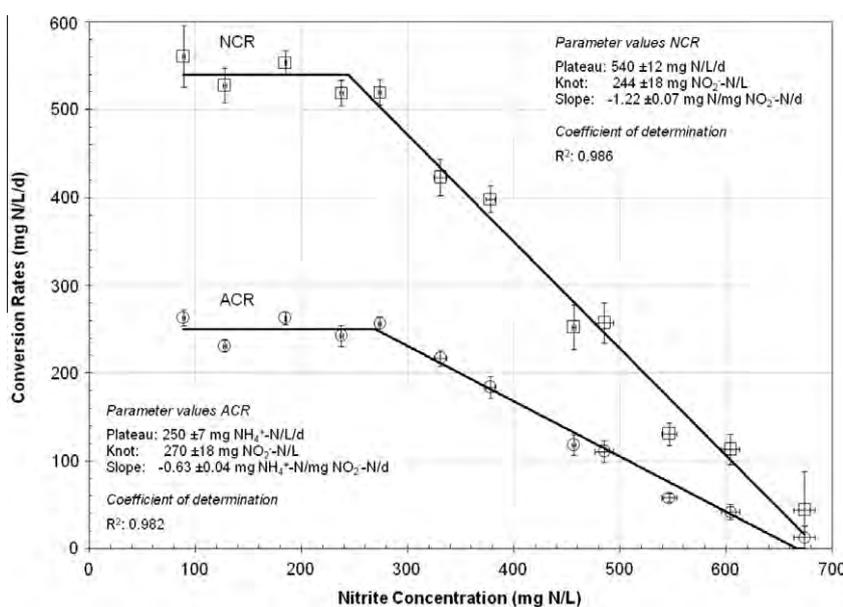
kg-pellets/d) and was similar to the NCR rate obtained under continuous-flow operation the same day (502 ± 10 mg N/L-reactor/d). The nitrogen conversion efficiencies (NCE) of the immobilized PVA pellets under batch conditions (94 ± 1%, Table 2) were higher than those obtained under continuous flow (<69%, Table 1). In addition, low final concentrations were attained for NH<sub>4</sub><sup>+</sup> (3 mg N/L) and NO<sub>2</sub><sup>-</sup> (5 mg N/L) under zero-order kinetics. Strous et al. (1999) indicated that the anammox process is not rate-limited by substrate availability up to quite low concentrations. Similarly, Ni et al. (2009) modeled granular anammox process and according to Monod kinetics calculated half-saturation constants K<sub>S</sub> (concentration of substrate at which the transformation rate is half the maximum value) of 0.73 mg NH<sub>4</sub><sup>+</sup>-N/L and 0.55 mg NO<sub>2</sub><sup>-</sup>-N/L. Fig. 5 shows that most of the concentration profile N data were higher than these K<sub>S</sub>. In this situation, a zero-order fit is particularly successful over a Monod (Bekins

et al., 1998). The results obtained indicate that the substrate diffusion into the PVA-pellets was optimal under batch operation and sufficient time and that under these conditions high removal efficiencies seem to be possible at maximum conversion rates without substrate-transport limitations.

### 3.6. Effect of nitrite on anammox activity

Nitrite is a substrate for anammox bacteria but it becomes an inhibitor of the anammox reaction at high concentrations. Continuous feeding tests were conducted to determine acceptable and inhibitory nitrite concentration levels for the immobilized anammox at the high concentrations. To accomplish this goal, more nitrite than needed for the anammox reaction was added into the reactor (nitrite concentration varied from 150 to 700 mg NO<sub>2</sub><sup>-</sup>-N/L while ammonium was unchanged 150 mg NH<sub>4</sub><sup>+</sup>-N/L). Fig. 6 shows the influence of nitrite concentration inside the reactor on anammox activity as represented by the nitrogen conversion rates (NCR) and the ammonium conversion rates (ACR). The pH inside the reactor during the overall test was measured in the range of 7.52–8.30. The nitrite concentration inside the reactor was higher than 89 mg NO<sub>2</sub><sup>-</sup>-N/L throughout the test. No negative effects in anammox activity were observed until the nitrite concentration reached a threshold concentration where increased nitrite concentration resulted in a linear decrease of activity. Such experimental behavior was best fitted by plateau-linear functions that determined the threshold inhibitory concentration (knot value) for NCR (R<sup>2</sup> = 0.986) and ACR (R<sup>2</sup> = 0.982). For NCR (nitrite + ammonium consumption), the knot value was 244 ± 18 mg NO<sub>2</sub><sup>-</sup>-N/L (confidence interval at 95%: 204–284 mg NO<sub>2</sub><sup>-</sup>-N/L). From this point forward, the plateau NCR, estimated as 540 ± 12 mg N/L/d, decreased according to a slope of -1.22 ± 0.07 mg N/mg NO<sub>2</sub><sup>-</sup>-N/d. Thus, 50% decrease in activity occurred at nitrite concentrations of 465 mg NO<sub>2</sub><sup>-</sup>-N/L and complete inhibition occurred at 687 mg NO<sub>2</sub><sup>-</sup>-N/L. Similar threshold and 50% inhibitory nitrite values were obtained from the ACR data (Fig. 6); the maximum nitrite concentration without anammox inhibition was 270 ± 18 mg NO<sub>2</sub><sup>-</sup>-N/L (confidence interval at 95%: 230–310 mg NO<sub>2</sub><sup>-</sup>-N/L) and a 50% decrease in activity occurred at 468 mg NO<sub>2</sub><sup>-</sup>-N/L.

Results obtained with immobilized anammox using strain NRRL B-50286 are in agreement with previous studies reporting



**Fig. 6.** Inhibition of anammox activity by nitrite concentration in continuous feeding tests. Data show average nitrogen conversion rates (NCR) and ammonium conversion rates (ACR) ± standard error. Regression lines are plateau-linear model indicating anammox inhibition at concentrations higher than the estimated knot value.

high resilience of the anammox process to nitrite (Dapena-Mora et al., 2007; Kimura et al., 2010). For example, Kimura et al. (2010) reported anammox inhibition only at nitrite concentrations higher than 274 mg N/L, with a 37% activity decrease when the nitrite concentration was 430 mg N/L, and cease of activity at nitrite levels higher than 750 mg  $\text{NO}_2^-$ -N/L. Dapena-Mora et al. (2007) found anammox activity decreases by 50% when nitrite concentration in a batch experiment was 350 mg N/L. Other results were plausible: total inactivation of anammox cultures have been reported at lower nitrite concentrations of 44–100 mg N/L (Egli et al., 2001; Strous et al., 1999). Kimura et al. (2010) indicated that differences in seed cultures and cultivation conditions may explain the large differences in sensitivity or tolerance of anammox to high nitrite. In the case of anammox sludge NRRL B-50286 (Candidatus *Brocadia caroliniensis*) used in this study, it grew in the parent reactor using nitrite concentrations in the synthetic medium of about 150 mg N/L during the preceding two years and 280 mg N/L the year before that. Thus, the high inhibitory nitrite boundaries of 244–270 mg N/L for anammox inhibition determined in this study support the Kimura et al. (2010) conclusion that long-term acclimation during cultivation causes tolerance to highly concentrated nitrite.

#### 4. Conclusion

This study showed that it was feasible to immobilize anammox bacteria in PVA cryogel carriers obtained by physical cross-linking through the freezing-thawing procedure. The anammox cryogels had an elastic, rubber-like appearance and were porous to anammox substrates and  $\text{N}_2$  by-product. They stayed dispersed in synthetic and swine wastewater inside continuous stirred-tank reactors without need of returning sludge. The anammox stoichiometric activity was successfully retained after immobilization. In mass balance, they achieved >80% of the potential N conversion activity. They appear to provide protection from colloidal solids in partially nitrified swine wastewater. The process was not limited by substrate availability until very low concentration achieving 95% ammonium removal efficiency, and it was resilient to high nitrite concentrations.

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#### Appendix A

Figs. A.1–A.4.

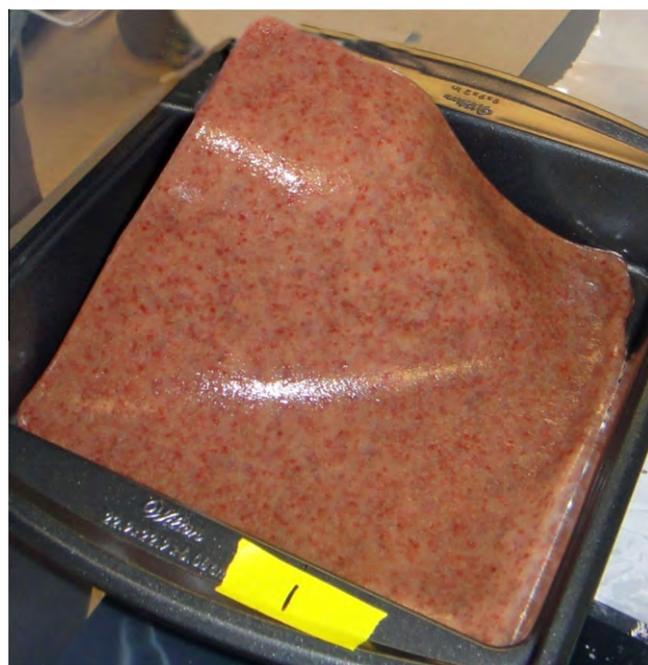


Fig. A.2. PVA-anammox gel sheet formed after freezing.

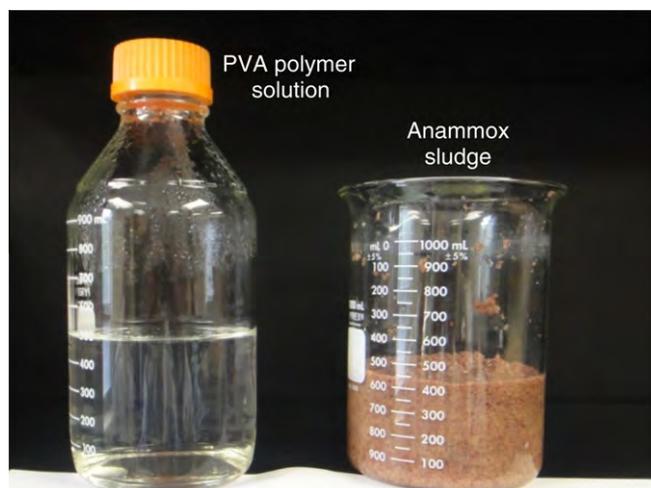


Fig. A.1. PVA polymer solution and anammox sludge used to make the immobilized anammox pellets.

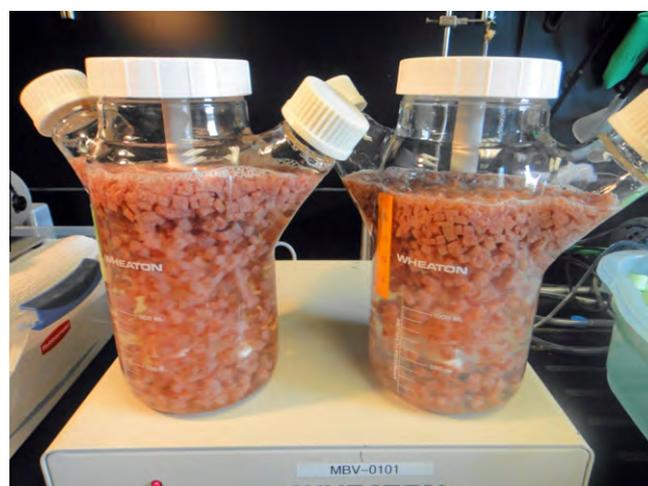


Fig. A.3. Pellets remained dispersed in the stirred reactors.



Fig. A.4. Formation of gas bubbles by anammox pellets.

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