Chemosphere 159 (2016) 300–307

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Effects of organic matter in livestock manure digester liquid on microbial community structure and *in situ* activity of anammox granules

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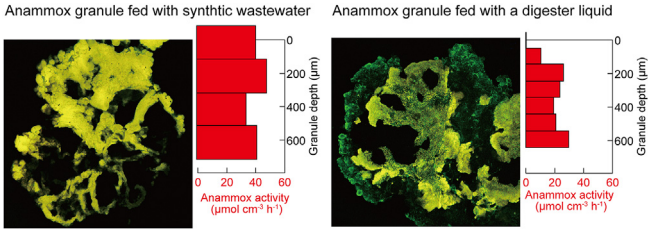
HIGHLIGHTS

- Digester liquids were fed to an anammox reactor.
- Anammox activity and bacterial density were lower in the outer layer of granules.
- Number of coexisting non-anammox bacteria increased in the outer layer of granules.
- Anammox activity in the inner part of the granules was maintained.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 24 February 2016
Received in revised form
23 May 2016
Accepted 5 June 2016
Available online 14 June 2016

Handling Editor: Y Liu

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

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.

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http://dx.doi.org/10.1016/j.chemosphere.2016.06.018
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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., 2015). 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 non- or slow-biodegradable compounds. Such organic matter can influence the anammox activity, although some organic matter is oxidized during pre-treatment.

The microbial community properties and anammox aggregate activities (i.e., biofilms and granules), and the reactor performance, can be elucidated by process monitoring, optimization, and control; this is essential for applying the anammox process to digester liquids. A combination of 16S rRNA gene cloning for community structure analysis and fluorescence *in situ* hybridization (FISH), along with microelectrode measurements, has been applied to anammox biofilms cultured with an inorganic nutrient medium (Kindaichi et al., 2007; Tsushima et al., 2007), and complete autotrophic nitrogen removal over nitrite aggregates (CANON) cultured with a mineral salt medium (Nielsen et al., 2005). However, knowledge of the properties of the microbial community structure and *in situ* activity when real digester liquids are fed to anammox aggregates remains limited.

We hypothesized that when a digester liquid is fed to anammox granular reactors (i) the anammox activity will be suppressed through out-competition of anammox bacteria by non-anammox bacteria (coexisting heterotrophic bacteria) that can utilize the organic matter in digester liquids in anammox granules or (ii) the outer granule layer may be covered by organic matter and/or coexisting bacteria, but anammox bacteria will still be present and maintain the anammox activity in the granule interiors.

The objective of this study was therefore to investigate the use of anammox granules in an anammox reactor fed with a real digester liquid at four different concentrations (Phases 1 to 4) when the reactor performance deteriorated during feeding with digester liquid. We fed an up-flow anammox granule reactor with a livestock manure digester liquid, which was diluted with a synthetic nutrient medium at four different ratios (0%, 33%, 66%, and 100% digester liquid). We directly measured the anammox bacteria and coexisting bacteria distributions using FISH and 16S rRNA gene cloning analyses, and NH_4^+ consumption activity using a microelectrode in the anammox granules after a decrease in the nitrogen removal rate through feeding with digester liquid was observed. The potential anammox activity and carbon uptake by the anammox granules were measured in batch experiments with or without ^{14}C-labeled bicarbonate at different acetate concentrations.

2. Materials and methods

2.1. Reactor operation

A control reactor, which was fed with a synthetic wastewater (reactor-SW), and a diluted-digester-liquid-fed reactor (reactor-DL), were operated in parallel at 37 °C as shown in Fig. 1. The reactors had a liquid volume of 14 mL (inner diameter 15 mm, height 80 mm). We obtained an anammox granule inoculum from a fixed-bed biofilm column reactor that had been operated for more than five years; detailed information has been reported previously (Tsushima et al., 2007). The anammox granules were inoculated at approximately 30% (v/v) of the liquid volume. The hydraulic retention times (HRTs) of the reactors were set at 0.85 h. Reactor-SW was operated with a synthetic wastewater (van de Graaf et al., 1996) containing 200 mg-N L^{-1} of $(NH_4)_2SO_4$ and 190 mg-N L^{-1} of $NaNO_2$, and reactor-DL was operated under four different sets of conditions (Phases 1 to 4); the operating parameters are summarized in Table 1. The ratio of synthetic wastewater to diluted digester liquid in the influent was varied. The nitrogen (NH_4^+ plus NO_2^-)-loading rate was set at 11 g-N L^{-1} day^{-1} for both reactors. Digester liquid from an anaerobic digester used for treating livestock manure in a dairy farm in Sapporo, Japan was used. Table 2 shows the digester liquid physicochemical properties. The diluted digester liquid was prepared as follows: (i) a supernatant was obtained from the digester liquid through continuous centrifugation (Kokusan Co., Ltd., Tokyo, Japan), (ii) the supernatant was filtered with 0.45 μm membranes (Advantec Co., Ltd., Tokyo, Japan), (iii) the filtrate was diluted with deionized water, to an NH_4^+ concentration of 200 mg-N L^{-1}, and (iv) sodium nitrite was added to a final

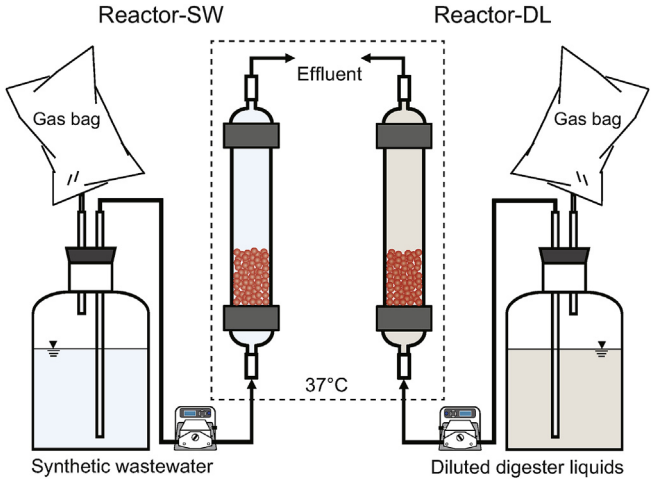

Fig. 1. Schematic diagrams of up-flow granular-bed anammox reactor fed with synthetic wastewater (reactor-SW) and diluted digester liquids (reactor-DL).

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