Rapid and successful start-up of anammox process by immobilizing the minimal quantity of biomass in PVA-SA gel beads

By

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ABSTRACT

Rapid start-up of anaerobic ammonium oxidation (anammox) process in up-flow column reactors was successfully achieved by immobilizing minimal quantity of biomass in polyvinyl alcohol (PVA)-sodium alginate (SA) gel beads. The changes in the reactor performance (*i.e.*, nitrogen removal rate; NRR) were monitored with time. The results demonstrate that the reactor containing the immobilized biomass concentration of 0.33 g-VSS L⁻¹ achieved NRR of 10.8 kg-N m⁻³ d⁻¹ after 35-day operation, whereas the reactor containing the granular biomass of 2.5 g-VSS L⁻¹ could achieve only NRR of 3.5 kg-N m⁻³ d⁻¹. This indicates that the gel immobilization method requires much lower seeding biomass for start-up of anammox reactor. To explain the better performance of the immobilized biomass, the biological and physicochemical properties of the immobilized biomass were characterized and compared with the naturally aggregated granular biomass. Effective diffusion coefficient (D_e) in the immobilized biomass was directly determined by microelectrodes and found to be three times higher than one in the granular biomass. High anammox activity (*i.e.*, NH₄⁺ and NO₂⁻ consumption rates) was evenly detected throughout the gel beads by microelectrodes due to faster and deeper substrate transport. In contrast, anammox activity was localized in the outer layers of the granular biomass, indicating that the inner biomass could not contribute to the nitrogen removal. This difference was in good agreement with the spatial distribution of microbes analysed by fluorescence *in situ* hybridization (FISH). Based on these results, PVA-SA gel immobilization is an efficient strategy to initiate anammox reactors with minimal quantity of anammox biomass.

Keywords: anaerobic ammonium oxidation; startup of anammox process; gel immobilization; effective diffusion coefficient

25 1. INTRODUCTION

26 Anaerobic ammonium oxidation (anammox) process has been regarded as an efficient, cost
27 effective and environmental friendly alternative to conventional nitrogen removal process due to
28 lower oxygen demand, no external carbon source requirement, lower sludge production and less N₂O
29 gas emission (Kartal et al., 2010; Kuenen, 2008; Okabe et al., 2011). Anammox process has been
30 installed for various wastewaters treatment, and there are presently more than 100 full-scale
31 anammox plants around the world (Lackner et al., 2014). One of the remaining important challenges
32 in practical application of anammox process is slow and unstable start-up due to their slow growth
33 rate (Ali et al., 2014a; Awata et al., 2013; Jetten et al., 1999; Oshiki et al., 2011; Park et al., 2010;
34 van der Star et al., 2007). To further promote the full-scale anammox installations, it is required to
35 establish a more rapid and reliable start-up method. For rapid and successful start-up of anammox
36 process, a sufficient amount of seeding biomass is essential, and the inoculated biomass must be
37 efficiently retained in the reactor. Biomass washout due to production of N₂ gas bubbles was another
38 cause of the failure of process start-up (Chen et al., 2010; Dapena-Mora et al., 2004).

39 In order to secure enough seeding biomass, the preservation of anammox biomass could be one
40 of possible solutions (Ali et al., 2014b; Heylen et al., 2012; Rothrock et al., 2011; Vlaeminck et al.,
41 2007). Ali et al. (2014b) recently reported that anammox biomass could be stored for five months at
42 room temperature in nutrient medium containing 3 mM molybdate with periodical supplementation
43 of NH₄⁺ and NO₂⁻. However there is a limitation of biomass storage capacity in practice. It is
44 therefore desirable to determine the necessary minimal quantity of anammox biomass for successful
45 start-up.

46 In order to efficiently retain the inoculated biomass in a reactor, biomass could be immobilized in
47 gel beads, which allows us to operate the reactor at high nitrogen loading rates (*i.e.*, at short
48 hydraulic retention time) without nitrite inhibition and biomass washout (Isaka et al., 2006; Magrí et
49 al., 2012; Vogelsang et al., 1999). This reactor operation is suitable for cultivation of anammox

bacteria (Tsushima et al., 2007a). Anammox bacteria were successfully immobilized either in polyvinyl alcohol (PVA) (Magrí et al., 2012), sodium alginate (SA) (Zhu et al. 2009), mixture of PVA and SA (Ali et al., 2014b; Quan et al., 2011), or polyethylene glycol (PEG) (Isaka et al., 2011), and the reactors consisting of these immobilized gel beads could achieve the nitrogen removal rates (NRRs) of $0.58 \sim 12 \text{ kg-N m}^{-3} \text{ d}^{-1}$ within time periods of $35 \sim 180$ days. In these studies, the initial biomass concentrations were varied; $1.8 \text{ g-volatile suspended solids (VSS) (L of reactor volume)}^{-1}$ (Magrí et al., 2012), 3.5 g-VSS L^{-1} (Zhu et al., 2009), 5 g-SS L^{-1} (Quan et al., 2011), and 5.2 g-VSS L^{-1} (Isaka et al., 2011). Although the minimal biomass concentration, that is necessary for successful start-up of anammox process, has not been studied and reported so far. The minimal biomass concentration must be experimentally determined to reduce the required seeding biomass. In addition, a detailed comparison of biological and physicochemical properties between naturally aggregated granular and artificially immobilized anammox biomass has not been characterized in the previous studies.

The objective of this study was, therefore, to determine the minimal concentration of anammox biomass immobilized in gel beads that is necessary for rapid and successful start-up of anammox process. The criteria of the rapid and successful start-up of anammox reactor in this study was defined as follows; an acceptable start-up time period of anammox reactors is about a month and a target volumetric nitrogen removal rate is $10 \text{ kg-N m}^{-3} \text{ d}^{-1}$ because the first full-scale anammox reactor in Rotterdam, NL has been operated at around $7 \text{ kg-N m}^{-3} \text{ d}^{-1}$ (Lackner et al., 2014; van der Star et al., 2007) and some pilot- and lab-scale experiments using similar immobilized biomass were conducted at around $10 \text{ kg-N m}^{-3} \text{ d}^{-1}$ (Ali et al., 2014; Quan et al., 2011). The minimal concentration of anammox biomass that is necessary for meeting these criteria was determined in this study. For this purpose, different amounts of anammox biomass were immobilized in a mixture gel of PVA and SA, and the immobilized gels were packed in up-flow column reactors. The influence of the initial biomass concentration on the start-up and performance of anammox process were investigated and

compared with the reactor containing the naturally aggregated granular biomass. Furthermore, the detailed comparison of biological and physicochemical properties such as effective diffusion coefficient and anammox activity in the immobilized gel beads and granules was made on the basis of various *in situ* analyses: fluorescence *in situ* hybridization (FISH) analysis, microelectrode measurements, and ^{13}C -bicarbonate incorporation analysis by secondary ion mass spectrometry (SIMS).

2. MATERIALS AND METHODS

2.1. Anammox biomass

Naturally aggregated granular anammox biomass (average 5-mm of diameter) (hereafter referred as granular biomass), was harvested from an up-flow anammox reactor (hereafter referred as parent reactor) (Tsushima et al., 2007b) (**Fig. 1A**). The granular biomass contains mono species of anammox bacterium, “*Candidatus Brocadia sinica*”, and they accounted for more than 94% of total bacterial cells as determined by FISH (Oshiki et al., 2011).

2.2. Biomass immobilization

Granular biomass was dispersed by a mortar-pestle style tissue homogenizer (50cm³, $\Phi 6 \times 290 \times 195$ mm) into small floccular biomass (< 100 μm diameter) and diluted with culture medium (described below in section 2.3) to six different biomass concentrations *i.e.* 4.8, 4.1, 3.3, 2.3, 0.9 and 0.5 g-VSS L⁻¹. Autoclaved immobilization support solutions of 6% (w/v) PVA and 2% (w/v) SA were mixed with an equal volume of the prepared biomass suspension as described previously (Ali et al., 2014b). The mixture was dropped into a solution of 4% (w/v) CaCl₂ by using a peristaltic pump (Eyela Roller Pump RP, Tokyo, Japan) equipped with a PharMed BPT tube (ID 3.1 mm), forming spherical gel beads with 4~5-mm diameter (**Fig. 1B**). The immobilized gel beads were cured in the same CaCl₂ solution for 12 h to enhance their mechanical stability. The immobilized anammox biomass (hereafter referred as immobilized biomass) were collected and rinsed thrice with deionized

100 water and inoculated in up-flow column reactors.

101 **2.3. Reactor establishment and operation**

102 Immobilized biomass with different initial biomass concentrations were separately inoculated
103 into six up-flow glass column reactors (10-mL, $\Phi 18 \times 80$ mm, FujiRika, Osaka, Japan) with a
104 packing ratio of 70 % (v/v) (**Fig. 1C**). Anammox biomass concentrations in the reactors were 1.67,
105 1.45, 1.15, 0.82, 0.33 and 0.16 g-VSS L⁻¹ (“L⁻¹” here and hereafter referred to the volume of the
106 reactor) in reactor 1 to 6, respectively (**Table S1**). Likewise, granular biomass (Φ 4~5 mm) was
107 harvested from the parent reactor and transferred to the same 10-ml glass column reactor with a
108 packing ratio of 30% that corresponds to the biomass concentration of 2.5 g-VSS L⁻¹ (**Fig. 1D**).
109 These column reactors were fed with synthetic medium and operated for 35 days at 37°C. The
110 synthetic medium used in the present study contained; NH₄⁺ (2.5~7.5) mM, NO₂⁻ (2.5~7.5) mM,
111 CaCl₂ 100 mg L⁻¹, MgSO₄ 300 mg L⁻¹, KH₂PO₄ 30 mg L⁻¹, KHCO₃ 500 mg L⁻¹ and trace element
112 solution (van de Graaf et al., 1995). Concentrations of NH₄⁺ and NO₂⁻ were set at 2.5 mM for 0~14
113 days, and increased to 5.0 and 7.5 mM after 15- and 30-day operation, which resulted in nitrogen
114 loading rates (NLRs) of 4.3, 8.1 and 12.1 kg-N m⁻³ d⁻¹, respectively (**Table S1**). Hydraulic retention
115 time (HRT) was set at 0.42 h. Concentrations of NH₄⁺, NO₂⁻ and NO₃⁻ in the influent and effluent of
116 the reactors during operation were determined using ion chromatographs (IC-2010, TOSOH, Tokyo,
117 Japan) equipped with TSKgel IC-Anion HS and TSKgel IC-Cation columns (TOSOH) after filtration
118 using 0.45- μ m-pore-size membranes (Okabe et al., 2011).

119 **2.4. Specific anammox activity**

120 Specific anammox activity (SAA) was determined by measuring ²⁹N₂ production rate as
121 previously described (Ali et al., 2014b; Oshiki et al., 2013). Briefly, granular and immobilized
122 biomass were suspended into the anammox culture medium (without NH₄⁺ and NO₂⁻), and then 5 ml
123 of each biomass suspension was dispensed into serum vials (12.5 ml, Nichiden-Rika Glass, Kobe,
124 Japan) separately and sealed with butyl rubber stoppers and aluminium caps. To avoid oxygen

contamination, all biomass was handled in an anaerobic chamber (Coy Laboratory Products, Grass Lake Charter Township, MI, USA), and the headspace gas was exchanged with ultra-pure He gas (>99.9999%) by using a gas exchange machine (model IP-8, SANSHIN, Yokohama, Japan). The vials were pre-incubated at 37°C for at least 6 hr, and then $^{15}\text{N-NH}_4\text{Cl}$ and $^{14}\text{N-NaNO}_2$ were supplemented to each vial at a final concentration of 2.5 mM each and incubated at 37°C. Fifty microliter of headspace gas was collected after every hour by a gas tight syringe (VICI, Baton Rouge, LA, USA) and injected to a gas chromatograph (GCMS-QP2010SE, Shimadzu, Japan) equipped with a CP-Pora Bond Q fused silica capillary column (Agilent Technologies, Santa Clara, CA, USA), and $m/z = 28, 29$ and 30 were monitored. Concentrations of $^{28}\text{N}_2$, $^{29}\text{N}_2$ and $^{30}\text{N}_2$ gas were determined by gas chromatography mass spectrometry analysis (Amano et al., 2007; Waki et al., 2010). Specific ammonium oxidation rate was determined by measuring $^{29}\text{N}_2$ gas production rate. Amount of $^{29}\text{N}_2$ gas was quantified by a standard curve prepared with $^{30}\text{N}_2$ standard gas (>98% purity, Cambridge Isotope Laboratories, Tewksbury, MA, USA) (Oshiki et al., 2013). This experiment was performed in triplicate for each type of biomass. Biomass concentration was determined as protein concentration and converted to g-VSS L^{-1} as previously described (Oshiki et al., 2011). Briefly, biomass was pelleted by centrifugation (18,200 g for 10 min), and the biomass pellet was suspended in 10% (w/v) sodium dodecyl sulfate solution. After boiling for 10 min, the suspension was centrifuged at 18,200 g for 10 min. Protein concentration in the supernatant was determined by Lowry method (Lowry et al., 1951) using DC Protein Assay Kit (Bio-Rad) as described in the manufacture instruction.

2.5. FISH analysis

Granular biomass and immobilized biomass (harvested from reactor 3) were fixed with 4% (w/v) paraformaldehyde in phosphate-buffer saline (PBS; 10mM sodium phosphate buffer, 130 mM sodium chloride; pH 7.2) at 4°C for 6 hr. Biomass was then washed thrice with PBS and embedded in Tissue-Tek optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA) at -30°C. Thin sections (20 μm in thickness) of biomass were prepared using a cryostat (Reichert-Jung Cryout

1800, Leica, Bensheim, Germany) and pasted on teflon coated glass slides. FISH was performed using the probes EUB mix (FITC) and AMX 820 (TRITC) as described previously (Okabe et al., 1999b) to identify total and anammox bacterial population, respectively. Hybridized samples were observed using confocal laser-scanning microscope (TCS SP8, Leica, Japan) equipped with Ar ion and He-Ne laser. Later, obtained microscopic images were processed and analysed by Leica Application Suite Advanced Fluorescence, (LAS AF, version 4.4).

2.6. Microelectrode measurements

The steady state concentration micro-profiles of NH_4^+ , NO_2^- , NO_3^- and pH in granular and immobilized biomass were measured using liquid ion exchanger (LIX) type microelectrodes (de Beer et al., 1997a). The microelectrodes were prepared, calibrated, and operated as previously described (Okabe et al., 1999a; Rathnayake et al., 2013; Satoh et al., 2007). The synthetic medium used for microelectrode measurements was of same composition as described in section 2.3. The granular and immobilized biomass (harvested from reactor 3) were incubated in a flow chamber (2.5 L) containing anoxic medium for at least 3 h before microelectrode measurements to ensure that steady-state profiles were obtained. Microelectrodes were inserted almost perpendicular to the surface of the biomass. The liquid-biofilm interface was identified by a high precision stereo microscope (Stemi 2000, Carl Zeiss, Germany). Temperature of the anoxic medium was maintained at 37°C throughout the experiments. At least three profiles were measured for each chemical species using different aggregate as previously described (Song et al., 2013).

2.7. Effective diffusion coefficient

Effective diffusion coefficients of the granular and immobilized biomass were directly measured by using LIX-type NH_4^+ specific microelectrode as suggested previously (Stewart, 2003). Briefly, the granular and immobilized biomass were washed thrice with deionized water before measurements. Both the granular and immobilized biomass were incubated in a small flow chamber (300-mL volume) filled with 100 mL of medium containing 1 mM NH_4^+ (without NO_2^- and NO_3^-).

175 An ammonium specific microelectrode was inserted perpendicular to the respective biomass at 500
 176 μm depth from the surface. At steady microelectrode reading, 10 mL of 100 mM NH_4^+ solution was
 177 added to increase NH_4^+ concentration of bulk solution to 10 mM. The steady reading was defined as
 178 the point at which fluctuation in reading for 20 seconds became lower than 0.1 mV. The time was
 179 recorded to attain the reading corresponding to 90% of bulk solution concentration (9 mM NH_4^+).
 180 Strict anoxic condition was maintained by purging N_2 gas to avoid oxidation of NH_4^+ by nitrifiers.
 181 Three measurements were recorded for each type of biomass. Effective diffusion coefficients were
 182 calculated by using the following equation:

$$183 \quad D_e = 0.37 \times d^2 / t_{90\%} \quad (\text{Eq. 1})$$

184 Where, D_e is effective diffusion coefficient in the biomass, d is the penetration depth and $t_{90\%}$
 185 is the time required for the solute to attain 90% of the bulk solution concentration at the penetration
 186 depth d . The values of (D_{aq}), diffusion coefficients in pure water for NH_4^+ , NO_2^- and NO_3^- , were
 187 taken from literature (Yi et al., 2011). The relative effective diffusivity, described as a ratio D_e/D_{aq} ,
 188 was determined for NH_4^+ . Same relative effective diffusivity was used to determine effective
 189 diffusion coefficient for NO_2^- and NO_3^- . Furthermore, volumetric NH_4^+ and NO_2^- consumption and
 190 NO_3^- production rates were estimated from the respective solute concentration profiles by using the
 191 Fick's second law of diffusion as described previously (Lorenzen et al., 1998; Nakamura et al., 2004).
 192 Following equation (Eq. 2), derived from the Fick's second law of diffusion, was used to simulate
 193 concentration profiles on the basis of volumetric NH_4^+ and NO_2^- consumption and NO_3^- production
 194 rates, ($A_n \times D_e$). The derivation and terms used in this equation was previously explained elsewhere
 195 (Lorenzen et al., 1998).

$$196 \quad C_{n+1} = C_n + h \times [\partial C / \partial z_{n-1} + h \times A_{n-1}] \quad (\text{Eq. 2})$$

197 **2.8. Isotope microscopic analysis**

198 Granular and immobilized biomass were separately inoculated in up-flow column reactors
 199 and operated under the same operating conditions as previously mentioned in section 2.3. Biomass

concentrations in the granular and immobilized biomass reactors were 1.15 and 2.5 g-VSS L⁻¹, respectively. These reactors were continuously fed with ¹³C-NaHCO₃ (97% chemical purity, Cambridge Isotope Laboratories, MA, USA) as a sole carbon source. After 35-day operation, granular and immobilized biomass were harvested and washed thrice with phosphate buffered saline solution. Washed biomass were embedded in Tissue-Tek OCT compound and sectioned into slices (10 µm in thickness) by using a cryostat (Reichert-Jung Cryout 1800, Leica, Bensheim, Germany). These thin sections were pasted on silica wafers. An *in situ* survey of spatial distribution of ¹³C-carbon incorporation by anammox bacterial cells in the granular and immobilized biomass was performed by line profiling analysis using a secondary ion mass spectrometry SIMS (ims-1270, CAMECA, France) equipped with an ion detector (stacked CMOS-type active pixel sensor; SCAPS)(Sakamoto et al., 2007).

211

3. RESULTS AND DISCUSSIONS

3.1. Influence of initial biomass content on start-up of anammox process

Six up-flow column reactors packed with the immobilized anammox biomass (containing biomass concentrations of 0.16~1.67 g-VSS L⁻¹) were operated for 35 days at 37°C. As for comparison, a column reactor containing the granular biomass of 2.5 g-VSS L⁻¹ was also operated under the same conditions. Nitrogen loading rates were increased when the effluent NO₂⁻ concentrations in all reactors except for reactor 6 became below 15 mg L⁻¹ to avoid nitrite inhibition (**Fig. S1**). NRRs increased with time in all the reactors (**Fig. 2**). Stoichiometric ratios of consumed NO₂⁻ to consumed NH₄⁺ ($\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$) and produced NO₃⁻ to consumed NH₄⁺ ($\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$) in the reactors were in the range of 1.1~1.4 and 0.16~0.22, respectively. Those stoichiometric ratios are close to the theoretical stoichiometric ratios of anammox reaction (*i.e.* 1.15 and 0.16 for $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$ and $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$, respectively) (Lotti et al., 2014), indicating anammox was responsible for nitrogen removal in these reactors. During the operation of the column reactors, the

225 color of the immobilized biomass was changed from pale white to reddish color, indicating the
 226 growth of anammox bacteria (**Fig. 1E**). For initial few days, NRRs were dependent on the initial
 227 biomass content: the higher initial biomass concentrations resulted in higher NRRs (**Fig. 2**). The
 228 reactor 1 (containing biomass concentration of $1.67 \text{ g-VSS L}^{-1}$) could achieve NRR of $3 \text{ kg-N m}^{-3} \text{ d}^{-1}$
 229 just after 7-day operation, whereas the NRR of the reactor 5 (containing $0.33 \text{ g-VSS L}^{-1}$) was about
 230 50% of the reactor 1. However, the difference in NRR gradually became small as time passed.
 231 Almost the same NRR (approximately $7 \text{ kg-N m}^{-3} \text{ d}^{-1}$) was achieved for all reactors after 25-day
 232 operation and then reached above $10 \text{ kg-N m}^{-3} \text{ d}^{-1}$ after 33 days, except for the reactor 6 (containing
 233 the lowest biomass concentration of $0.16 \text{ g-VSS L}^{-1}$) and granular biomass reactor (containing 2.5
 234 g-VSS L^{-1}). The NRR of the reactor 6 was slowly but steadily increased to $5.8 \text{ kg-N m}^{-3} \text{ d}^{-1}$, whereas
 235 the granular biomass reactor could attain only NRR of $3.5 \text{ kg-N m}^{-3} \text{ d}^{-1}$ at the end of operation. Based
 236 on these experimental results, the minimal concentration of anammox biomass that is necessary for
 237 meeting the criteria of rapid and successful start-up of anammox process was $0.33 \text{ g-VSS L}^{-1}$ in this
 238 study. It should be noted that the granular reactor containing the highest biomass (2.5 g-VSS L^{-1})
 239 could not meet the criteria and only about 13% of the granular biomass ($0.33 \text{ g-VSS L}^{-1}$) was good
 240 enough to achieve rapid and successful start-up when the biomass is immobilized in PVA-SA gel
 241 beads.

242

243 **3.2. Specific anammox activity of immobilized biomass**

244 To explain the better performance of the immobilized biomass reactor, specific anammox
 245 activity (SAA) was determined after 35-day operation by measuring the production of mixed labelled
 246 $^{14+15}\text{N}_2$ from the cultures incubated with ^{15}N -ammonium and ^{14}N -nitrite. Reactor 3 was chosen as a
 247 representative of immobilized anammox biomass due to its stable reactor performance. The specific
 248 anammox activity (SAA) was measured only for the biomass harvested from the reactor 3 and
 249 granular biomass reactor. Production of $^{29}\text{N}_2$ is the true evidence of anammox process and can be

distinguished from the heterotrophic denitrification and other biological reactions (Holtappels et al., 2011; Kartal et al., 2007). The SAA of the immobilized biomass was $278.5 \pm 30.9 \mu\text{mol-}^{29}\text{N}_2 \text{ g-VSS}^{-1} \text{ h}^{-1}$, which was significantly higher than that of the granular biomass ($184.7 \pm 30.9 \mu\text{mol-}^{29}\text{N}_2 \text{ g-VSS}^{-1} \text{ h}^{-1}$) ($P < 0.05$) (**Table 1**). These values were within the range of previously determined SAA values for anammox biomass (Dapena-Mora et al., 2007; Isaka et al., 2008; Magrí et al., 2012). The higher SAA of the immobilized biomass supported the higher NRRs of the immobilized biomass reactors with lower biomass concentration.

3.3. Effective diffusion coefficient in immobilized biomass

The effective diffusion coefficients, D_e , in the immobilized and granular biomass were determined to explain the higher SAA in the immobilized biomass. Just like the SAA measurement, the gel beads harvested from the reactor 3 was subjected to this measurement due to its stable reactor performance. It is speculated that D_e in the immobilized biomass is different from that in the granular biomass because D_e is highly dependent on the biomass matrix such as gel polymer or bacterial biomass including microbial exopolymers (de Beer et al., 1997b; Stoodley et al., 1994; Stewart, 1998). The D_e of NH_4^+ at 37°C in the granular and immobilized biomass were determined to be $8.6 \pm 2.3 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ and $29.0 \pm 6.7 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, respectively (**Table 2**). Accordingly, the ratios of D_e/D_{aq} were calculated as 0.23 and 0.77, respectively. These values of D_e/D_{aq} were comparable to the values for biofilms (Stewart, 2003). The determined D_e values were used for the following calculation of volumetric NH_4^+ and NO_2^+ consumption rates and NO_3^- production rate on the basis of concentration profiles determined by microelectrode measurements (**Fig. 3**).

3.4. Spatial distribution and activity of anammox bacteria

In order to explain the higher SAA in the immobilized biomass than the granular biomass, the spatial distributions of anammox activity and bacteria within the immobilized and granular biomass were analyzed using microelectrode measurement and FISH analysis. The steady state concentration profiles of NH_4^+ , NO_2^- , NO_3^- , and pH in the granular and immobilized biomass were

275 measured up to a depth of 1100 μm with 100 μm step size (**Fig. 3A** and **B**). The NH_4^+ and NO_2^-
276 concentrations concomitantly decreased while the concentration of NO_3^- and pH increased inside the
277 granular and immobilized biomass, which indicates typical evidence of anammox activity.

278 In the granular biomass, high NH_4^+ and NO_2^+ consumption rates were observed only in the
279 outer 600 μm (**Fig. 3C**). Even though NH_4^+ and NO_2^- concentrations still remained at high levels, no
280 significant anammox activity was observed below 600 μm depth. This is probably because pH was
281 increased up to 8.5 and higher in the inner layer (below 600 μm), which probably inhibited the
282 anammox activity. It was reported that anammox activity was reduced to half (Awata et al., 2013;
283 Oshiki et al., 2011) and even to quarter (Ali et al., 2014a) at pH 8.5. Similar localizations of
284 anammox activity, NH_4^+ and NO_2^- consumptions, NO_3^- production and increase in pH mainly in the
285 outer 0 ~ 500 μm layer, were observed in the anammox biofilms (Cho et al., 2011; Kindaichi et al.,
286 2007; Tsushima et al., 2007a).

287 In contrast, moderate NH_4^+ and NO_2^- consumption and NO_3^- production rates were evenly
288 observed throughout the immobilized biomass (down to 1,100 μm) (**Fig. 3D**). pH around 7.6~7.8
289 was unchanged throughout the immobilized biomass. These experimental data suggested that
290 substrates and bicarbonate were rapidly replenished from the bulk solution since the effective
291 diffusion coefficient in the immobilized biomass was three times higher than one in the granular
292 biomass (**Table 2**). To our knowledge this is the first report presenting experimental evidence to
293 explain the high anammox activity in the immobilized biomass.

294 Substrate transport limitation due to the low diffusivity in the granular biomass resulted in
295 the localization of anammox activity (**Fig. 3A** and **3C**) and consequently bacterial cells (biomass) in
296 the outer layer (**Fig. 4** and **Fig. S2**). Similar localizations of bacterial cells or biomass density in the
297 anammox granules and biofilms were reported previously (Kindaichi et al., 2007; Lin et al., 2013;
298 Tsushima et al., 2007b). High biomass density and low effective diffusion of substrates created the
299 sharp decrease in NH_4^+ and NO_2^+ concentrations and pH increase in the granular biomass (**Fig. 3A**).

Anammox biomass in the inner part of the granule biomass did not contribute to the overall nitrogen removal. In contrast, due to the higher diffusivity the substrate transport was accelerated to sustain the activity of anammox bacteria throughout the immobilized biomass, which leads to the homogeneous distribution of anammox biomass (**Fig. 4B** and **Fig. S2**) and consequently the higher SAA than the granular biomass (**Table 1**). It should be noted that biomass concentration is important for the immobilized biomass reactors whereas specific surface area is more important than biomass concentration for the granular biomass reactor due to substrate transport limitation.

3.5. ^{13}C -bicarbonate incorporation

The spatial distributions of *in situ* anammox activity of the immobilized and granular biomass were further investigated by measuring ^{13}C -bicarbonate incorporation of anammox bacterial cells by using a secondary ion mass spectrometry (SIMS) (**Fig. 5**). Much higher incorporation of ^{13}C -bicarbonate was observed in the immobilized biomass than the granular biomass. Anammox bacteria present even in the inner part of the immobilized biomass significantly incorporated ^{13}C -bicarbonate, but not in the granular biomass. Adsorption and entrapment of ^{13}C -bicarbonate in gel matrix was not observed for the immobilized gel beads incubated as negative control. These results were in good agreement with the results of the microelectrode measurements and FISH analysis.

For this experiment, selection of isotope-labelled substrate and incubation time were important to avoid cross-feeding of isotope (Kindaichi et al., 2004; Okabe et al., 2005). ^{13}C -bicarbonate was used as a labelling substrate (carbon source) in this study, because anammox bacteria dominated in both the biomass (more than 94% of total bacteria) and all reactors were operated under anoxic condition to exclude the contribution of ammonium-oxidizing bacteria (AOB). Considering the doubling time and growth yield of *Ca. Brocadia sinica* (Oshiki et al., 2011) and preliminary experimental results, 35-day incubation period was chosen, in which the ^{13}C cross-feed to heterotrophic bacteria likely did not occur.

325

326 4. CONCLUSION

- 327 ● For rapid and successful start-up of anammox process, anammox biomass was immobilized in a
328 mixture gel of 6% (w/v) PVA and 2% (w/v) SA, which were then packed in up-flow column
329 reactors. The minimal initial concentration of anammox biomass that is necessary for meeting
330 the criterion of rapid and successful start-up (achieving the nitrogen removal rate (NRR) of
331 higher than $10 \text{ kg-N m}^{-3} \text{ d}^{-1}$ within a month) was determined.
- 332 ● The column reactor containing the immobilized biomass ($0.33 \text{ g-VSS L}^{-1}$) was started up much
333 faster than the reactor containing the granular biomass (2.5 g-VSS L^{-1}) and achieved higher NRR
334 ($10.8 \text{ kg-N m}^{-3} \text{ d}^{-1}$) after 35-day operation. This result indicates that PVA-SA gel immobilization
335 is an efficient strategy to initiate anammox reactors with minimal quantity of anammox biomass.
- 336 ● The effective diffusion coefficient in the immobilized biomass (gel beads) was directly
337 determined by microelectrodes and found to be more than three times higher than one in the
338 naturally aggregated granular biomass. The substrate transport was accelerated to sustain the
339 activity of anammox bacteria in the inner part of the immobilized biomass, which consequently
340 resulted in the higher nitrogen removal rate than the granular biomass.

341 A considerably small amount of anammox biomass is enough for successful establishment
342 of anammox reactors if PVA-SA gel immobilization technology is used. It often needs long periods
343 of time and many efforts to grow anammox granules. Even though granules were developed,
344 anammox biomass inside of the granules would not contribute to nitrogen removal due to diffusion
345 limitation as presented in this study. Although the gel immobilization is an efficient method to
346 establish anammox reactors, the hydrodynamic behaviour and durability of the immobilized biomass
347 for long-term operation should be investigated at pilot-scale in future study. Furthermore, this gel
348 immobilization technology should be tested for mixed AOB and anammox populations for a
349 single-stage nitrification/anammox process to promote practical application in wastewater treatment.

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Tables

Table 1: Specific anammox activity (SAA) of granular and immobilized biomass harvested from up-flow column after 35 days of operation. Anammox activity was measured based on ^{29}N - N_2 production rate from ^{15}N -ammonium and ^{14}N -nitrite.

Description		Granular Biomass	Immobilized Biomass
$^{29}\text{N}_2$ Production rate ($\mu\text{mole h}^{-1} \text{ vial}^{-1}$)	AVG	0.23	0.27
	SD	0.03	0.03
Biomass concentration ³	mg-protein vial^{-1}	0.74	0.59
	mg-VSS vial^{-1}	1.22	0.97
Specific anammox activity ($\mu\text{mole-}^{29}\text{N}_2 \text{ g-VSS}^{-1} \text{ h}^{-1}$)	AVG	184.73	278.48
	SD	30.92	30.94
	n^1	3	3
	t	-3.71	
	df^2	4	
	$p\text{-value}$	0.020	

¹ “ n ” indicates number of samples.

² “ df ” represents degree of freedom.

³ Conversion of protein concentration to VSS concentration was performed with a conversion factor of 1.64.

507 **Table 2:** Effective diffusion coefficients, D_e , and relative effective diffusivity, D_e/D_{aq} , for granular
508 and immobilized biomass.

Description	Effective diffusion coefficient, D_e ($\times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$)	
	Granular biomass	Immobilized biomass
NH_4^+	8.61	28.99
NO_2^-	8.31	27.96
NO_3^-	8.26	27.82
	Relative effective diffusivity	
D_e/D_{aq} ¹	0.23	0.77

509 ¹ “ D_e/D_{aq} ” indicates relative effective diffusivity and has no units.

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513 **Fig. 1. A)** Naturally grown granular biomass (diameter 4~5 mm) harvested from a parent anammox
514 reactor (Tsushima et al., 2007b). **B)** The homogenized anammox biomass were immobilized in
515 PVA-SA gel beads (PVA 6 % w/v, SA 2 % w/v, diameter 4~5 mm). **C)** Up-flow column reactors
516 packed with the gel beads immobilized different initial biomass concentrations at the beginning of
517 operation. More reddish gel beads indicate higher anammox biomass content. **D)** Up-flow column
518 reactor packed with the naturally aggregated granular biomass at the start of operation. **E)** The
519 up-flow column reactor after several days of operation. The gel beads exhibited vivid red color,
520 indicating anammox bacteria have grown actively in the reactor.

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522

523 **Fig. 2.** Time courses of nitrogen loading rates (NLRs) and removal rates (NRRs) of six up-flow
524 column reactors containing immobilized anammox biomass and granular biomass. These reactors
525 were operated under the same conditions at 37°C for 35 days.

526

Fig. 3. Steady state concentration profiles of ammonium (triangle), nitrite (diamond), nitrate (square) and pH (circle) in granular biomass (A) and immobilized biomass (B). Dashed line represents a liquid-solid interface of granular or immobilized biomass. These measurements were performed under anoxic condition that was maintained by purging N_2 gas. Three profiles were measured for each chemical species using three granules. Error bars indicate the range of standard deviations (SD) derived from triplicate measurements. Spatial distributions of the volumetric consumption and production rates of ammonium (black filled bars), nitrite (empty bars), and nitrate (grey filled bars) of the granular and immobilized biomass are calculated from the average measured concentration

536 profiles (**A** and **B**) and the newly determined effective diffusion coefficients (D_e) (**Table 2**) using
537 Microsoft EXCEL 2013 add-ins solver, respectively (**C** and **D**). Positive and negative values indicate
538 the consumption and production rates, respectively.

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543 **Fig. 4.** Confocal laser-scanning microscope image of thin cross-section of the granular anammox
544 biomass (**A**) and immobilized anammox biomass (**B**), showing *in situ* spatial organization of
545 anammox bacteria (yellow) and coexisting other bacteria (green). Fluorescence *in situ* hybridization
546 was performed with FITC-labeled EUB mix probe composed of equimolar EUB338, EUB338II, and
547 EUB338III (green) for most members of Eubacteria and TRITC-labeled amx820 probe (red) for
548 anammox bacteria. Both probes hybridized with anammox bacteria, resulting in yellow signal.
549 Higher abundance of anammox bacteria is detected in the outer layer of the granular biomass,
550 whereas more homogeneous distribution was found in the immobilized biomass. Scale bars represent
551 100 µm.

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Fig. 5. Carbon isotope ratios ($^{13}\text{C}/^{12}\text{C}$) of the granular (A) and immobilized biomass (B) analysed by secondary ion mass spectrometry (SIMS) through line profiling technique with a step size of 100 μm . Dashed lines (0 μm depth) represent the surface of the granular and immobilized biomass. Positive depths indicate inside of the biofilm. The values of $^{13}\text{C}/^{12}\text{C}$ at negative depths are considered as natural abundance of carbon isotope ratio. In preliminary experiments, adsorption/entrapment of ^{13}C -bicarbonate in gel matrix was not observed for the immobilized biomass. Granular and immobilized biomass were separately inoculated in up-flow column reactor and fed with nutrient medium containing ^{13}C -bicarbonate as sole carbon source for 35 days. Triplicate measurements were taken for each type biomass represented by triangle, circle and square.

PVA-SA gel immobilization is an efficient way to initiate anammox process with less biomass