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Effects of Organic Matter in Livestock Manure Digester Liquid on Microbial Community Structure and *In Situ* Activity of Anammox Granules

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ABSTRACT

Anaerobic ammonium oxidation (anammox) is a promising process for NH_4^+-rich wastewaters such as anaerobic digester liquids. In the present study, we investigated various properties of an up-flow column reactor containing anammox granules and fed with a real digester liquid at four different concentrations (Phases 1 to 4). The efficiencies of NH_4^+ and NO_2^- removal decreased by up to 32% and 42%, respectively, in the digester-liquid-fed reactor (reactor-DL). When the performance of reactor-DL deteriorated, the community structure, spatial distribution, and *in situ* anammox activity in the two reactors were further investigated using 16S rRNA gene-based phylogenetic analysis, fluorescence *in situ* hybridization (FISH), and microelectrode measurements. The phylogenetic analysis and FISH results showed that non-anammox bacteria were predominant in the granule outer layers in reactor-DL, whereas anammox bacteria still dominated the granule interiors. Microelectrode measurements showed clear evidence of NH_4^+ oxidation activity in the interiors of granules from reactor-DL. Batch experiments using anammox granules at different acetate concentrations indicated that concentrations up to 50 mM had no effects on the anammox activity, whereas inorganic carbon uptake decreased in the presence of acetate. The present study clearly shows that the anammox activity and anammox bacterial density in the granules were maintained after feeding the digester liquid to the reactor for 140 days.

Keywords: Anaerobic ammonium oxidation (anammox); Digester liquid; fluorescence *in situ* hybridization (FISH); Phylogenetic analysis; Microelectrodes

1. Introduction

Biological nitrogen removal from NH_4^+-rich wastewater (i.e., low C/N ratios) can be efficiently and economically performed using anaerobic ammonium oxidation (anammox) (van Dongen et al., 2001; Morales et al., 2016). Anammox is a biologically mediated process with the following stoichiometry (Strous et al., 1998):

$$1NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (1)$$

Recently, an alternative stoichiometry for the anammox process was proposed by Lotti et al. (2014):

$$1NH_4^+ + 1.146NO_2^- + 0.071HCO_3^- + 0.057H^+ \rightarrow 0.986N_2 + 0.161NO_3^- + 0.071CH_{1.74}O_{0.31}N_{0.20} + 2.002H_2O \quad (2)$$

The following bacterial types have been proposed: *Candidatus* Brocadia, *Candidatus* Kuenenia, *Candidatus* Scalindua, *Candidatus* Jettenia, *Candidatus* Anammoxoglobus, and *Candidatus* Anammoximicrobium (Ali and Okabe, 2015). Although they have not yet been isolated as pure cultures, detailed information regarding the physiological characteristics of *Candidatus* Brocadia, *Candidatus* Kuenenia, *Candidatus* Scalindua, and *Candidatus* Jettenia is available (Strous et al., 1999; Oshiki et al., 2011; Awata et al., 2013; Ali et al., 2015a).

Water discharged from sludge digestion or digester liquid from livestock manure are suitable wastewater sources for the anammox process; their low C/N ratios (0.3 in the diluted digester liquid used in the present study) (Ni et al., 2012) make them difficult to treat using conventional nitrification–denitrification processes without external carbon sources. Until now, anammox processes have been used to treat various wastewater sources (summarized by Terada et al., 2011) such as water discharged from sludge digestion (Furukawa et al., 2009) and digester liquids from livestock manure (Molinuevo et al., 2009), combined with partial nitrification in various reactor types. Digester liquids contain organic matter, including suspended solids and

67 non- or slow-biodegradable compounds. Such organic matter can influence the anammox
68 activity, although some organic matter is oxidized during pre-treatment.
69 The microbial community properties and anammox aggregate activities (i.e., biofilms
70 and granules), and the reactor performance, can be elucidated by process monitoring,
71 optimization, and control; this is essential for applying the anammox process to digester liquids.
72 A combination of 16S rRNA gene cloning for community structure analysis and fluorescence *in*
73 *situ* hybridization (FISH), along with microelectrode measurements, has been applied to
74 anammox biofilms cultured with a inorganic nutrient medium (Kindaichi et al., 2007; Tsushima
75 et al., 2007), and complete autotrophic nitrogen removal over nitrite aggregates (CANON)
76 cultured with a mineral salt medium (Nielsen et al., 2005). However, knowledge of the
77 properties of the microbial community structure and *in situ* activity when real digester liquids are
78 fed to anammox aggregates remains limited.
79 We hypothesized that when a digester liquid is fed to anammox granular reactors (i)
80 the anammox activity will be suppressed through out-competition of anammox bacteria by
81 non-anammox bacteria (coexisting heterotrophic bacteria) that can utilize the organic matter in
82 digester liquids in anammox granules or (ii) the outer granule layer may be covered by organic
83 matter and/or coexisting bacteria, but anammox bacteria will still be present and maintain the
84 anammox activity in the granule interiors.
85 The objective of this study was therefore to investigate the use of anammox granules in
86 an anammox reactor fed with a real digester liquid at four different concentrations (Phases 1 to
87 4) when the reactor performance deteriorated during feeding with digester liquid. We fed an
88 up-flow anammox granule reactor with a livestock manure digester liquid, which was diluted
89 with a synthetic nutrient medium at four different ratios (0%, 33%, 66%, and 100% digester
90 liquid). We directly measured the anammox bacteria and coexisting bacteria distributions using
91 FISH and 16S rRNA gene cloning analyses, and NH_4^+ consumption activity using a

92 microelectrode in the anammox granules after a decrease in the nitrogen removal rate through
93 feeding with digester liquid was observed. The potential anammox activity and carbon uptake
94 by the anammox granules were measured in batch experiments with or without ^{14}C-labeled
95 bicarbonate at different acetate concentrations.
96
97 **2. Materials and methods**
98 *2.1. Reactor operation*
99 A control reactor, which was fed with a synthetic wastewater (reactor-SW), and a
100 diluted-digester-liquid-fed reactor (reactor-DL), were operated in parallel at 37 °C as shown in
101 Fig. 1. The reactors had a liquid volume of 14 mL (inner diameter 15 mm, height 80 mm). We
102 obtained an anammox granule inoculum from a fixed-bed biofilm column reactor that had been
103 operated for more than five years; detailed information has been reported previously (Tsushima
104 et al., 2007). The anammox granules were inoculated at approximately 30% (v/v) of the liquid
105 volume. The hydraulic retention times (HRTs) of the reactors were set at 0.85 h. Reactor-SW
106 was operated with a synthetic wastewater (Van de Graaf et al., 1996) containing 200 mg-N L^{-1}
107 of $(NH_4)_2SO_4$ and 190 mg-N L^{-1} of $NaNO_2$, and reactor-DL was operated under four different
108 sets of conditions (Phases 1 to 4); the operating parameters are summarized in Table 1. The ratio
109 of synthetic wastewater to diluted digester liquid in the influent was varied. The nitrogen (NH_4^+
110 plus NO_2^-)-loading rate was set at 11 g-N L^{-1} day^{-1} for both reactors. Digester liquid from an
111 anaerobic digester used for treating livestock manure in a dairy farm in Sapporo, Japan was used.
112 Table 2 shows the digester liquid physicochemical properties. The diluted digester liquid was
113 prepared as follows: (i) a supernatant was obtained from the digester liquid through continuous
114 centrifugation (Kokusan Co., Ltd., Tokyo, Japan), (ii) the supernatant was filtered with 0.45 μm
115 membranes (Advantec Co., Ltd., Tokyo, Japan), (iii) the filtrate was diluted with deionized water,
116 to an NH_4^+ concentration of 200 mg-N L^{-1}, and (iv) sodium nitrite was added to a final

concentration of 190 mg-N L^{-1} because of the lack of NO_2^- required for the anammox process in the digester liquid. Oxygen was excluded from the medium tank by connecting a gasbag filled with N_2 (GL Science, Tokyo, Japan) to the influent liquid tank (Tsushima et al., 2007). The pH was adjusted to 7.0–7.5 by the addition of 1 N H_2SO_4.

2.2. Batch experiments

Batch experiments were performed to measure the anammox activity and the growth potential of the anammox bacteria. The anammox granules from reactor-SW (SW-granules) were gently homogenized and washed twice in the synthetic nutrient medium. A biomass suspension was dispensed in 7 mL serum vials and diluted to a final concentration of 0.2–0.25 mg dry-weight per milliliter with a synthetic nutrient medium containing 100 mg-N L^{-1} of NH_4^+ and NO_2^-, and 0–50 mM of acetate. The final incubation volume was 4 mL. He gas (>99.99995%) was used to replace the headspace by repeated evacuating and purging. The vials were then statically incubated at 37 °C for 24 h. To determine the growth potential of anammox bacteria, sodium [^{14}C]bicarbonate was added to give a final concentration of 10 μCi $vial^{-1}$ (370 kBq $vial^{-1}$) and the samples were incubated for 4 or 24 h. All experiments were conducted in triplicate. The anammox activity was expressed as the specific NH_4^+ consumption during incubation for 24 h. The ^{14}C uptake was calculated based on the amount of ^{14}C incorporated into cells that were washed with a phosphate-buffered saline lacking ^{14}C.

2.3. Analytical methods

The NH_4^+ concentration was determined using the phenate method (APHA 1998). After filtration of the liquid samples with 0.45 μm membranes (Advantec), ion-exchange chromatography (ICS-1000, Thermo Fisher Scientific Inc., Sunnyvale, CA, USA) was used to measure the NO_2^- and NO_3^-concentrations using an IonPac AS22 anion column. The samples

were filtered with 0.2 μm membranes (Advantec) and then acetate was determined by liquid chromatography (HPLC 10Avp, Shimadzu, Kyoto, Japan) using a Shim-packSCR-102H column (Shimadzu) and a conductivity detector (CDD-6A, Shimadzu). A total organic carbon analyzer (TOC-V CSH, Shimadzu) was used to measure the dissolved organic carbon (DOC) concentration after filtration of the samples with 0.45 μm glass-fiber filters (Advantec). Standard methods were used to determine the total dissolved solids and volatile dissolved solids in the digester liquid (APHA 1998). A pH meter (D-51, Horiba, Kyoto, Japan) was used for pH measurements. The humic acid absorbance at 260 nm (E_{260}) was determined using a spectrophotometer (UV-1800, Shimadzu). Radioactivity was measured using a liquid scintillation counter (LSC-5100, Hitachi-Aloka Medical, Tokyo, Japan).

2.4. Fluorescence in situ hybridization

Granule samples collected from reactor-SW (SW-granules) and reactor-DL (DL-granules) after 145 days of operation were fixed and embedded as described previously (Okabe et al., 1999). The procedure described by Amann (1995) was used for *in situ* hybridization. The oligonucleotide probes used in this study were EUB338 (Amann et al., 1990), EUB338II (Daims et al., 1999), and EUB338III (Daims et al., 1999) for most bacteria, and Amx820 (Schmid et al., 2000) for anammox bacteria. Fluorescein isothiocyanate (FITC) or tetramethylrhodamine 5-isothiocyanate (TRITC) was used to label the 5′ ends of the probes. A confocal laser-scanning microscope (LSM5 PASCAL, Carl Zeiss, Oberkochen, Germany) was used for observations. The relative abundance of probe-defined anammox bacteria in granule cross-sections granules was quantitatively determined using image analysis software (ImageJ) (Collins, 2007) and custom-made macros (Mielczarek et al., 2012).

2.5. Microelectrode measurements

167 NH_4^+ concentration profiles of the SW- and DL-granules at steady-state were obtained
168 using a liquid ion-exchange membrane (LIX) microelectrode for NH_4^+. The granule samples
169 were taken from reactor-SW and reactor-DL after operation for 145 days. NH_4^+ microsensors
170 were produced and calibrated as using previously reported methods (Rathnayake et al., 2013).
171 The media used for NH_4^+ concentration profile determination in the SW- and DL-granules were
172 the same as the media fed to reactor-SW and reactor-DL at 145 days of operation. The granules
173 were incubated for at least 3 h at 37 °C in a flow chamber (2.5 L) under anoxic conditions before
174 the microelectrode measurements to obtain steady-state profiles (Ali et al., 2015b). The specific
175 NH_4^+ consumption rate was calculated based on the profiles (Rathnayake et al., 2013).
176
177 *2.6. Cloning and sequencing of 16S rRNA gene and phylogenetic analysis*
178 Total DNA was extracted from the SW- and DL-granules after 145 days of operation,
179 using a FastDNA spin kit (MP Biomedicals, Irvine, CA, USA). The 16S rRNA genes were
180 amplified using a bacteria-specific primer set, EUB338f-univ1390r (Kindaichi et al., 2011). A
181 clone library was constructed using previously described methods (Cao et al., 2015). ARB
182 software was used to collect sequences with a similarity of 97% or greater into operational
183 taxonomic units (OTUs) (Ludwig et al., 2004). A phylogenetic tree was constructed using the
184 ARB software with the database SSU Ref NR release 119 (Pruesse et al., 2007) and the
185 following methods: neighbor joining (distance matrix) with Jukes–Cantor correction, maximum
186 parsimony (Phylip DNAPARS), and maximum likelihood (RAxML) with the GTR Gamma
187 model. The sequence data were deposited in the GenBank/EMBL/DDBJ databases (accession
188 numbers LC091345 to LC091367).
189
190 **3. Results and discussion**
191 *3.1. Reactor performance*

192 NH_4^+ and NO_2^- were simultaneously removed, with greater than 80% NH_4^+ and NO_2^-
193 removal efficiencies from the first day of reactor operation for both reactors (Fig. 2A and 2B).
194 The average stoichiometric ratios of consumed NO_2^- to consumed NH_4^+ ($\Delta NO_2^-/\Delta NH_4^+$) and
195 produced NO_3^- to consumed NH_4^+ ($\Delta NO_3^-/\Delta NH_4^+$) were 1.11 ± 0.13 (standard deviation) and
196 0.13 ± 0.04, respectively, for reactor-SW, and 0.96 ± 0.06 and 0.13 ± 0.03, respectively, for
197 reactor-DL. These values were similar to previously reported ratios (1.146 and 0.161,
198 respectively) obtained using the recalculated stoichiometry for the anammox process (Lotti et al.,
199 2014); these results support the involvement of the anammox process in both reactors. The
200 average NH_4^+ and NO_2^- removal efficiencies were 82 ± 4% and 96 ± 4%, respectively, for
201 reactor-SW, and 86 ± 3% and 95 ± 1%, respectively, during Phase 1 for reactor-DL. Stable
202 nitrogen removal was attained from the first day with both reactors (Fig. 2A and 2B). The
203 average nitrogen removal rates were 9.3 ± 0.8 g-N L^{-1} day^{-1} for reactor-SW and 10.1 ± 0.7 g-N
204 L^{-1} day^{-1} during Phase 1 for reactor-DL. High anammox performances were achieved from the
205 first day because of the use of highly active anammox granules as the inoculum in the reactors. A
206 stable anammox performance was achieved with reactor-SW over 160 days. The high NH_4^+
207 removal efficiency on day 152 was caused by oxygen contamination of the influent tank.
208 The effect of organic matter in the digester liquid on the anammox activity, after
209 operation of reactor-DL (Phase 1) for 22 days, was investigated by replacing one-third of the
210 influent (v/v) was replaced by a diluted digester liquid in Phase 2 (Table 1). During Phase 2, the
211 average NO_2^- removal efficiency increased slightly (99 ± 1%), and the average NH_4^+ removal
212 efficiency decreased slightly (80 ± 4%, Fig. 2B). These results can be attributed to stimulation of
213 coexisting heterotrophic denitrifying bacterial activity by organic matter in the digester liquid
214 and a reduction in anammox activity. The average nitrogen removal rate was similar (9.5 ± 0.7
215 g-N L^{-1} day^{-1}) to that during Phase 1 (10.1 ± 0.4 g-N L^{-1} day^{-1}), implying that an increase in the
216 denitrification rate compensated for a decrease in the anammox rate. After stable reactor

operation for 50 days during Phase 2, a further one-third of the influent (v/v) was replaced by the diluted digester liquid (Table 1). During Phase 3, the nitrogen removal efficiencies and nitrogen removal rate were maintained for 45 days. The average NH_4^+ and NO_2^- removal efficiencies were 75 ± 4% and 99 ± 1%, respectively, and the nitrogen removal rate was 9.0 ± 0.7 g-N L^{-1} day^{-1} (Fig. 2B), but the average DOC and E_{260} were about six and three times higher, respectively, than those in Phase 1 (Table 1). The average NH_4^+ removal efficiencies during Phase 3 in reactor-SW and reactor-DL were significantly different ($P < 0.05$, Welch's *t*-test), whereas the average NO_2^- removal efficiencies were not significantly different ($P > 0.05$, Welch's *t*-test). The influent to reactor-DL was completely replaced by the diluted digester liquid at 117 days (Table 1). During Phase 4, although the anammox activity was maintained for the initial 12 days; the NH_4^+ and NO_2^- removal efficiencies had decreased to 32−38% and 42−51%, respectively, at 134 days. The average NH_4^+ and NO_2^- removal efficiencies during Phase 4 for reactor-SW and reactor-DL were significantly different ($P < 0.05$, Welch's *t*-test). The NH_4^+ and NO_2^- removal efficiencies did not recover during 30 days of operation in Phase 4. The anammox activity in Phase 4 was not lost completely, but the nitrogen removal rate was half that seen in the other phases. This indicates that the anammox bacteria were partially active in the DL-granules during Phase 4. The decrease in anammox activity was possibly related to inadequate input of metals from the digester liquid. Ferrous iron is a key trace metal for anammox bacteria, because it is incorporated into heme c protein and affects the anammox activity (Bi et al., 2014); anammox bacterial cells contain a large amount of iron, accounting for 40% (w/w) of the total metal content (Ali et al., 2015a).

The average DOC concentration in the synthetic wastewater fed to reactor-SW was 6.5 ± 1.2 mg-C L^{-1} (Fig. 2C and Table 1); this was calculated based on the ethylenediaminetetraacetic acid (EDTA) in the trace element solutions (Van de Graaf et al., 1996). The effluent DOC concentrations were slightly higher (7.5 ± 1.2 mg-C L^{-1}) than the

influent concentrations. DOC production in an anammox reactor and enrichment culture have been reported previously (Kindaichi et al., 2007; Tsushima et al., 2007). For reactor-DL during Phases 2–4, the DOC concentrations in the influent and effluent steadily increased with increasing ratio of diluted digester liquid to synthetic wastewater (Table 1). The DOC concentrations in the influent and effluent were similar during Phase 2 for reactor-DL (Fig. 2D), whereas the DOC concentration in the effluent decreased slightly during Phases 3–4, indicating DOC consumption by coexisting heterotrophic bacteria (e.g., denitrifying bacteria). The increase in the average stoichiometric ratios ($\Delta NO_2^-/\Delta NH_4^+$) from 0.96 ± 0.06 (Phase 1) to 1.19 ± 0.11 (Phase 3) and 1.35 ± 0.23 (Phase 4) can be explained by stimulation of coexisting heterotrophic bacteria.

3.2. Potential anammox activity and carbon uptake

To investigate the potential anammox activity and carbon uptake by the anammox granules, the anammox and ^{14}C-uptake rates were measured in batch experiments performed at various concentrations of acetate, as a model organic compound, in the digester liquid. After incubation for 24 h, simultaneous consumption of NH_4^+ and NO_2^- was observed at 0–50 mM acetate concentrations. The anammox activity was significantly unchanged ($P > 0.05$, Welch's *t*-test) over a wide range (0–50 mM) of acetate concentrations (Fig. 3A). This clearly indicates that acetate in concentrations up to 50 mM has little effect on the anammox activity. Inorganic carbon uptake, which is an indicator of the growth potential of anammox bacteria, was not significantly affected ($P > 0.05$, Welch's *t*-test) by acetate during incubation for 4 h. In contrast, the growth potential clearly decreased significantly ($P < 0.05$, Welch's *t*-test) with increasing acetate concentration during incubation for 24 h (Fig. 3B). These results indicate that longer exposure of anammox granules to acetate decreases the growth potential of anammox bacteria. Magrí et al. (2013) pointed out that the presence of organic compounds enhances the growth of

heterotrophic bacteria in anammox consortia because of differences among the maximum growth rates (μ_{max}). However, Awata et al. (2015) reported that acetate in concentrations up to 10 mM has little effect on inorganic carbon uptake by *Candidatus* Scalindua sp. The effect of organic compounds on anammox bacterial growth may depend on the dominant anammox species.

3.3. Spatial distributions of bacteria and their activities in anammox granules

The *in situ* spatial distributions of anammox bacteria and non-anammox bacteria (i.e., coexisting bacteria) in SW- and DL-granules after 145 days of operation were directly visualized by FISH using anammox-specific and general bacterial probes (Fig. 4A and 4B). As previously reported, both types of granule consisted of biomass that was densely packed with interstitial voids (Kindaichi et al., 2007; Tsushima et al., 2007). Cells hybridized with the Amx820 probe (i.e., anammox bacteria) were distributed throughout the SW-granules (Fig. 4A). In contrast, anammox bacteria were only present inside the granules, and non-anammox bacteria (coexisting bacteria) covered the outer 100 μm layers of the DL-granules (Fig. 4B). The relative abundances of anammox bacteria in the SW- and DL-granules were approximately 98% and 59%, respectively. These results clearly show that the SW- and DL-granules have different structures.

Microsensor measurements showed that NH_4^+ consumption (i.e., anammox) occurred throughout the SW-granules (Fig. 5A) and anammox rates were higher in the SW-granule outer layers, probably because of the higher concentrations of substrates there (Fig. 5B). Similar trends have been observed in anammox granular biomass (Rathnayake et al., 2013; Lv et al., 2016) and anammox biofilms (Kindaichi et al., 2007). The anammox rates in the DL-granules were lower than those in the SW-granules, especially at the surfaces; this can be attributed to a lower density of anammox bacteria (Fig. 4B). The lower anammox rates during Phase 4 for reactor-DL could therefore be explained by a decrease in the specific anammox rate resulting from a decrease in

292 the anammox bacterial density. The retention of a higher density of anammox bacteria in the
293 granule outer layers is necessary for preserving anammox activity.
294
295 *3.4. Bacterial community structure of anammox granules*
296 Bacterial clone libraries (library-SW and library-DL) were constructed to investigate
297 the difference between the bacterial community structures in reactor-SW and reactor-DL. We
298 obtained 77 and 86 clones from the SW- and DL-clone libraries, respectively. At least six phyla,
299 including 23 different OTUs, were identified (Fig. 6). The phylum-level tree topologies
300 generated using the three methods did not differ. The distribution of OTUs from the SW-clone
301 library among phyla was as follows: Planctomycetes, 86%; Chloroflexi, 6%;
302 Bacteroidetes/Chlorobi, 4%; Proteobacteria, 3%; and Candidate division WS6, 1%. In contrast,
303 the distribution of OTUs from reactor-DL phyla was as follows: Planctomycetes, 44%;
304 Chloroflexi, 22%; Bacteroidetes/Chlorobi, 26%; Proteobacteria, 7%; and Acidobacteria, 1%. A
305 comparison of the two clone libraries shows that the detection frequency of Planctomycetes in
306 library-DL was significantly lower, and thus the population of coexisting bacteria was higher,
307 indicating that the population of anammox bacteria decreased as a result of feeding digester
308 liquid to reactor-DL.
309 The most frequently detected OTUs in both reactors were SW-a1 and DL-a2, affiliated
310 with *Candidatus* Jettenia *caeni* (Ali et al., 2015a), with 100% sequence identity, indicating that
311 the dominant anammox bacterial species did not change in either reactor, but the relative
312 abundance of anammox bacteria decreased during Phase 4. In contrast to the decrease in the
313 anammox bacterial population, the detection frequencies of some other OTUs increased: DL-a6
314 in Proteobacteria, DL-a10 and DL-d10 in Bacteroidetes/Chlorobi, and DL-a1 in Chloroflexi.
315 The OTUs SW-c12 and DL-a6 were closely related to the uncultured bacterial clone
316 KIST-JJY033, obtained from an anammox reactor, with 100% sequence identity, and

317 *Denitratisoma oestradiolicum*, which was isolated from an anammox reactor, with 98.4%
318 sequence identity. *D. oestradiolicum* is capable of denitrification and can grow on volatile fatty
319 acids such as acetates, propionates, and butyrates (Fahrbach et al., 2006). Furthermore,
320 *Pseudomonas* spp. have denitrification abilities; therefore, it could be speculated that acetate is
321 the carbon source used for denitrification by OTUs DL-a6 and DL-b7. The OTUs SW-g4 and
322 DL-a10 were closely related to the uncultured bacterial clone Dok19, which was obtained from
323 an anammox reactor, with 99.6% sequence identity, and a pure culture strain, *Ignavibacterium*
324 *album* (Iino et al., 2010), with 93.0% sequence identity. The ecophysiological role of *I. album* in
325 reactor-DL is not clear at present. The OTUs SW-e1 and DL-a1 were closely related to an
326 isolated bacterium in the phylum Chloroflexi, *Anaerolinea thermophila* (Sekiguchi et al., 2003),
327 with 81.0% sequence identity. This indicates that the OTUs SW-e1 and DL-a1 detected in the
328 granules are probably different species at the genus level, therefore the physiological role of the
329 bacteria related to OTUs SW-e1 and DL-a1 is not clear at present. The OTUs SW-e1 and DL-a1
330 were also closely related to the uncultured bacterial clone HUY-A12 detected in an anammox
331 biofilm reactor (Kindaichi et al., 2012), with 99.8% sequence identity. Kindaichi et al. (2012)
332 reported that *Chloroflexi*-like bacteria coexisting in anammox biofilms could take up organic
333 compounds produced by anammox bacteria. Although the details of the ecophysiological
334 functions of these bacteria are not clear, they can at least degrade and scavenge organic
335 compounds in the anammox reactor. It should be noted that the functions of other OTUs
336 mentioned above are not clear, but most have been detected in anammox or nitrogen removal
337 reactors in previous studies (e.g., Kindaichi et al., 2012).
338
339 **4. Conclusions**
340 We investigated the microbial community structure, spatial distribution, and *in situ*
341 activity of anammox granules fed with a livestock manure digester liquid. When the digester

liquid was fed to anammox reactors, the NH_4^+ and NO_2^- removal efficiencies decreased by up to 32% and 42%, respectively. Coexisting heterotrophic bacteria were predominant in the DL-granule outer layers (at a depth of 200 μm), and the density of anammox bacteria was relatively low. The NH_4^+ consumption rate at the surfaces of DL-granules was lower than that for the SW-granules. Our observations support hypothesis (ii) (see section 1), i.e., when a digester liquid is fed to an anammox reactor, the outer layers of the granules are covered with organic matter and/or coexisting bacteria, but anammox bacteria are still present, and maintain the anammox activity in the granule interiors. Maintenance in the granule outer layers of the anammox population and activity is the key point in anammox reactors receiving digester liquids.

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Figure captions

Fig. 1. Schematic diagrams of up-flow granular-bed anammox reactor fed with synthetic wastewater (reactor-SW) and diluted digester liquids (reactor-DL).

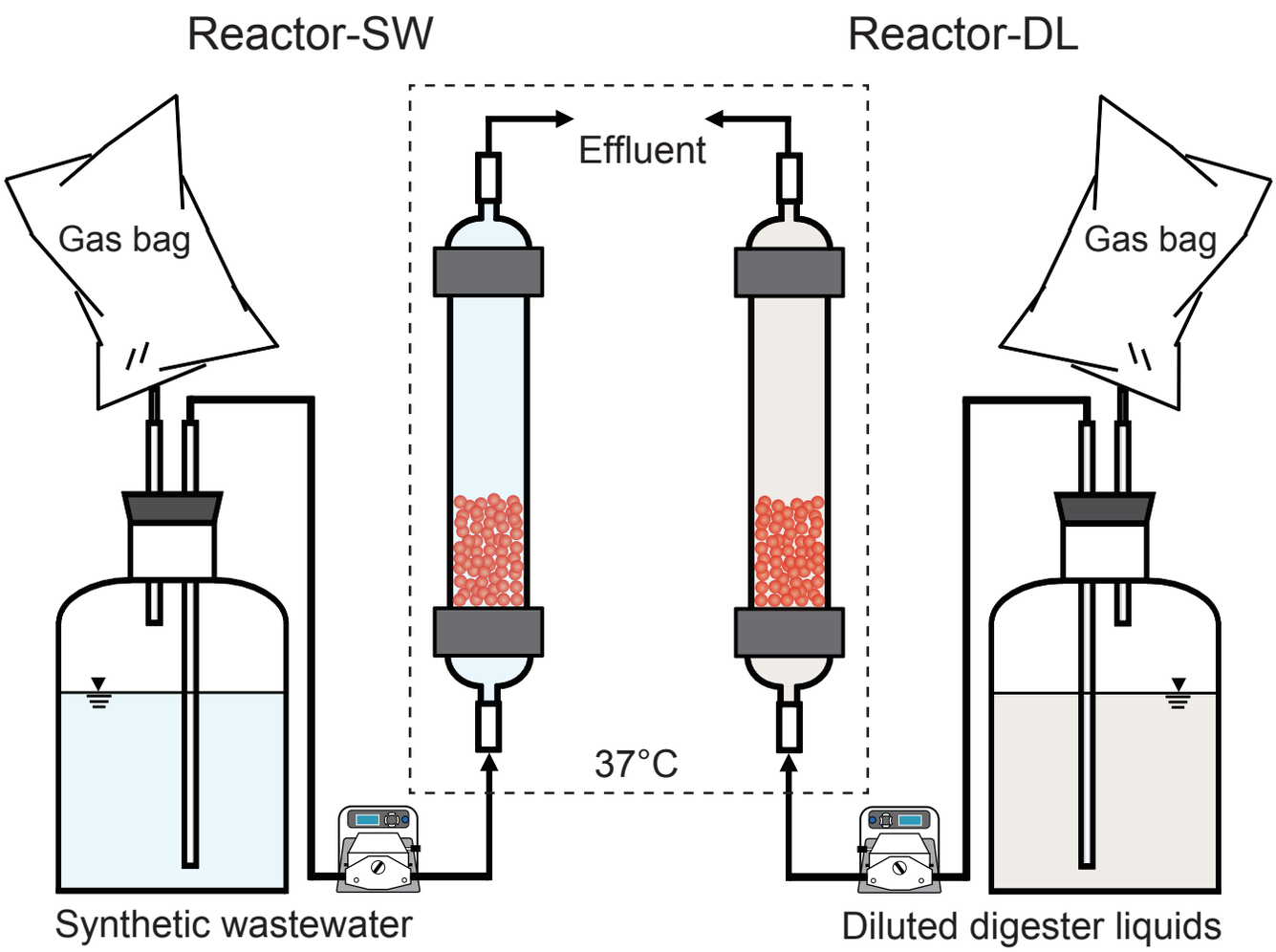
Fig. 2. NH_4^+ and NO_2^- removal efficiencies (filled and open triangles), and nitrogen loading and removal rates (filled and open circles) for reactor-SW as control (A) and reactor-DL fed with diluted digester liquid (B). DOC concentrations in the influent (filled diamonds) and effluent (open diamonds) in reactor-SW (C) and reactor-DL (D). Dotted lines indicate change in operational phase.

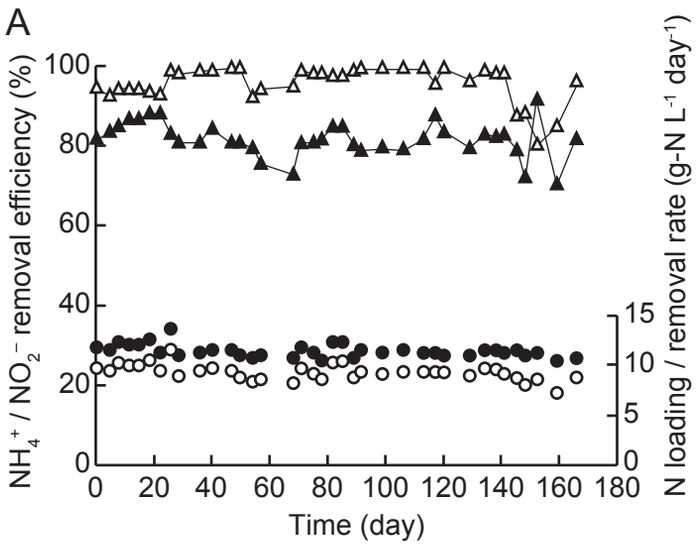
Fig. 3. Effect of acetate on anammox activity (A) and ^{14}C uptake (B). Anammox activity and ^{14}C uptake are expressed as relative percentage of 0 mM acetate and the mean ± standard deviation (n = 3). Open circles and open triangles in (B) represent ^{14}C uptake for 4 and 24 h incubations, respectively.

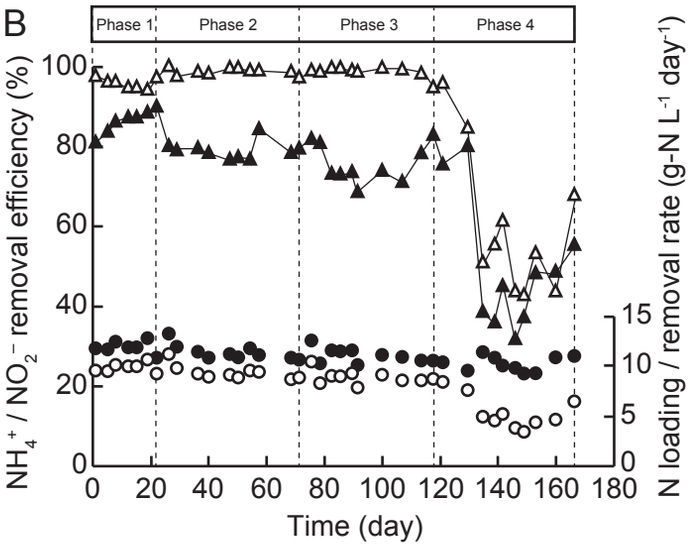
Fig. 4. FISH micrographs of vertical section (20 μm thick) of anammox granules fed with synthetic wastewater (A) and digester liquid (B). A combination of FITC-labeled mixed EUB338mix probes and TRITC-labeled probe Amx820 was used for *in situ* hybridizations. Anammox bacterial cells are yellow and non-anammox bacteria (coexisting bacteria) are green. Bars represent 100 μm.

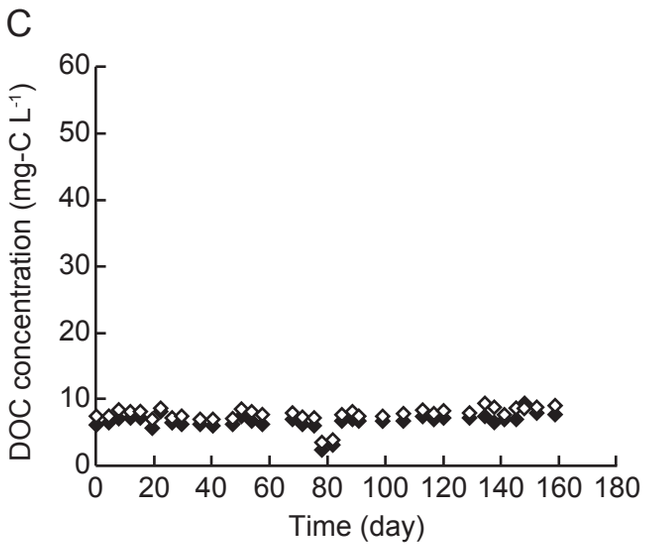
Fig. 5. Steady-state concentration profiles of NH_4^+ in SW- (circles) and DL- (open triangles) granules (A). Estimated spatial distributions of specific consumption rates of NH_4^+ in SW- (circles) and DL- (triangles) granules (B). Granule surface is at depth of 0 μm. Error bars show standard deviations (n = 3).

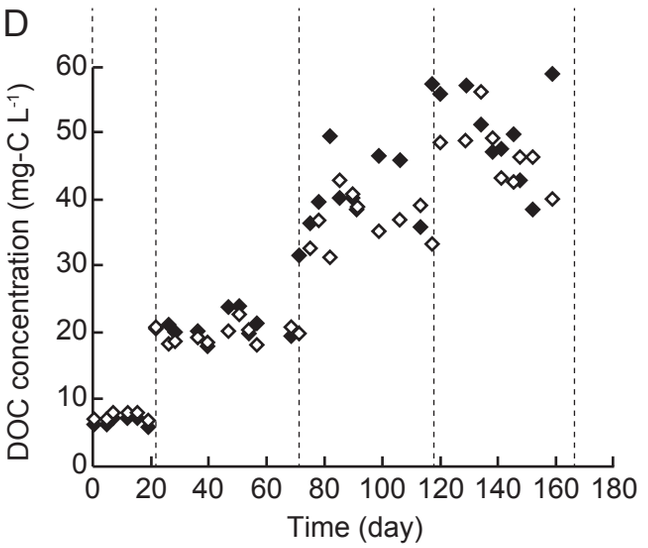
Fig. 6. Maximum likelihood phylogenetic tree of bacterial clones from reactors, based on 16S rRNA gene sequence. SW and DL represent OTUs from control reactor fed with synthetic wastewater (reactor-SW) and reactor fed with diluted digester liquid (reactor-DL), respectively. Frequencies of identical clones analyzed are indicated by numbers in parentheses, and scale bar indicates number of nucleotide changes per sequence position. Reproducible nodes are indicated as closed circles (>90% bootstrap support for three inferences used in this study: neighbor joining, maximum parsimony, and maximum likelihood methods, with 1000 replicates). Accession numbers of OTUs obtained in this study and sequences from public data are also indicated. Phylum-level designations (dotted line) are bracketed on right. *Thermotoga* spp. sequence served as outgroup for rooting tree.

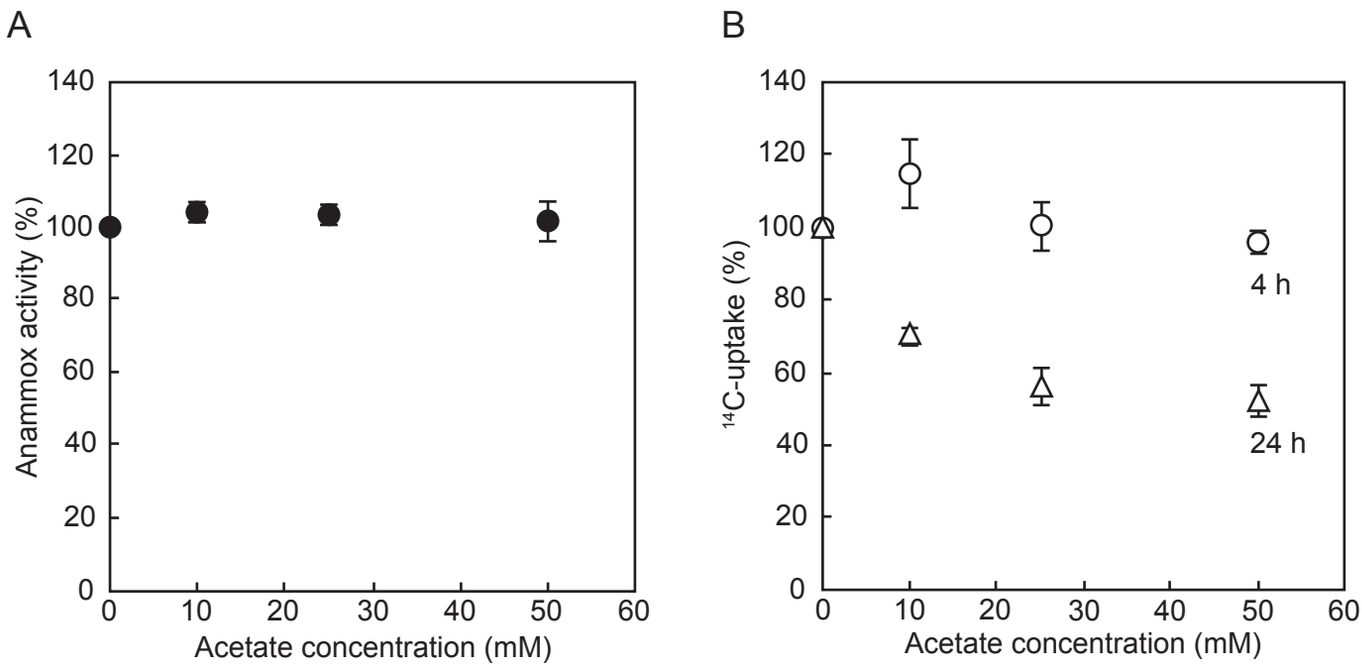

Reactor-SW
Reactor-DL
Effluent
Gas bag
Gas bag
37°C
Synthetic wastewater
Diluted digester liquids

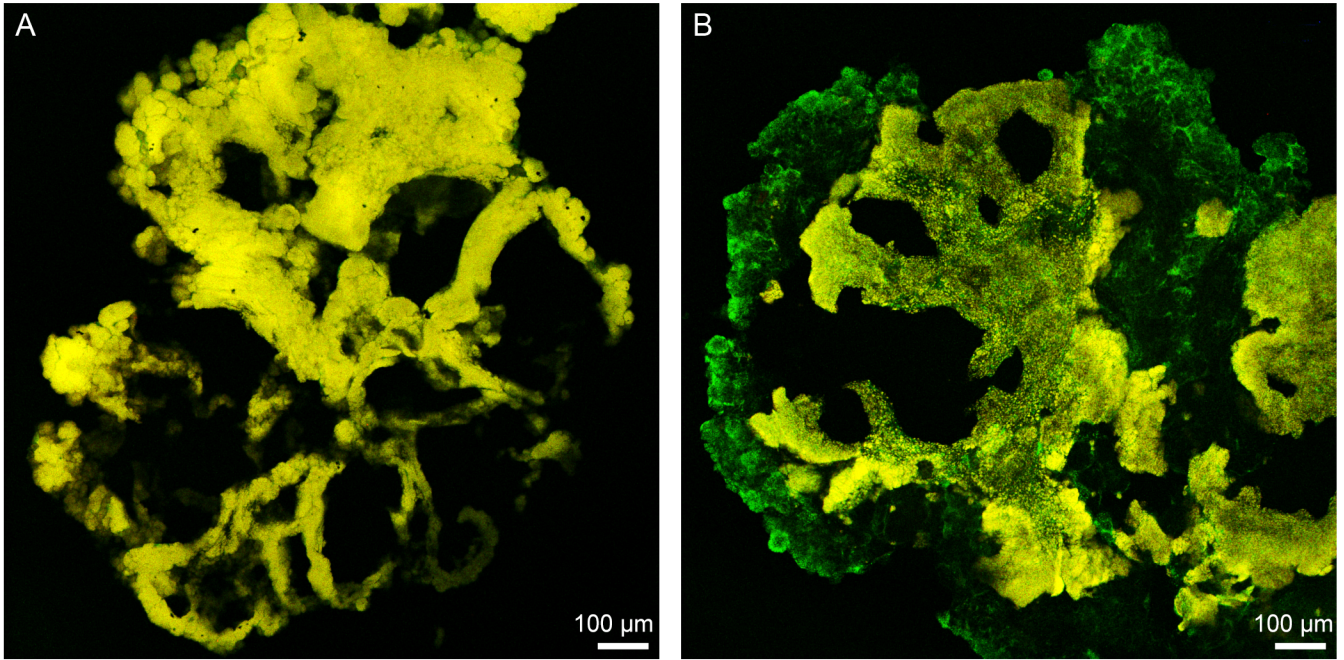

A
NH_4^+ / NO_2^- removal efficiency (%)
N loading / removal rate (g-N L^{-1} day^{-1})
Time (day)


B
Phase 1
Phase 2
Phase 3
Phase 4
NH_4^+ / NO_2^- removal efficiency (%)
N loading / removal rate (g-N L^{-1} day^{-1})
Time (day)


C
DOC concentration (mg-C L^{-1})
Time (day)


D
DOC concentration (mg-C L^{-1})
Time (day)


A
B
Anammox activity (%)
Acetate concentration (mM)
^{14}C-uptake (%)
4 h
24 h
0
20
40
60
80
100
120
140
0
10
20
30
40
50
60


A
100 µm
B
100 µm

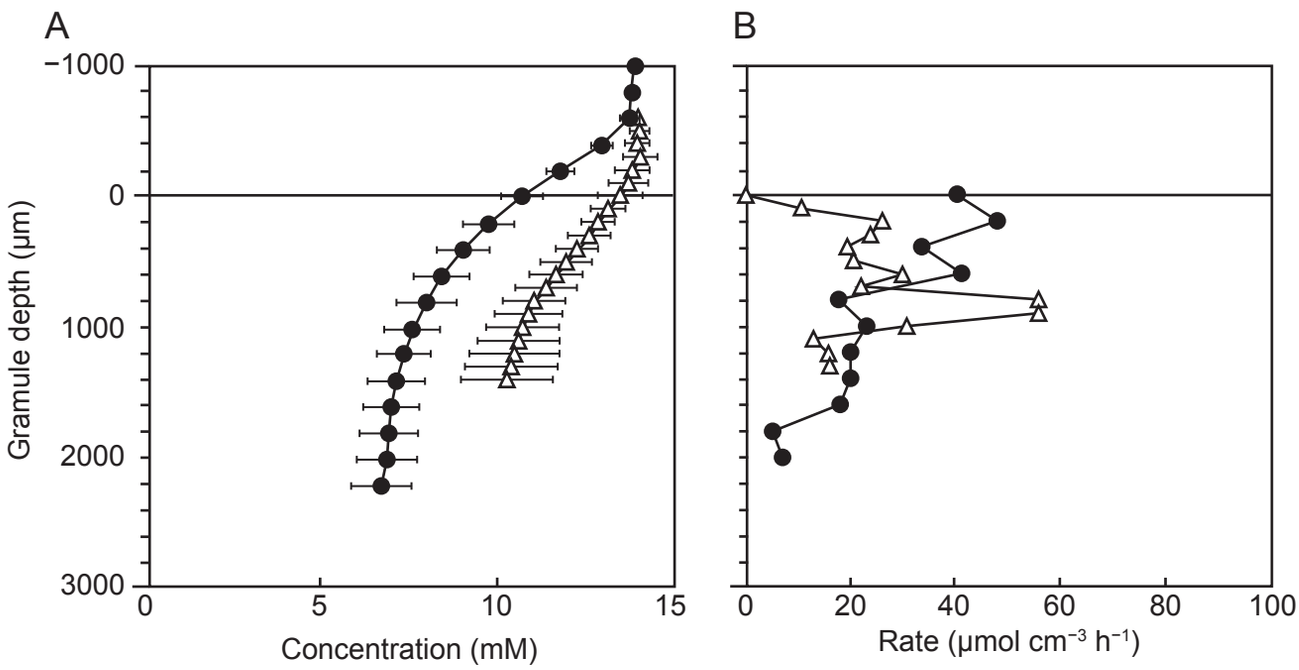

A
B
−1000
0
1000
2000
3000
Gramule depth (µm)
0
5
10
15
Concentration (mM)
0
20
40
60
80
100
Rate (µmol cm⁻³ h⁻¹)

Table 1

Operating conditions and characteristics of diluted digester liquids in each operational phase.

	Reactor-SW	Reactor-DL			
Parameter		Phase 1	Phase 2	Phase 3	Phase 4
Period (day)	0–166	0–22	22–71	71–117	117–166
Ratio between SW and DL (v/v)	1:0	1:0	2:1	1:2	0:1
NH_4^+-N (mg L^{-1})[a]	204 ± 17	220 ± 18	197 ± 13	200 ± 18	170 ± 23
NO_2^--N (mg L^{-1})[a, b]	192 ± 13	195 ± 4	198 ± 19	182 ± 12	185 ± 10
HRT (h)	0.85	0.85	0.85	0.85	0.85
Loading rate (g-N L^{-1} day^{-1})[a]	12 ± 1	12 ± 1	11 ± 1	11 ± 1	10 ± 1
DOC (mg-C L^{-1})[a]	6.5 ± 1.2	6.5 ± 0.6	21.9 ± 3.7	42.0 ± 7.2	50.7 ± 6.8
E_{260} (cm^{-1})[a]	0.3 ± 0.1	0.3 ± 0.1	0.5 ± 0.0	1.0 ± 0.1	1.5 ± 0.2
DOC/E_{260}[a]	20 ± 3	20 ± 3	20 ± 4	36 ± 4	34 ± 3

[a] Average value and standard deviation during each operational phase.

[b] Sodium nitrite was added to the digester liquids because of the lack of nitrite in diluted digester liquids.

Table 2

Physicochemical characteristics of digester liquid after filtration with 0.45 µm membranes.

Parameter	Range	Average ± standard deviation (n=5)
Total dissolved solids (g L^{-1})	7.2–9.1	8.1 ± 0.7
Volatile dissolved solids (g L^{-1})	2.0–3.2	2.6 ± 0.4
DOC (mg-C L^{-1})	675–1238	969 ± 208
Acetate (mg-C L^{-1})	7–18	11 ± 6
NH_4^+-N (mg L^{-1})	1490–1872	1736 ± 136
NO_2^--N (mg L^{-1})	2–27	7 ± 13
NO_3^--N (mg L^{-1})	14–19	8 ± 10
pH	7.9–8.7	8.4 ± 0.3
E_{260} (cm^{-1})	27–35	31 ± 4