For the submission to Water Research

Revised manuscript WR26050

**Simple, rapid and effective preservation and reactivation
of an anaerobic ammonium oxidizing bacterium
“*Candidatus Brocadia sinica*”**

By

Muhammad Ali^a, Mamoru Oshiki^a, and Satoshi Okabe^{a,1}

Category: Original research paper

^a *Division of Environmental Engineering, Faculty of Engineering, Hokkaido University,
North-13, West-8, Kita-ku, Sapporo, Hokkaido 060-8628, Japan.*

¹ *Corresponding author: Satoshi Okabe,*

Division of Environmental Engineering, Faculty of Engineering, Hokkaido University

North-13, West-8, Kita-ku, Sapporo, Hokkaido 060-8628, Japan

Phone & Fax: +81-(0)11-706-6266

E-mail: sokabe@eng.hokudai.ac.jp.

Abstract

It is still the biggest challenge to secure enough seeding biomass for rapid start-up of full-scale (anaerobic ammonium oxidation) anammox processes due to slow growth. Preservation of active anammox biomass could be one of the solutions. In this study, biomass of anammox bacterium, “*Candidatus Brocadia sinica*”, immersed in various nutrient media were preserved at -80°C, 4°C and room temperature. After 45, 90 and 150 days of preservation, specific anammox activity (SAA) of the preserved anammox biomass was determined by measuring $^{29}\text{N}_2$ production rate and transcription levels of *hzsA* gene encoding hydrazine synthase alpha subunit. Storage in nutrient medium containing 3 mM of molybdate at room temperature with periodical (every 45 days) supply of NH_4^+ and NO_2^- was proved to be the most effective storage technique for “*Ca. Brocadia sinica*” biomass. Using this preservation condition, 96, 92 and 65% of the initial SAA was sustained after 45, 90 and 150 days of storage, respectively. Transcription levels of *hzsA* gene in biomass correlated with the SAA ($R^2 = 0.83$), indicating it can be used as a genetic marker to evaluate the anammox activity of preserved biomass. Furthermore, the 90-day-stored biomass was successfully reactivated by immobilizing in polyvinyl alcohol (6%, w/v) and sodium alginate (2%, w/v) gel and then inoculated to up-flow column reactors. Total nitrogen removal rates rapidly increased to $7 \text{ kg-N m}^{-3} \text{ d}^{-1}$ within 35 days of operation. Based on these results, the room temperature preservation with molybdate addition is simple, cost-effective and feasible at a practical scale, which will accelerate the practical use of anammox process for wastewater treatment.

Keywords:

Preservation of anammox biomass; start-up of anammox reactor; *hzsA* gene expression;

45   **1   INTRODUCTION**

46   Anaerobic Ammonium Oxidation (anammox) bacteria oxidize ammonium (NH_4^+)
47   using nitrite (NO_2^-) as an electron acceptor under anoxic condition, resulting in the
48   production of nitrogen gas (N_2) (Kartal et al., 2013). Anammox has revolutionized
49   treatment of wastewater streams highly concentrated in $\text{NH}_4\text{-N}$, due to lower oxygen, no
50   external carbon source demand and less N_2O production (Kartal et al., 2010; Kuenen,
51   2008; Okabe et al., 2011a; Mulder et al., 1995). On the other hand, integration of
52   anammox process into full-scale wastewater treatment system is still a challenge, due to
53   its slow growth rate with doubling time typically 1-2 weeks (Awata et al., 2013; Jetten
54   et al., 2005; Oshiki et al., 2011; van der Star et al., 2008), causing slow startup of the
55   process (Cho et al., 2011; Park et al., 2000; Schmid et al., 2005; Strous et al., 1998;
56   Tsushima et al., 2007b; van der Star et al., 2007).

57   Inoculation of sufficient amount of active anammox biomass is essential to achieve
58   the rapid and stable start-up of anammox process for wastewater treatment. However, it
59   is difficult to cultivate and maintain a large amount of active biomass in laboratory or
60   industry in advance. In order to secure enough seeding biomass, inoculation with
61   preserved anammox biomass could be one of possible solutions. Several techniques are
62   available for the preservation of microbial cultures e.g. refrigeration, freezing (-20°C to
63   -200°C), lyophilization, or any combination of these. Although these techniques have
64   been successfully used to preserve the cultures of various micro-organisms, only a few
65   researches have attempted preserving anammox bacteria. Vlaeminck et al. (2007)
66   demonstrated that two-month storage of anammox biomass obtained from an oxygen
67   limited autotrophic nitrification denitrification (OLAND) process at -20°C resulted in
68   irreversible inactivation of anammox bacteria. Rothrock et al. (2011) have reported that

69 pre-freezing with liquid nitrogen (-200°C) was required for long-term preservation of
70 anammox bacteria via lyophilization. Recently, Magri et al. (2012) preserved anammox
71 biomass immobilized in polyvinyl alcohol (PVA) gel at -8 °C for 17 h but anammox
72 activity was severely damaged.

73 In addition, anammox activity was not accurately evaluated in these researches
74 because only NH_4^+ , NO_2^- or NO_3^- concentration was measured to evaluate the anammox
75 activity. Anammox activity can be evaluated accurately by culturing with ^{15}N -
76 ammonium and ^{14}N -nitrite, which produces mixed labeled $^{14+15}\text{N}_2$. Production of $^{14+15}\text{N}_2$
77 is the true evidence of anammox reaction and distinguished from the heterotrophic
78 denitrification which produces only $^{14+14}\text{N}_2$ (Kartal et al., 2007). More recently, Heylen
79 et al. (2012) introduced a protocol for long-term cryopreservation of single cell
80 anammox biomass using dimethyl sulfoxide (DMSO) as a cryoprotective agent (CPA).
81 Unfortunately, lyophilization and cryopreservation with CPA seems to be impracticable
82 at mass scale due to complex procedures and costly chemicals. Therefore, a simple,
83 effective and economical long-term preservation and storage method of anammox
84 biomass is urgently needed.

85 The aim of the present study was to develop a simple, rapid and effective
86 preservation method for anammox biomass of “*Ca. Brocadia sinica*”, which is feasible
87 at a practical scale. Granular anammox biomass and anammox biomass immobilized in
88 PVA and sodium alginate (SA) were preserved in various culture media for up to 150
89 days under -80°C, 4°C and ambient temperature (15~25°C). In addition, the influence of
90 the periodical nutrient (NH_4^+ and NO_2^-) supply and replacement of the entire culture
91 media during the preservation periods on the maintenance of anammox activity was
92 examined. The anammox activity was measured after 45, 90 and 150 days of

93 preservation by determining 1) specific $^{14+15}\text{N}_2$ production rates and 2) transcription
94 levels of *hzsA* gene encoding hydrazine synthase alpha subunit. Furthermore, anammox
95 biomass preserved for 90 days was immobilized in PVA and SA gel and then inoculated
96 in up-flow column reactors, and the nitrogen removal performances were monitored for
97 35 days to demonstrate whether the preserved biomass can be reactivated rapidly and
98 used as a seeding biomass.

99

100 **2 MATERIALS AND METHODS**

101 **2.1 Anammox biomass**

102 Granular anammox biomass was harvested from a continuous up-flow column
103 bioreactor being operated in our laboratory for many years (Tsushima et al., 2007a).
104 Operating conditions and other details about the parent reactor were presented
105 previously (Tsushima et al., 2007b). In the granular anammox biomass, an anammox
106 bacterium, “*Ca. Brocadia sinica*”, accounted for more than 90% of total biomass as
107 determined by fluorescence *in-situ* hybridization analysis (Cho et al., 2010; Kindaichi et
108 al., 2007) with AMX820 and EUB mix oligonucleotide probes (Oshiki et al., 2011).

109 The immobilized anammox biomass was harvested from another up-flow column
110 reactor (volume; 220 ml) (Bio column reactor, Fujisaki, Osaka, Japan) operated at 37°C
111 with continuous supply of inorganic media containing NH_4^+ and NO_2^- (5mM each).
112 Immobilized anammox biomass was prepared from granular anammox biomass as
113 described in section 2.3., and inoculated in the up-flow reactor with 70% (v/v) packing
114 ratio. The up-flow column reactor was operated at a hydraulic retention time (HRT)
115 ranged between 3.0~0.4 h, resulting in the nitrogen removal rate of $6.1 \text{ kg-N m}^{-3} \text{ d}^{-1}$ at 27
116 days of operation. Immobilized anammox biomass was harvested at 27 days of

operation by using a spatula and subjected to preservation experiment.

2.2 Inorganic media

Inorganic media used in the present study contained; NH_4^+ , NO_2^- , CaCl_2 (100 mg L^{-1}), MgSO_4 (300 mg L^{-1}), KH_2PO_4 (30 mg L^{-1}), KHCO_3 (500 mg L^{-1}) and trace element solution (van de Graaf et al., 1996). Concentrations of NH_4^+ and NO_2^- were set in the range 0~5 mM. Prepared media was purged by N_2 for more than 30 min to remove dissolved oxygen.

2.3 Gel immobilization of anammox bacterial cells

Granular anammox biomass was manually dispersed into floccular biomass (<100 μm diameter) by a mortar-pestle style tissue homogenizer (Thomas Scientific, Swedesboro, NJ, USA) and suspended in inorganic media at concentration of 4.0 g-VSS L^{-1} . The biomass suspension was mixed well with an equal volume of immobilization support solution containing PVA (6%, w/v) and sodium alginate (SA) (2%, w/v). The gel beads were prepared by dropping mixed solutions into a solution of CaCl_2 (4% w/v) by using a peristaltic pump (Eyela Roller Pump RP, Tokyo, Japan) equipped with a Pharm Med BPT tube (ID 3.1 mm). Those procedures enabled to form spherical gel beads with average diameter of 4~5 mm (**Fig. S1**). The gel beads were cured in the same CaCl_2 solution for 12 h to enhance physical strength of the gel beads. The prepared immobilized anammox biomass were rinsed with sterilized water three times, and then used for inoculation.

2.4 Preservation experiments

Granular anammox biomass and immobilized anammox biomass were washed with inorganic media without NH_4^+ and NO_2^- to remove any residual nutrients prior to preservation. The washed biomass (2.5 g-VSS) was dispensed into 10-ml glass serum

141 vials (Nichiden-Rika Glass, Kobe, Japan), which were sealed with butyl rubber stoppers
142 and aluminum caps and preserved under conditions indicated in **Table 1**. As for
143 biomasses preserved at 4°C and ambient temperature, 7.0 ml of anoxic inorganic media
144 containing 5 mM of NH_4^+ and NO_2^- was dispensed into vials. In addition, NO_3^- (5.7
145 mM) and sodium molybdate (3 mM) were supplemented to some vials as redox buffer
146 and an inhibitor of microbial sulfate reduction, respectively. Inorganic media was not
147 dispensed into vials preserved at -80°C. During preservation, periodical exchange of
148 entire inorganic media and supplementation of NH_4^+ and NO_2^- were further conducted
149 for some vials. For granular anammox biomass preserved at 4°C, entire inorganic
150 nutrient media was exchanged every 15 days. NH_4^+ and NO_2^- (final concentration each
151 5 mM) was supplemented to granular anammox biomass preserved at ambient
152 temperature after 45 days of incubation. Triplicate vials were prepared for each
153 preservation condition.

154 **2.5 Activity test**

155 Biomass preserved for 45, 90 and 150 days was harvested from vials and subjected to
156 SAA measurement as previously described by Oshiki et al. (2013). Briefly, the
157 preserved vials were left on desk until temperature inside of vials was equivalent to
158 room temperature (20~25°C). No heating of vials were undertaken to relieve heat shock
159 stress of biomass. Biomass collected from the vials were dispersed by mortar-pestle
160 style tissue homogenizer into small floccular biomass (< 100 μm diameter) and diluted
161 with inorganic media without NH_4^+ and NO_2^- to final concentrations of 0.5~1.0 mg-VSS
162 ml^{-1} . Two millilitre of biomass suspension was dispensed into 10-ml glass serum vials,
163 which were sealed with butyl rubber stoppers and aluminum caps. All these procedures
164 were performed in an anaerobic chamber (Coy Laboratory Products, Grass Lake Charter

165 Township, MI) where oxygen concentration was < 1 ppm. Headspace in the vials was
166 exchanged with highly pure He gas (>99.9999%) by vacuuming and purging. ¹⁵N-
167 NH₄Cl and ¹⁴N-NaNO₂ (each 2.5 mM) were added from anoxic stock solutions to each
168 vial, and then the vials were incubated under anoxic condition at 37°C for 5 h. Fifty
169 microliter of headspace gas was collected after every hour by a gas tight syringe (VICI,
170 Baton Rouge, LA, USA) and injected to a gas chromatograph. All the batch tests were
171 performed in triplicate for each preservation condition.

172 **2.6 Transcription analysis of *hzsA* gene**

173 Total RNA was extracted by using Rneasy Mini Kit (Qiagen, Valencia, CA, USA)
174 with minor modification. Biomass sample was suspended in 0.5 ml of PBS buffer and
175 0.75 ml of phenol:chloroform:isoamyl alcohol (125:24:1, pH 4.0) and transferred into a
176 lysing matrix E tube (MP Biomedicals, Tokyo, Japan). The tube was agitated in a
177 FastPrep bead-beating system (MP Biomedicals, Tokyo, Japan) for 90s at 5.5 m/s. After
178 centrifugation at 15,000 g for 10 min at 4°C, the supernatant was mixed with 0.4 ml of
179 buffer RLT and 0.8 mL of 70% EtOH. The suspension was passed through a Rneasy
180 column, and the column was subsequently washed with buffer RW1 and buffer RPE.
181 Finally, extracted total RNA was eluted from the column by using Dnase-free distilled
182 water. All buffers were prepared and used as following an instruction manual provided
183 by manufactures (Qiagen, Valencia, CA, USA). Contaminating DNA molecules were
184 removed by using Rnase-free Dnase Set included in a Rneasy Mini Kit (Qiagen,
185 Valencia, CA, USA). RNA concentration was determined by using a NanoVue Plus
186 spectrophotometer (GE Healthcare, Tokyo, Japan).

187 Reverse transcription was performed using PrimeScript^{TM} II 1st strand cDNA
188 Synthesis Kit (TaKaRa Bio, Otsu, Japan) with random hexamers, in accordance with

189 manufacturer's instructions. The synthesized cDNA was subjected to quantitative PCR
190 analysis using Applied Biosystems 7500 Real-time PCR System (Applied Systems,
191 California, USA). PCR mixture consisted of 12.5 μ l of 2 x SYBR® Premix Ex Taq™
192 (Perfect Real Time; TaKaRa Bio), 0.2 μ mol/l of each primer, and template cDNA (2.5
193 ng) at a final volume of 25 μ L. Primers used for *hzsA* gene expression analysis were
194 *hzsA_F* (5'-CACGTGACCGGTAATTCTCT-3') and *hzsA_R* (5'-
195 GGTATTTTGAAGGAGACTGG-3') (Harhangi et al., 2012). The thermal profile used
196 for PCR started with an initialization step of 10 min at 94°C, followed by 35 cycles (15s
197 at 94°C and 1min at 60°C), followed by melting curve analysis. All samples were
198 quantified in triplicate. Amplification of *hzsA* was ascertained by determining the
199 nucleotide sequences of PCR amplicon by Sanger sequencing method.

200 In order to normalize the level transcription of *hzsA*, abundance of *hzsA* in total
201 genomic DNA was also determined by quantitative PCR analysis. Total genomic DNA
202 was extracted by using Fast DNA SPIN kit (MP Biomedicals, Tokyo, Japan) according
203 to the standard protocol provided by the manufacturer. Concentration of DNA molecules
204 was determined spectrophotometrically, and 2.5 ng of total genomic DNA was used in
205 25 μ l PCR mixture and subjected to quantitative PCR analysis.

206 **2.7 Start up of anammox reactor by using preserved biomass**

207 Granular anammox biomass preserved at ambient temperature for 90 days was
208 immobilized into gel bead as described in the section 2.3, and inoculated to up-flow
209 column reactors (volume; 10 ml) with a packing ratio of 70 % (v/v). The column
210 reactors were operated at 37°C with continuous supply of inorganic media containing
211 NH_4^+ and NO_2^- . The nitrogen removal rate was monitored for 35 days of operation. The
212 reactors were operated with a hydraulic retention time of 0.42 h. Concentrations of

213 NH_4^+ and NO_2^- in the inorganic media were set at 2.5 mM until 24 days of operation,
214 and then increased to 5 mM, which corresponded to the nitrogen loading rates of 4.5
215 and $9.0 \text{ kg-N m}^{-3} \text{ d}^{-1}$, respectively.

216 **2.8 Chemical Analysis**

217 Concentrations of $^{29}\text{N}_2$ and $^{30}\text{N}_2$ gas were determined by gas chromatography mass
218 spectrometry analysis (Waki et al., 2010; Yoshinaga et al., 2011). Headspace gases were
219 analyzed by a gas chromatograph GCMS-QP2010SE (Shimadzu, Japan) equipped with
220 a CP-Pora Bond Q fused silica capillary column (Agilent Technologies, Santa Clara, CA,
221 USA), and $m/z = 29$ and 30 were monitored. Amount of $^{29}\text{N}_2$ gas was quantified by a
222 standard curve prepared with $^{30}\text{N}_2$ standard gas (>98% purity) (Cambridge Isotope
223 Laboratories, Tewksbury, MA, USA) (Oshiki et al., 2013).

224 Biomass concentration was determined as protein concentration as previously
225 described (Oshiki et al. 2011). Briefly, biomass was pelleted by centrifugation (18,200 g
226 for 10 min), and the biomass pellet was suspended in 10% (w/v) sodium dodecyl sulfate
227 solution. After boiling for 10 min, the suspension was centrifuged at 18,200 g for 10
228 min. Protein concentration in the supernatant was determined by Lowry method (Lowry
229 *et al.*, 1951) using DC Protein Assay Kit (Bio-Rad, Tokyo, Japan) and bovine serum
230 albumin as protein standard. Conversion of protein concentration to VSS concentration
231 was performed with conversion coefficient 1.64 (Oshiki et al. 2011).

232 Concentrations of NH_4^+ , NO_2^- and NO_3^- were determined using ion
233 chromatographs (IC-2010, TOSOH, Tokyo, Japan) equipped with TSKgel IC-Anion HS
234 or TSKgel IC-Cation columns (TOSOH, Tokyo, Japan) after filtration using $0.2 \mu\text{m}$ -
235 pore-size membranes (Okabe et al., 2011b).

236 **2.9 linear regression analysis**

237 Linear regression analysis was performed to examine a correlation between
238 transcription level of *hzsA* and specific anammox activities. The correlations were
239 examined using Microsoft Excel 2013.

240

241 **3 RESULTS AND DISCUSSIONS**

242 **3.1 Storage at -80°C**

243 The specific anammox activities (SAAs) of fresh granular and immobilized
244 biomass before storage were ascertained as 302.7 ± 57.6 and 554.0 ± 61.6 $\mu\text{mole-N g-}$
245 $\text{VSS}^{-1} \text{ h}^{-1}$, respectively. The percentage of SAA recovered from the granular and
246 immobilized anammox biomass after storage at -80°C was determined (**Fig 1**). Granular
247 biomass showed 59% recovery of SAA after 45 days but no activity was observed after
248 90 and 150 days. Heylen et al. (2012) observed about 40% recovery of SAA of granular
249 biomass of “*Ca. Kuenenia stuttgartiensis*” after storing at -80°C for about 200 days.
250 However, it was also reported that preservation success was always strain dependent as
251 entirely different SAA recoveries were observed for “*Ca. Kuenenia*” and “*Ca.*
252 *Scalindua*” even under the same preservation condition (Heylen et al., 2012).

253 Immobilized anammox biomass stored at -80°C could recover < 5% of SAA after
254 preservation. Previously, it was observed that anammox biomass immobilized in PVA
255 gel lost about 90% of SAA after storing at -8°C only for 17 h (Magri et al., 2012).
256 Freezing at -80°C could result in concentration of dissolved solutes in the immobilizing
257 gel around the cell, which would cause a leakage of water from the cell to maintain
258 identical solutes concentration around the cell membrane (Pegg, 2007). This would be a
259 possible reason why the freezing of immobilized anammox biomass at -80°C was
260 unsuccessful.

261 **3.2 Storage at 4°C**

262 Granular biomass stored under condition 6 showed a maximum SAA recovery of
263 89% after 45 days, whereas less than 15% of SAA was observed after 90 days (**Fig 2**).
264 Similarly, SAA could not be maintained under any condition at 4°C after 150 days of
265 storage. On the contrary, Vlaeminck et al. (2007) showed anammox activity was
266 increased by 25 to 30% during storage from 60 and 150 days under condition similar to
267 condition 5. This is probably because anammox activity was over estimated due to an
268 increase in denitrification activity since the anammox activity was not determined by
269 ¹⁵N isotopic labeling experiment. In our study, significant increase in heterotrophic
270 denitrification activity was observed during the storage (**Fig. S2**).

271 Although the entire nutrient media in vials was exchanged after every 15 days
272 (condition 3, 4 and 5), relatively low recoveries of SAA (about 51, 37 and 38%,
273 respectively) were observed. Exchange of entire media most likely caused
274 contamination of oxygen into the vials which led to the decrease in SAA. Therefore,
275 entire media exchange strategy was not applied to any of the storage conditions for
276 immobilized anammox biomass. Immobilized anammox biomass stored under condition
277 10 showed the SAA recovery of 60 and 33% after 45 and 90 days of storage,
278 respectively (**Fig 3**). Recovery of SAA from immobilized biomass is relatively low as
279 compared to granular biomass. These experimental results suggest that the storage of
280 granular biomass is better due to the good recovery of SAA and no immobilization steps.

281 **3.3 Storage at room temperature**

282 In the previous storage experiments, it was observed that heterotrophic
283 denitrification activity increased significantly (**Fig. S2**). To suppress the heterotrophic
284 denitrification activity during storage, Penicillin G was added as an inhibitor (van de

285 Graaf et al., 1996) under condition 13. Granular biomass stored under condition 12
286 showed the maximum SAA recovery of 96, 92 and 65% after 45, 90 and 150 days of
287 storage, respectively (**Fig 4**). Under condition 12, sodium molybdate was added as
288 inhibitor for sulfate-reducing bacteria (SRB) at a final concentration of 3 mM as
289 recommended by Fukui et al. (1997) and NH_4^+ and NO_2^- were supplemented after every
290 45 days at a final concentration of 5 mM. The color of anammox biomass, except for
291 biomass stored under condition 12, turned into black after 90 days, indicating sulfate
292 reduction occurred without molybdate addition (**Fig. S1**). It was reported previously that
293 1~2 mM of sulfide reduced SAA by 60% and the sulfide concentration above 5 mM
294 completely inhibited anammox activity (Dapena-Mora et al., 2007). Addition of 3 mM
295 of molybdate effectively inhibited sulfate reduction, resulting in the salvation of
296 anammox activity at room temperature for even 150 days. Nitrate addition, instead of
297 molybdate addition, could be an alternative way to inhibit sulfate reduction. For
298 example, about 30 and 31% of anaerobic ammonium removal activity were recovered
299 after 60 and 150 days of storage, respectively, under the condition similar to condition
300 14 with nitrate addition (80 mg-N/L) every 15 days (Vlaeminck et al., 2007).

301 Instead of the entire media exchange, only NH_4^+ and NO_2^- were supplemented to
302 each vial after every 45 days at a final concentration of 5 mM, which lead to maintain
303 high SAA even after 150 days of storage. Addition of nitrate after every 15 days was
304 more laborious as compared to preservation condition of our study in which molybdate
305 was added only once at the start of preservation. Storage at room temperature in nutrient
306 medium (with periodical supply of NH_4^+ and NO_2^- every 45 days) containing molybdate
307 (3 mM) was proved to be the most effective condition among those tested in this study.

308 **3.4 Comparison of *hzsA* gene expression with $^{29}\text{N}_2$ production rate**

309 Total RNA was extracted from preserved biomass under the conditions 1 and 3 ~ 7,
 310 and the transcription level of *hzsA* was determined by quantitative RT-PCR. **Fig. 5**
 311 represented a correlation between the transcription level of *hzsA* normalized by DNA
 312 and specific anammox activity. In the **Fig.5**, the transcription level of *hzsA* was
 313 expressed as Ct values, where the Ct values are inversely proportional to the log 10 of
 314 the copy number present; *i.e.*, lower values of $2^{\text{Ct(RNA-DNA)}}$ means that *hzsA* was more
 315 transcribed in biomass. Linear regression analysis revealed a significant correlation (R^2
 316 = 0.83) between the transcription level of *hzsA* and specific anammox activities,
 317 showing that the transcription level of *hzsA* was higher in active biomass (*i.e.*, the
 318 biomass showing higher specific anammox activities). Along with several other
 319 methods, the real-time PCR is a very useful technique to detect the presence and activity
 320 of anammox bacteria (Tsushima et al. 2007a). Though, 16S rRNA gene is not always
 321 related to the physiology or activity of the target microorganism, and the current
 322 anammox primers do not capture all diversity (Schmid et al., 2005). However, hydrazine
 323 synthase (*hzsA*) primers are more specific than the primers targeting the 16S rRNA,
 324 hydrazine oxidoreductase (*hzo*) or nitrite reductase (*nirS*) genes, because these genes are
 325 also present in other bacteria (Harhangi et al., 2012; Li et al., 2010). Hence, the *hzsA*
 326 gene expression analysis validated the results of SAA obtained through $^{29}\text{N}_2$ production
 327 rate.

328 **3.5 Reactivation of the stored biomass**

329 Gel immobilization technique for anammox biomass provides various advantages,
 330 *i.e.*, the high nitrogen loading rate can be achieved at short hydraulic retention times
 331 without nitrite inhibition and washout of cells, it is easy to separate liquid and solid
 332 phase in the reactor, and a small amount of initial biomass is required (Isaka et al., 2011;

Kimura et al., 2013; Magri et al., 2012; Tsushima et al., 2007b). Therefore, 90-day-stored biomass under conditions 11, 12, 13 and 14 (SAA of 133.0 ± 9.7 , 208.6 ± 6.8 , 21.4 ± 3.4 , and 69.8 ± 13.2 $\mu\text{mole-N g-VSS}^{-1} \text{ h}^{-1}$, respectively) were immobilized in PVA-SA gel and inoculated in the reactor 1, 2, 3 and 4, respectively (**Fig. S1**). Time courses of nitrogen removal rates (NRR) of these reactors were presented in **Fig 6**. The stoichiometric ratios of the consumed NO_2^- to the consumed NH_4^+ ($\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$) and produced NO_3^- to the consumed NH_4^+ ($\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$) were in the range of 1.1~1.4 and 0.21~0.27, respectively. These stoichiometric ratios are close to theoretical values of 1.32 and 0.26 for $\Delta\text{NO}_2^-/\Delta\text{NH}_4^+$ and $\Delta\text{NO}_3^-/\Delta\text{NH}_4^+$, indicating anammox was responsible for nitrogen removal in these reactors.

After 20 days of operation, the NRR of each anammox reactor became comparable, regardless of their different preservation conditions, namely different SAA. The NRRs increased significantly and ultimately reached $7 \text{ kg-N m}^{-3} \text{ d}^{-1}$ at the end of operation (after 35 days), showing the rapid reactivation of 90-day-stored anammox biomass. Likewise, Magri et al. (2012) revealed that about 70% of SAA was lost during storage, but the SAA was recovered after one month of reactivation. Based on this experimental result, anammox activity lost during storage at room temperature can be recovered within a short time period, suggesting that the stored anammox biomass could be directly used for a rapid start-up of anammox process without any additional reactivation steps.

353

354 **4 CONCLUSION**

In this study, it was shown that anammox biomass can be stored effectively for longer period of time at room temperature in nutrient medium containing molybdate (3

357 mM) with only supplementation of NH_4^+ and NO_2^- every 45 days. Using this
358 preservation condition, 96, 92 and 65% of the initial specific anammox activity (SAA)
359 were recovered after 45, 90 and 150 days, respectively. Storage at room temperature
360 does not require any special equipment and skill and thus is a simple, easy and cost-
361 effective method for biomass preservation. Furthermore, rapid reactivation of the
362 preserved biomass could be achieved by immobilizing in PVA-SA gel. The combination
363 of room temperature storage and PVA-SA immobilization techniques would be feasible
364 at a practical scale and thus accelerate the practical use of anammox process for
365 wastewater treatment. However, since these preservation and reactivation conditions
366 were tested only for *Ca. Brocadia sinica*, further study is required for phylogenetically
367 different anammox bacteria to establish a general storage protocol for anammox bacteria.

368

369 **ACKNOWLEDGEMENT**

370 Muhammad Ali expresses his utmost gratitude to the Ministry of Education,
371 Culture, Sports, Science and Technology (MEXT) for providing Ph.D. scholarship. M.
372 Oshiki was supported by a grant-in-aid for JSPS Fellows from the Japan Society for the
373 Promotion of Science (JSPS). This research was financially supported by Core Research
374 for Evolutional Science and Technology (CREST) project from Japan Science and
375 Technology Agency (JST).

376

377 REFERENCES

- 378 Awata, T., Oshiki, M., Kindaichi, T., Ozaki, N., Ohashi, A., Okabe, S. 2013. Physiological
379 Characterization of an Anaerobic Ammonium-Oxidizing Bacterium Belonging to the
380 "Candidatus Scalindua" Group. *Appl. Environ. Microbiol.* 79(13), 4145-4148.
- 381 Cho, S., Takahashi, Y., Fujii, N., Yamada, Y., Satoh, H., Okabe, S. 2010. Nitrogen removal
382 performance and microbial community analysis of an anaerobic up-flow granular bed
383 anammox reactor. *Chemosphere.* 78(9), 1129-1135.
- 384 Dapena-Mora, A., Fernandez, I., Campos, J.L., Mosquera-Corral, A., Mendez, R., Jetten, M.S.M.
385 2007. Evaluation of activity and inhibition effects on Anammox process by batch tests
386 based on the nitrogen gas production. *Enzyme Microb. Technol.* 40(4), 859-865.
- 387 Fukui, M., Suh, J., Yonezawa, Y., Urushigawa, Y. 1997. Major substrates for microbial sulfate
388 reduction in the sediments of Ise Bay, Japan. *Ecol. Res.* 12(2), 201-209.
- 389 Harhangi, H.R., Le Roy, M., van Alen, T., Hu, B.-I., Groen, J., Kartal, B., Tringe, S.G., Quan,
390 Z.-X., Jetten, M.S.M., Op den Camp, H.J.M. 2012. Hydrazine Synthase, a Unique
391 Phylomarker with Which To Study the Presence and Biodiversity of Anammox Bacteria.
392 *Appl. Environ. Microbiol.* 78(3), 752-758.
- 393 Heylen, K., Ettwig, K., Hu, Z.Y., Jetten, M., Kartal, B. 2012. Rapid and Simple
394 Cryopreservation of Anaerobic Ammonium-Oxidizing Bacteria. *Appl. Environ.*
395 *Microbiol.* 78(8), 3010-3013.
- 396 Isaka, K., Itokawa, H., Kimura, Y., Noto, K., Murakami, T. 2011. Novel autotrophic nitrogen
397 removal system using gel entrapment technology. *Bioresour. Technol.* 102(17), 7720-
398 7726.
- 399 Jetten, M.S.M., Cirpus, I., Kartal, B., van Niftrik, L., van de Pas-Schoonen, K.T., Sliekers, O.,
400 Haaijer, S., van der Star, W., Schmid, M., van de Vossenberg, J., Schmidt, I., Harhangi,
401 H., van Loosdrecht, M., Kuenen, J.G., den Camp, H.O., Strous, M. 2005. 1994-2004: 10
402 years of research on the anaerobic oxidation of ammonium. *Biochem. Soc. Trans.* 33,
403 119-123.

404 Kartal, B., de Almeida, N.M., Maalcke, W.J., Op den Camp, H.J.M., Jetten, M.S.M., Keltjens,
 405 J.T. 2013. How to make a living from anaerobic ammonium oxidation. *FEMS Microbiol.*
 406 *Rev.* 37(3), 428-461.

407 Kartal, B., Kuenen, J.G., van Loosdrecht, M.C.M. 2010. Sewage Treatment with Anammox. *Sci.*
 408 328(5979), 702-703.

409 Kartal, B., Kuypers, M.M.M., Lavik, G., Schalk, J., den Camp, H.J.M.O., Jetten, M.S.M., Strous,
 410 M. 2007. Anammox bacteria disguised as denitrifiers: nitrate reduction to dinitrogen gas
 411 via nitrite and ammonium. *Environ. Microbiol.* 9(3), 635-642.

412 Kimura, Y., Itokawa, H., Noto, K., Murakami, T., Isaka, K. 2013. Stability of autotrophic
 413 nitrogen removal system under four non-steady operations. *Bioresour. Technol.* 137,
 414 196-201.

415 Kindaichi, T., Tsushima, I., Ogasawara, Y., Shimokawa, M., Ozaki, N., Satoh, H., Okabe, S.
 416 2007. In Situ Activity and Spatial Organization of Anaerobic Ammonium-Oxidizing
 417 (Anammox) Bacteria in Biofilms. *Appl. Environ. Microbiol.* 73(15), 4931-4939.

418 Kuenen, J.G. 2008. Anammox bacteria: from discovery to application. *Nat. Rev. Microbiol.* 6(4),
 419 320-326.

420 Li, M., Hong, Y., Klotz, M., Gu, J.-D. 2010. A comparison of primer sets for detecting 16S
 421 rRNA and hydrazine oxidoreductase genes of anaerobic ammonium-oxidizing bacteria
 422 in marine sediments. *Appl. Microbiol. Biotechnol.* 86(2), 781-790.

423 Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the
 424 folin phenol reagent. *J. Biol. Chem.* 193(1), 265-275.

425 Magri, A., Vanotti, M.B., Szogi, A.A. 2012. Anammox sludge immobilized in polyvinyl alcohol
 426 (PVA) cryogel carriers. *Bioresour. Technol.* 114, 231-240.

427 Mulder, A., Vandegraaf, A.A., Robertson, L.A., Kuenen, J.G. 1995. Anaerobic ammonium
 428 oxidation discovered in a denitrifying fluidized-bed reactor. *FEMS Microbiol. Rev.*
 429 16(3), 177-183.

430 Okabe, S., Oshiki, M., Takahashi, Y., Satoh, H. 2011a. N₂O emission from a partial nitrification–

431 anammox process and identification of a key biological process of N₂O emission from
 432 anammox granules. *Water Res.* 45(19), 6461-6470.

433 Okabe, S., Oshiki, M., Takahashi, Y., Satoh, H. 2011b. Development of long-term stable partial
 434 nitrification and subsequent anammox process. *Bioresour. Technol.* 102(13), 6801-6807.

435 Oshiki, M., Ishii, S., Yoshida, K., Fujii, N., Ishiguro, M., Satoh, H., Okabe, S. 2013. Nitrate-
 436 Dependent Ferrous Iron Oxidation by Anaerobic Ammonium Oxidation (Anammox)
 437 Bacteria. *Appl. Environ. Microbiol.* 79(13), 4087-4093.

438 Oshiki, M., Shimokawa, M., Fujii, N., Satoh, H., Okabe, S. 2011. Physiological characteristics
 439 of the anaerobic ammonium-oxidizing bacterium 'Candidatus Brocadia sinica'.
 440 *Microbiol.* 157(6), 1706-1713.

441 Pegg, D. 2007. Principles of Cryopreservation. in: *Cryopreservation and Freeze-Drying*
 442 *Protocols.* (Eds.) J. Day, G. Stacey. Vol. 368, Humana Press, pp. 39-57.

443 Rothrock, M.J., Jr., Vanotti, M.B., Szoegi, A.A., Garcia Gonzalez, M.C., Fujii, T. 2011. Long-
 444 term preservation of anammox bacteria. *Appl. Microbiol. Biotechnol.* 92, 147-157.

445 Schmid, M.C., Maas, B., Dapena, A., de Pas-Schoonen, K.V., de Vossenberg, J.V., Kartal, B.,
 446 van Niftrik, L., Schmidt, I., Cirpus, I., Kuenen, J.G., Wagner, M., Damste, J.S.S.,
 447 Kuypers, M., Revsbech, N.P., Mendez, R., Jetten, M.S.M., Strous, M. 2005. Biomarkers
 448 for in situ detection of anaerobic ammonium-oxidizing (anammox) bacteria. *Appl.*
 449 *Environ. Microbiol.* 71(4), 1677-1684.

450 Strous, M., Heijnen, J.J., Kuenen, J.G., Jetten, M.S.M. 1998. The sequencing batch reactor as a
 451 powerful tool for the study of slowly growing anaerobic ammonium-oxidizing
 452 microorganisms. *Appl. Microbiol. Biotechnol.* 50(5), 589-596.

453 Tsushima, I., Kindaichi, T., Okabe, S. 2007a. Quantification of anaerobic ammonium-oxidizing
 454 bacteria in enrichment cultures by real-time PCR. *Water Res.* 41(4), 785-794.

455 Tsushima, I., Ogasawara, Y., Kindaichi, T., Satoh, H., Okabe, S. 2007b. Development of high-rate
 456 anaerobic ammonium-oxidizing (anammox) biofilm reactors. *Water Res.* 41(8), 1623-1634.

457 van de Graaf, A.A., de Bruijn, P., Robertson, L.A., Jetten, M.S.M., Kuenen, J.G. 1996.

458 Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized
 459 bed reactor. *Microbiol.* 142(8), 2187-2196.

460 van der Star, W.R.L., Abma, W.R., Blommers, D., Mulder, J.-W., Tokutomi, T., Strous, M.,
 461 Picioreanu, C., Van Loosdrecht, M.C.M. 2007. Startup of reactors for anoxic
 462 ammonium oxidation: Experiences from the first full-scale anammox reactor in
 463 Rotterdam. *Water Res.* 41(18), 4149-4163.

464 van der Star, W.R.L., Miclea, A.I., van Dongen, U.G.J.M., Muyzer, G., Picioreanu, C., van
 465 Loosdrecht, M.C.M. 2008. The membrane bioreactor: A novel tool to grow anammox
 466 bacteria as free cells. *Biotechnol. Bioeng.* 101(2), 286-294.

467 Vlaeminck, S.E., Geets, J., Vervaeren, H., Boon, N., Verstraete, W. 2007. Reactivation of
 468 aerobic and anaerobic ammonium oxidizers in OLAND biomass after long-term storage.
 469 *Appl. Microbiol. Biotechnol.* 74(6), 1376-1384.

470 Waki, M., Yasuda, T., Suzuki, K., Sakai, T., Suzuki, N., Suzuki, R., Matsuba, K., Yokoyama, H.,
 471 Ogino, A., Tanaka, Y., Ueda, S., Takeuchi, M., Yamagishi, T., Suwa, Y. 2010. Rate
 472 determination and distribution of anammox activity in activated sludge treating swine
 473 wastewater. *Bioresour. Technol.* 101(8), 2685-2690.

474 Yoshinaga, I., Amano, T., Yamagishi, T., Okada, K., Ueda, S., Sako, Y., Suwa, Y. 2011.
 475 Distribution and Diversity of Anaerobic Ammonium Oxidation (Anammox) Bacteria in
 476 the Sediment of a Eutrophic Freshwater Lake, Lake Kitaura, Japan. *Microbes Environ.*
 477 26(3), 189-197.

478 **Table legends**

479

480 **Table 1:** Preservation conditions tested in this study.

481

482 **Figure legends**

483 **Fig. 1:** Per cent recovery of the specific anammox activity (SAA) of biomass stored at -
484 80°C after 45, 90 and 150 days. Anammox activity was measured based on $^{29}\text{N-N}_2$
485 production rate from ^{15}N -ammonia and ^{14}N -nitrite. (*) represents no activity was
486 observed. Error bars indicate the standard deviations (SD) of triplicate samples.

487

488 **Fig. 2:** Per cent recovery of the specific anammox activity (SAA) of granular biomass
489 stored at 4°C after 45, 90 and 150 days. Anammox activity was measured based on $^{29}\text{N-}$
490 N_2 production rate from ^{15}N -ammonia and ^{14}N -nitrite. (*) represents no activity was
491 observed. Error bars indicate the standard deviations (SD) of triplicate samples.

492

493 **Fig. 3:** Per cent recovery of the specific anammox activity (SAA) of immobilized
494 biomass stored at 4°C after 45, 90 and 150 days. Anammox activity was measured based
495 on $^{29}\text{N-N}_2$ production rate from ^{15}N -ammonia and ^{14}N -nitrite. (*) represents no activity
496 was observed. Error bars indicate the standard deviations (SD) of triplicate samples.

497

498 **Fig. 4:** Per cent recovery of the specific anammox activity (SAA) of granular biomass
499 stored at room temperature (15 ~ 25°C) after 45, 90 and 150 days. Anammox activity
500 was measured based on $^{29}\text{N-N}_2$ production rate from ^{15}N -ammonia and ^{14}N -nitrite. (*)
501 represents no activity was observed. Error bars indicate the standard deviations (SD) of

502 triplicate samples.

503

504 **Fig. 5:** Relationship between the relative transcription levels of *hzsA* gene and specific
505 anammox activity derived from $^{29}\text{N}_2$ production rate. This result was used to confirm
506 the SAA determined by $^{29}\text{N}_2$ production rate for storage conditions 1 and 3 ~ 7 (**Fig. 1** &
507 **Fig. 2**, respectively).

508

509 **Fig. 6:** Reactivation of the 90-day-stored biomass under conditions 11~14. Each
510 biomass was immobilized in PVA-SA gel and packed in up-flow column reactors 1 ~ 4,
511 respectively. Nitrogen removal rate (NRR) of each reactor was monitored for 35 days.
512 Hydraulic retention time was 0.4 h and nitrogen loading rate was maintained at 4.5 and
513 $9.0 \text{ kg-N m}^{-3} \text{ d}^{-1}$ in each reactor. Operating temperature was 37°C .

514

Table 1: Ali *et al.* (2013)

Condition No.	Temp.	Biomass	NH ₄ ⁺ , NO ₂ ⁻ (mM)	NO ₃ ⁻ (mM)	Molybdate (mM)	Penicillin G (mM)	Media exchange
1	-80°C	Granule	-	-	-	-	-
2		Immobilized	-	-	-	-	-
3		Granule	5	-	-	-	EM*
4		Granule	5	-	3 mM	-	EM*
5		Granule	5	5.7	-	-	EM*
6	4°C	Granule	5	-	-	-	-
7		Granule	5	-	3 mM	-	-
8		Immobilized	5	-	-	-	-
9		Immobilized	5	-	3 mM	-	-
10		Immobilized	5	5.7	-	-	-
11	Room temp. (15 ~ 25°C)	Granule	5	-	-	-	+ NH ₄ ⁺ , NO ₂ ⁻
12		Granule	5	-	3 mM	-	+ NH ₄ ⁺ , NO ₂ ⁻
13		Granule	5	-	-	1.5	+ NH ₄ ⁺ , NO ₂ ⁻
14		Granule	5	5.7	-	-	+ NH ₄ ⁺ , NO ₂ ⁻

- : not applied

*: Entire media (EM) was exchanged after every 15 days

+ NH₄⁺, NO₂⁻: Only NH₄⁺ and NO₂⁻ were supplemented after 45 days

Fig. 1: Ali *et al.* (2013)

Fig. 2: Ali *et al.* (2013)

Fig. 3: Ali *et al.* (2013)

Fig. 4: Ali *et al.* (2013)

Fig. 5: Ali *et al.* (2013)

Fig. 6: Ali *et al.* (2013)