For submission to *Water Research* as a Full Paper

Revised manuscript WR6467

Development of High-Rate Anaerobic Ammonium-Oxidizing (ANAMMOX) Biofilm Reactors

by

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ABSTRACT

To promptly establish anammox (anaerobic ammonium oxidation) reactors, appropriate seeding sludge with high abundance and activity of anammox bacteria was selected by quantifying 16S rRNA gene copy numbers of anammox bacteria by real-time quantitative PCR (RTQ-PCR) and batch culture experiments. The selected sludge was then inoculated into up-flow fixed-bed biofilm column reactors with nonwoven fabric sheets as biomass carrier and the reactor performances were monitored over one year. The anammox reaction was observed within 50 days and a total nitrogen removal rate of 26.0 kg-N m^{-3} day^{-1} was obtained after 247 days. To our knowledge, such a high rate has never been reported before. Hydraulic retention time (HRT) and influent NH_4^+ to NO_2^- molar ratio could be important determinant factors for efficient nitrogen removal in this study. The higher nitrogen removal rate was obtained at the shorter HRT and higher influent NH_4^+/NO_2^- molar ratio. After anammox reactors were fully developed, the community structure, spatial organization and *in situ* activity of the anammox biofilms were analyzed by the combined use of a full-cycle of 16S rRNA approach and microelectrodes. *In situ* hybridization results revealed that the probe Amx820-hybridized anaerobic anammox bacteria were distributed throughout the biofilm (accounting for more than 70% of total bacteria). They were associated with *Nitrosomonas*-like aerobic ammonia-oxidizing bacteria (AAOB) in the surface biofilm. The anammox bacteria present in this study were distantly related to the Candidatus *Brocadia anammoxidans* with the sequence similarity of 95%. Microelectrode measurements showed that a high *in situ* anammox activity (i.e., simultaneous consumption of NH_4^+ and NO_2^-) of 4.45 g-N of (NH_4^+ + NO_2^-) m^{-2} day^{-1} was detected in the upper 800 μm of the biofilm, which was consistent with the spatial distribution of anammox bacteria.

Keywords; Anammox biofilm reactor, biofilm structure and function, 16S rRNA approach, microelectrodes.

INTRODUCTION

Anaerobic ammonium oxidation (anammox) is a biological process in which ammonium is directly converted to dinitrogen gas with nitrite as the electron acceptor under anoxic conditions (Jetten, *et al*., 1999). The anammox reaction was first discovered in a denitrifying pilot plant reactor in Delft, the Netherlands (Mulder *et al*., 1995), and today anammox reactions have been reported from several other treatment plants (Egli *et al*., 2001, Fux *et al*., 2002, van Dongen *et al*., 2001). Anammox is carried out by chemolithoautotrophic bacteria belonging to the order *Planctomycetales*. To date, two fresh water species, Candidatus *Brocadia anammoxidans* (Strous *et al*., 1999) and Candidatus *Kuenenia stuttgartiensis* (Schmid *et al*., 2000) and three marine species, Candidatus *Scalindua sorokinii* (Kuyper *et al*., 2003), Candidatus *Scalindua brodae* (Schmid *et al*., 2003), and Candidatus *Scalindua wagneri* (Schmid *et al*., 2003) have been proposed. Recently, a mixotrophic anammox bacterium Candidatus *Anammoxglobus propionicus* was also described (Kartal et al., 2006). Since these bacteria have not been isolated in pure culture yet, the current information about their physiology has been obtained from enrichment culture studies (Egli *et al*., 2001, Strous *et al*., 1998 and Toh *et al*., 2002).

The anammox process is a new and promising alternative to the conventional nitrogen removal processes. The application of anammox to nitrogen removal would lead to a significant reduction of costs for aeration and exogenous electron donor as compared to the conventional nitrification-denitrification process (van Dongen *et al*., 2001). However, one of the main drawbacks common to application of the anammox process is requirement of a long start-up period due to mainly slow growth rates of anammox bacteria (Egli *et al*., 2001 and van Dongen *et al*., 2001) (the doubling time was reported to be approximately 11 days) (Strous *et al*., 1998). Additionally, since anammox bacteria are strictly anaerobes and autotrophs, they are very difficult to be cultured. To promptly establish anammox reactors,

appropriate seeding sludge or starter cultures must be selected and used, and sufficient amounts of anammox bacteria must be efficiently retained in the reactors. However, rational procedures for start-up and optimization of anammox reactors have not been developed yet.

The objectives of this study were therefore (i) to select appropriate seeding sludge for the rapid start-up of anammox reactors; (ii) to promptly establish the anammox reactors with the selected sludge and optimize the reactor performance; and (iii) to characterize the microbial community structure and *in situ* activity of the anammox biofilms. To achieve these objectives, up-flow fixed-bed biofilm column reactors with nonwoven fabric sheets as biomass carrier were used for cultivation of anammox bacteria, and the reactor performance was monitored over one year. After anammox reactors were fully developed, the community structure, spatial organization and *in situ* activity of the anammox biofilms were analyzed by the combined use of a full-cycle of 16S rRNA approach and microelectrodes.

MATERIALS AND METHODS

Sludge samples

Sludge samples were collected from 11 different wastewater treatment plants (WWTPs) to select appropriate seeding sludge for establishment of up-flow fixed-bed biofilm reactors (**Table 1**). To quantify anammox bacterial population, the 16S rRNA gene copy numbers of anammox bacteria in the sludge samples were determined by real-time quantitative PCR (RTQ-PCR) with anammox specific primers as described below. Carbon to nitrogen ratios (C/N) (g/g) of influent wastewater were also measured because organic compounds effect the activity of anammox bacteria (van de Graaf *et al.*, 1996).

Quantification of 16S rRNA gene copy number of anammox bacteria by RTQ-PCR

Direct DNA extractions were performed using the Fast DNA spin kit for soil (BIO101,

Qbiogene Inc., Carlsbad, CA) according to the manufacturer's protocol. RTQ-PCR assay was performed for quantification of 16S rRNA genes of anammox bacteria, as previously described by Tsushima *et al*. (2007). Briefly, the RTQ-PCR assays were performed in duplicate with a total volume of 25 μL reaction mixture containing 12.5 μL of buffers supplied with a SYBR® green PCR master mix kit (Applied Biosystems, Forster City, CA), 300 nM of primers and 2.5 μL of sample DNA in MicroAmp Optical 96-well reaction plates with optical caps (Applied Biosystems). Specific primers for anammox bacteria were previously described by Tsushima *et al*. (2007). The template DNA was amplified and monitored with an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The PCR conditions were as follows: 2 min at 50°C and 10 min at 94°C, followed by 40 cycles of 15 sec at 94°C and 1 min at 60°C. All PCR runs included control reactions without template DNA to test possible non-specific amplification. Standard curves for anammox bacteria were constructed using a series of DNA concentrations prepared from the plasmid vector carrying a 16S rRNA gene of a anammox bacterium related clone, which was obtained from the previously constructed clone library (Tsushima *et al*., 2007).

Anammox activity test

Anammox activities of the sludges taken from the WWTPs were analyzed in standard batch cultures. Each sludge sample was diluted to approximately 20 mg-MLSS L^{-1} with the anammox nutrient medium, and 95 mL of the mixed liquid was transferred into 100-mL serum bottles. Oxygen was removed from the mixed liquid by purging with N_2 gas (99.99%) for 30 min. The serum bottles were sealed tightly with butyl rubber caps. The anammox nutrient medium consisted of $(NH_4)_2SO_4$ (30-84 mg-N L^{-1}), $NaNO_2$ (30-84 mg-N L^{-1}), $KHCO_3$ (500 mg L^{-1}), KH_2PO_4 (27 mg L^{-1}), $MgSO_4 \cdot 2H_2O$ (300 mg L^{-1}), $CaCl_2 \cdot 2H_2O$ (180 mg L^{-1}), and 1 mL of trace element solution I and II (van de Graaf *et al*., 1996). pH was adjusted

to 7.5 with 1N H_2SO_4. Each sample was incubated at 37°C in the dark and the concentrations of NH_4^+, NO_2^- and NO_3^- were periodically monitored during the incubation. The medium in the serum bottles was exchanged with fresh one in an anaerobic chamber when the total amount of nitrogen consumption exceeded 140 mg-N per 100-mL serum bottle (Tsushima *et al*., 2006).

Anammox biofilm reactors

Two up-flow fixed-bed glass biofilm column reactors (reactor I and II) were operated in parallel (**Fig. 1**). The reactors had an inner diameter of 50 mm, height of 500 mm and the liquid volume of 0.8 liter. Nonwoven fabric sheets (12.5 × 2.0 × 0.8 cm; Japan Vilene Co., Ltd., Tokyo, Japan) were used as support materials for biofilms. They comprised a total surface area of 500 cm^2 and an interstitial volume of 760 cm^3, corresponding to the filling ratio of 5% of total reactor volume.

Eighteen mg (dry weight) of the sludge with the highest 16S rRNA gene copy numbers of anammox bacteria among 11 sludge samples was inoculated to the reactor I. Two liters of the effluent from the reactor I was collected after 3 months of operation, concentrated to 50 mL (corresponding to approximately 5 mg-dry weight of solid) and inoculated into the reactor II. The temperature was maintained at 37°C for both the reactors. PharMed® tubing (made of thermoplastic elastomer polypropylene) was used to minimize oxygen penetration. Both the reactors were fed with the anammox nutrient medium as described above. The medium was flushed with N_2 gas from line A for at least 1 h to achieve dissolved oxygen concentration below detection limit (< ca. 0.5 mg L^{-1}) (**Fig. 1)**. Furthermore, a N_2-filled gas bag (GL science, Tokyo, Japan) was connected with line B to avoid oxygen getting into the medium reservoir (**Fig. 1**). The pH was adjusted in the range 7.0-7.5 with 1N H_2SO_4. The concentrations of NH_4^+ and NO_2^- were varied with time from 20 to 550 mg-N L^{-1} and from 20 to 460 mg-N L^{-1},

respectively, corresponding to the influent NH_4^+/NO_2^- molar ratios of 0.75 to 1.25. The hydraulic retention times (HRTs) of the reactors were reduced gradually from 8 h to 1.4 h in the reactor I and 8 h to 0.2 h in the reactor II, respectively. The nitrogen (NH_4^++ NO_2^-)-loading rates were 0.1-9.4 kg-N m^{-3} day^{-1} in the reactor I and 0.1-58.5 kg-N m^{-3} day^{-1} in the reactor II. Samples were obtained from the influent and effluent lines and analyzed for the concentrations of NH_4^+, NO_2^-, NO_3^- and dissolved organic carbon (DOC). The 16S rRNA gene copy numbers of anammox bacteria in the biofilms were determined by RTQ-PCR (see above) after anammox reaction was clearly observed.

Analytical procedure

The concentrations of NH_4^+, NO_2^-, and NO_3^- were determined by using an ion-exchange chromatography (DX-100, DIONEX, Sunnyvale, CA) with an IonPac CS3 cation column and IonPac AS9 anion column after filtration with 0.2-μm-pore size membranes (Advantec Co., Ltd., Tokyo, Japan). The concentration of dissolved organic carbon (DOC) was measured by a TOC-analyzer (TOC-5000A; SHIMADZU, Kyoto, Japan) after filtration with 0.45-μm-pore size glass fiber filters (Advantec Co., Ltd., Tokyo, Japan). Suspended solids (SS) concentration was determined according to Standard Methods (APHA, 1995).

DNA extraction and PCR amplification

DNA was extracted from biofilm samples (approximately 0.2 mL) taken from the reactors with the Fast DNA spin kit (BIO101, Qbiogene Inc., Carlsbad, CA) as described in the manufacturer's instructions. 16S rRNA gene fragments were amplified from the extracted total DNA with Taq DNA polymerase (TaKaRa Bio Inc., Otsu, Japan) using *Planctomycetals*-specific primer set pla46f (Neef *et al.*, 1998) and 1492r (Weisburg *et al.*, 1991), and bacterial primer set 11f (Kane *et al.*, 1993) and 1492r (Weisburg *et al.*, 1991),

respectively. The PCR conditions targeted for bacteria were as follows: 5 min initial denaturation at 94°C, 30 cycles of 1 min at 94°C, 1 min at 50°C, and 110 s at 72°C. Final extension was carried out for 4 min at 72°C. The PCR conditions targeted for anammox bacteria were as follows: 5 min initial denaturation at 94°C, 25 cycles of 1 min at 94°C, 1 min at 50°C, and 70 s at 72°C. Final extension was carried out for 4 min at 72°C. The PCR products were electrophoresed on a 1% (wt/vol) agarose gel.

Cloning and sequencing of 16S rRNA gene and phylogenetic analysis

PCR products were ligated into a pCR-XL-TOPO® vector and transformed into ONE SHOT *Escherichia coli* cells according to the manufacturer's instructions (TOPO XL PCR cloning; Invitrogen, Carlsbad, CA). Nucleotide sequencing was performed with an automatic sequencer (ABI Prism 3100 Avant Genetic Analyzer; Applied Biosystems). All sequences were checked for chimeric artifacts by the CHECK_CHIMERA program in the Ribosomal Database Project (Maidak *et al*., 1997). Almost full-length sequences (ca. 1,500 bp) were compared with similar sequences of the reference organisms by BLAST search (Altschul *et al*., 1990). Sequences with more than 97% sequence similarity were grouped into the same operational taxonomic unit (OTU) by using Similarity Matrix program from the Ribosomal Database Project (Maidak *et al*., 1997). The sequences of each representing OTU were aligned with the CLUSTAL W package (Thompson *et al*., 1994) and used for phylogenetic analysis. A phylogenetic tree was constructed by the neighbor-joining method (Saito and Nei, 1987). Bootstrap resampling analysis for 100 replicates was performed to estimate the confidence of tree topologies.

Microelectrode Measurements

The concentration profiles of O_2, NH_4^+, NO_2^-, NO_3^-, and pH were measured using

microelectrodes as described by Okabe *et al*. (1999). Clark-type microelectrodes for O_2 with a tip diameter of approximately 15 μm and 90% response time of shorter than 1 sec were prepared and calibrated as described by Revsbech (1989). The LIX-type microelectrodes for NH_4^+, NO_2^-, NO_3^-, and pH were constructed, calibrated, and used according to the protocol described by de Beer *et al*. (1997) and Okabe *et al*. (1999).

The microelectrode measurements in anammox biofilms were performed in a glass column reactor. The reactor volume was 64 cm^3 (length 13.5 cm, inner diameter 2.5 cm). A sampling port (1.0 cm height, inner diameter 1.0 cm) was installed in the reactor (at ca. 6 cm from the inlet side), which was filled with 5% agarose gel. The microelectrodes were directly inserted into the biofilm through this agarose gel-filled sampling port. Nonwoven fabric sheets (12.5 × 2.0 × 0.8 cm) with fully developed anammox biofilms were taken from the reactor I on day 392 and installed in the glass column reactor and pre-incubated for a week. The same anammox nutrient medium containing 100 mg L^{-1} of NH_4^+-N and NO_2^--N was fed into the reactor. The hydraulic retention time (HRT) of the reactor was 0.8 h and the nitrogen-loading rate was maintained at ca. 6.0 kg-N m^{-3} day^{-1} during the measurements. The temperature was maintained at 37°C.

Based on the measured NH_4^+, NO_2^- and NO_3^- concentration profiles, the total NH_4^+, NO_2^- and NO_3^- conversion rates (J (μg-N cm^{-2} h^{-1})) were calculated using Fick's first law of diffusion. Net volumetric NH_4^+, NO_2^- and NO_3^- conversion rates (R (μg-N cm^{-3} h^{-1})) in the biofilms were also calculated using Fick's second law of diffusion as described previously by Lorenzen *et al.* (1998). The molecular diffusion coefficients used for the calculations were 1.47×10^{-5} cm^2 s^{-1} for NH_4^+, 1.30×10^{-5} cm^2 s^{-1} for NO_2^-, and 1.30×10^{-5} cm^2 s^{-1} for NO_3^- in water at 37 °C.

Fixation and cryosectioning of biofilm samples

Biofilm samples obtained from the reactor were fixed in 4% paraformaldehyde solution for 24 h at 4°C, washed three times with phosphate-buffered saline (PBS) (10 mM sodium phosphate buffer, 130 mM sodium chloride; pH 7.2), and embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) overnight to infiltrate the OCT compound into the biofilm, as described previously (Okabe *et al*., 1999). After rapid freezing at -21°C, 30-µm-thick vertical thin sections were prepared with a cryostat (Reichert-Jung Cryocut 1800, Leica, Bensheim, Germany).

Oligonucleotide probes and fluorescence *in situ* hybridization (FISH)

The 16S rRNA-targeted oligonucleotide probes used in this study were EUB338 (Amann *et al*., 1990), EUB338-II (Daims *et al*., 1999), EUB338-III (Daims *et al*., 1999) for mostly *eubacteria*, Amx820 (Schmid *et al*., 2000) for anammox bacteria, Nso190 (Mobarry *et al*., 1996) and Nse1472 (Juretschko *et al*., 1998) for aerobic ammonia-oxidizing bacteria (AAOB). To detect all bacteria, the probes were used in the equimolar mixture together with probes EUB338, EUB338II, and EUB338III. The probes were labeled with fluorescein isothiocyanate (FITC) or tetramethylrhodamine 5-isothiocyanate (TRITC) at the 5' end. *In situ* hybridization was performed according to the procedure described by Amann *et al*. (1995) and Okabe *et al*. (1999). A model LSM510 confocal laser-scanning microscope (CLSM, Carl Zeiss, Oberkochen, Germany), equipped with an Ar ion laser (488 nm) and HeNe laser (543 nm), was used. The average surface area fraction of probe-hybridized cells was determined from at least 20 randomly chosen LSM projection images of each cross section of the biofilm samples using image analysis software provided by Zeiss (Okabe *et al*., 1999).

Nucleotide sequence accession numbers

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of the three OTU sequences used for the phylogenetic tree analysis are AB269933 to AB269935.

RESULTS

Screening of seeding sludge

To select an appropriate seeding sludge for rapid start-up of anammox reactors, the 16S rRNA gene copy numbers of anammox bacteria in the sludge samples obtained from 11 different treatment plants were quantified by RTQ-PCR assay (**Table 1**). The 16S rRNA gene copy numbers of anammox bacteria and their correlation with the average C/N ratios of the influent wastewater are summarized in **Table 1**. The 16S rRNA gene copy numbers were in the range of 10^6 to 10^8 copies per mg of dry sludge. The highest copy number (1.6×10^8 copies per mg of dry sludge) was detected in sludge K obtained from a denitrifying basin in a domestic WWTP located in Higashihiroshima, Japan. The influent C/N ratio of sludge K was the lowest (0.9).

Anammox activity tests revealed that sludge K completely consumed 30 mg-N L^{-1} of NH_4^+ and NO_2^-, respectively, within the initial 37 days, which was the fastest among 11 sludge samples analyzed (**Table.1**). The maximum nitrogen removal rate (MNRR) reached 0.083 kg-N m^{-3} day^{-1} after day 100, which was also the highest among 11 sludge samples. In the activity tests using sludge C that had the lowest copy number (1.7×10^6 copies per mg of dry sludge), no anammox reaction was detected during more than 100 days of incubation (**Table 1**). In addition, in the case of sludge J (9.6×10^6 copies per mg of dry sludge), no anammox reaction was detected at all. Thus, we decided to use sludge K as an inoculum for following the anammox reactor experiments.

Performance of anammox reactors

Reactor I, which was inoculated with the sludge K, was operated for 392 days (**Fig. 2A**). On day 40, the simultaneous removal of NH_4^+ and NO_2^- was clearly observed, indicating that occurrence of anammox reaction. The total nitrogen removal rate gradually increased with increasing the nitrogen-loading rate after 55 days. After around 60 days, the color of biomass changed from light brown to characteristic red color. The 16S rRNA gene copy number of anammox bacteria attached on the nonwoven fabric sheet was $8.3 \pm 1.2 \times 10^{12}$ copies cm^{-3} (n = 4) on day 392. The maximum total nitrogen removal rate of 6.2 kg-N m^{-3} day^{-1} was obtained on day 392 when the nitrogen-loading rate was 9.4 kg-N m^{-3} day^{-1} (HRT, 1.4 h).

In the reactor II, which was inoculated with the effluent biomass of the reactor I after 3-month operation, the simultaneous removal of NH_4^+ and NO_2^- was observed after 50 days (**Fig. 2B**). This is because a small amount of biomass (5 mg-dry weight of solid) was inoculated into the reactor II. Anammox bacteria were enriched at only the inlet side of nonwoven fabric sheets when HRT was 8 h, while anammox bacteria gradually proliferated throughout the reactor after HRT was reduced to 1 h (increasing the nitrogen-loading rate). As a result, the maximum total nitrogen removal rate of 26.0 kg-N m^{-3} day^{-1} was attained after 247 days when the nitrogen-loading rate was 58.5 kg-N m^{-3} day^{-1} (HRT, 0.24 h). Thereafter, the nitrogen removal rate decreased to 5.8 kg-N m^{-3} day^{-1} because HRT was increased to 1 h. The nitrogen removal rate gradually increased to 14.0 kg-N m^{-3} day^{-1} again by increasing the influent NH_4^+ and NO_2^- concentrations (HRT was fixed at 1 h). However, the nitrogen removal rate no longer increased (actually decreased to 9.8 kg-N m^{-3} day^{-1}) when the influent nitrogen (NH_4^+ + NO_2^-) concentration was increased up to 1,000 mg-N L^{-1} (the nitrogen-loading rate was 24.0 kg-N m^{-3} day^{-1}).

Figure 3 showed the nitrogen removal rates in the reactor II attained at various influent nitrogen concentrations during days 259 to 362 (HRT was fixed at 1 h). The nitrogen removal rate steadily increased with increasing the influent nitrogen concentration up to 900 mg-N L^{-1},

above which it decreased. This indicated that there was a limitation to enhance the nitrogen removal rate by only increasing the influent nitrogen concentration at a fixed HRT. **Figure 4** showed the nitrogen removal rates and produced DOC concentrations in the reactor II at different HRTs with a fixed influent nitrogen ($NH_4^+ + NO_2^-$) concentration (900 mg-N L^{-1}; NH_4^+/NO_2^- = ca. 1.2 (M/M)). As expected, the nitrogen removal rate increased as HRT was reduced (**Fig. 4**). Reduction of HRT resulted in decrease in the DOC concentrations in the reactor effluent due to a dilution effect. **Figure 5** showed the nitrogen removal rates at different influent NH_4^+/NO_2^- molar ratios at a fixed HRT (8 h) and a fixed influent nitrogen ($NH_4^+ + NO_2^-$) concentrations (800 mg-N L^{-1}). The total nitrogen removal rate increased from 1.2 to 1.5 kg-N m^{-3} day^{-1} with increasing the influent NH_4^+/NO_2^- molar ratio from 0.75 to 1.25 (M/M) ($p < 0.05$).

Phylogenetic analysis

Two 16S rRNA gene clone libraries (an anammox bacterial clone library and a bacterial clone library) were constructed from the biofilms taken from the reactor I on the day 392 using the *Planctomycetals* specific primer set (Pla46f and 1492r) and bacterial specific primer set (11f and 1492r), respectively. No chimeric sequence was observed in both clone libraries. Sixteen clones were randomly selected from the anammox bacterial clone library and sequenced. All clones were grouped into one OTU (OTU1 in **Fig. 6**) on the basis of more than 97% sequence similarity. The clones grouped into OTU1 were related to the Candidatus *Brocadia anammoxidans* belonging to the order *Planctomycetales* with 95% sequence similarity (**Fig. 6**). Moreover, forty-one clones were randomly selected from the bacterial clone library. Of these 41 clones, 24 clones were related to anammox bacteria and grouped into two OTUs (OTU2 and OTU3 in **Fig. 6**). The clones grouped into OTU2 were also related to the Candidatus *Brocadia anammoxidans* with 95% sequence similarity (**Fig. 6**). The clone

sequences of OTU1 and OTU2 were closely related each other (more than 99.6% similarity). OTU3 was also closely related to OTU1 and OTU2 with 98.9% and 97.4% sequence similarity, respectively. We also obtained clone sequences closely related to OTU1, OTU2, and OTU3 (more than 98.5 % similarity) from the original sludge K and the reactor II on the day 247. This indicates that the enriched anammox bacteria in the reactor I was originated from sludge K that was inoculated. The remaining 17 clones were affiliated with uncultured clones belonging to the *Betaproteobacteria* such as *Thauera* sp. and *Acidovorax* sp. with 90 to 99% sequence similarity (data not shown). These bacteria were not observed from other anammox enriched cultures (Fujii et al., 2002, Strous et al., 2006).

***In situ* anammox activity.**

Figure 7A shows the concentration profiles of O_2, NH_4^+, NO_2^-, NO_3^-, and pH in the anammox biofilm, which were determined under as realistic conditions (i.e., water flow, water chemistry, temperature and so on). The concentrations of NH_4^+, NO_2^-, and NO_3^- in the bulk water at the measuring point (at ca. 6 cm from the inlet port) were 25.6, 18.8, and 16.8 mg-N L^{-1}, respectively. The average nitrogen removal rate of the reactor was 3.0 kg-N m^{-3} day^{-1} during the measurements. O_2 concentration was under detection limit throughout the biofilm at this measuring point. NH_4^+ and NO_2^- concentration profiles indicated that both NO_2^- and NH_4^+ concentrations simultaneously decreased in the biofilm, indicating occurrence of anammox reaction. The NH_4^+ concentration, however, gradually increased below a depth of 800 μm due to probably anaerobic mineralization of organic compounds. The NO_3^- concentration steadily increased in the upper 800 μm. pH increased from 7.3 to 7.8 in the upper 700 μm of the biofilm. The similar concentration profiles were repeatedly obtained when we conducted several measurements under the same condition.

The spatial distributions of net volumetric NH_4^+, NO_2^- and NO_3^- consumption rates showed

occurrence of simultaneous NH_4^+ and NO_2^- consumption and NO_3^- production in the upper 800 μm (data not shown). Based on the microprofiles, the total consumption rates of NH_4^+, NO_2^- and NO_3^- were calculated to be 2.04, 2.41 and -0.67 g-N m^{-2} day^{-1} (1: 1.2: -0.33) in the anammox zone.

Spatial distribution of anammox bacteria.

The spatial distribution of anammox bacteria in the biofilm obtained from the reactor I on day 392 was examined by FISH using EUB338 probe mixture (EUB338mix) and Amx820 probe specific to anammox bacteria. A vertical cross-section image of the biofilm revealed a heterogeneous structure consisting of bacterial cells and void spaces (**Fig. 8A**). As shown in **Fig. 8B**, almost all bacteria detected with EUB338mix (EUB338 + EUB338II +EUB338III) probe were simultaneously hybridized with Amx820 probe and were distributed throughout the biofilm. The Amx820 probe-hybridized cells were mainly present in the form of spherical dense microcolonies (**Fig. 8C**). The cells hybridized with Amx820 accounted for 89%, 74% and 72% of the total bacteria detected with EUB338mix probe at depths of 0-1 mm, 1-3 mm and 3-4 mm from the biofilm surface, respectively. The bacteria hybridized with EUB338mix probe but not hybridized with Amx820 probe (shown in green in **Fig. 8B**) coexisted with anammox bacteria mainly in the surface layer and the biomass-liquid interface. To identify these bacteria, the probes specific for aerobic ammonia-oxidizing bacteria (AAOB) were applied. As a result, AAOB hybridized with NSO190 was detected mainly in the surface biofilm. In addition, the NSO190-hybridized AAOB were also hybridized with Nse1472, indicating that they were most likely *Nitrosomonas eutropha*, *N. europaea* or *N. Halophila* (shown in red in **Fig. 8C**). The presence of the unidentified *eubacteria* hybridized with only EUB338mix probe (except AAOB) became evident in the deeper part of the biofilm (shown in green in **Fig. 8D**).

DISCUSSION

Anammox capacity of the reactors

In this study, we have successfully developed anammox reactors promptly and achieved a total nitrogen removal rate of 26.0 kg-N m^{-3} day^{-1} within 250 days. To our knowledge, such a high volumetric nitrogen removal rate has never been reported before. To date, Sliekers *et al*., (2003) have reported a nitrogen conversion rate of 8.7 kg-N m^{-3} day^{-1} in a gas-lift reactor, which was one third of our rate. Such a high anammox rate was probably attributed to the high density of anammox bacteria (ca. 16 g VSS L^{-1}, more than 70 % of total bacteria was anammox bacteria) retained in the reactors by using nonwoven fabric sheets as biofilm carrier as well as applying the high total nitrogen loading rate (especially nitrite), compared to other reactors (van de Graaf et al., 1996, Sliekers et al., 2003). The high nitrogen-loading rate prevents substrate transport limitation in the biofilms throughout the reactor (Nicolella et al., 2000). The specific nitrogen removal rate in this study was calculated to be at 1.6 kg-N kg-VSS^{-1} day^{-1}, which is also higher than the rates reported in the literature (Third et al., 2005). This high activity should be correlated to the growth rate. In our previous study, we have determined the doubling time of the anammox bacteria enriched from the same sludge K to be 3.6 to 5.4 days (Tsushima et al., 2007), which were shorter than the previously reported value (ca. 11 days) (Strous et al., 1999). In addition, the appropriate seeding sludge was selected and used after quantifying 16S rRNA gene copy number of anammox bacteria followed by anammox activity test. HRT and influent NH_4^+/NO_2^- molar ratio could play a vital role in obtaining efficient nitrogen removal in this study. The nitrogen removal rate was decreased when the influent nitrogen concentration was increased to 1,000 mg L^{-1} (**Fig. 3**). This is probably due to the toxicity of the residual nitrite (224 ± 10 mg-N L^{-1}), which was above the inhibitory nitrite concentration levels (70 - 180 mg-N L^{-1}) suggested previously by

Strous et al. (1999), or possibly an accumulation of unknown by-products derived from the anammox reaction. Shortening HRT led to the higher nitrogen-loading rates, which could prevent a possible nitrite limitation. An alternative explanation could be that unknown by-products derived from anammox reaction, which possibly cause self-inhibition of anammox bacterial activity, were washed out at shorter HRTs (**Fig 4).** Other studies also reported that the performance of anammox reactors was enhanced by reducing HRT stepwise (Sliekers *et al*., 2003). In fact, although Rouse *et al*. (2003) operated an anammox reactor with recycling the effluent, the nitrogen removal rate could hardly increase even though sufficient nitrogen ($NH_4^+$and NO_2^-) loading-rate was maintained. Furthermore, the higher anammox rate could be achieved by the higher influent NH_4^+/NO_2^- molar ratio (**Fig. 5**). This is probably because the high influent NH_4^+/NO_2 ratio gave the lower nitrite-loading rate, which led to less nitrite inhibition as demonstrated by the lower nitrite concentration in the effluent (**Fig. 5**). In addition, AAOB present in the surface biofilm could convert a part of NH_4^+ to NO_2^-, and then anammox bacteria convert the remaining NH_4^+ and produced NO_2^- to N_2. Maintaining NH_4^+ concentration high also prevents further oxidation of NO_2^- to NO_3^-.

***In situ* anammox activity**

The microelectrode measurements clearly demonstrated that anammox reaction (simultaneous consumption of NH_4^+ and NO_2^-) occurred in the biofilm, which was consistent with the results of 16S rRNA gene cloning and FISH analyses. There was, however, on partial nitrification (visible peak of NO_2^-) within the surface biofilm although AAOB were present (**Fig. 8C**). This is because the rate of aerobic ammonia oxidation was lower than the rate of anammox reaction due to the limitation of O_2 flux. Similarly, no visible peak of NO_2^- was detected in anammox bacterial granules in oxygen-limited sequencing batch reactors (SBRs) (Nielsen *et al*., 2005). The stoichiometric ratio of total NH_4^+ consumption, NO_2^- consumption

and NO_3^- production in the biofilm was 1: 1.2: 0.33, which was similar to the previously reported value for the anammox reaction (1: 1.31±0.06: 0.22±0.02) (van de Graaf *et al.*, 1996). The slightly lower NO_2^- consumption could be attributed to partial nitrification (i.e., conversion of NH_4^+ to NO_2^-) in the biofilm.

The NO_2^- consumption rate (2.41 g-N m^{-2} day^{-1}) in the biofilm in this study was higher than the rates (0.2 to 0.5 g-N m^{-2} day^{-1}) of anammox bacterial granules in the oxygen-limited sequencing batch reactors (SBRs) as determined by microelectrodes (Nielsen *et al.*, 2005). The NH_4^+ consumption rate of the biofilm (2.04 g-N m^{-2} day^{-1}) was in the same order of magnitude as aerobic NH_4^+ oxidation rats of nitrifying biofilms in the previous studies, in which 2.4 g-N of (NO_2^- plus NO_3^-) m^{-2} day^{-1} (Schramm *et al.*, 1996) and 0.4 g-N of NH_4^+ m^{-2} day^{-1} (Okabe *et al.*, 1999) were detected, respectively. These high rates were probably attributed to the inherent high specific conversion rate of anammox bacteria (Jetten *et al.*, 1999) and the higher density of anammox bacteria in the reactors. In addition, the electron acceptor for anammox reaction (i.e., NO_2^-) could not be limited in the biofilm due to much higher solubility of NO_2^- than that of O_2 for AAOB, which could be advantageous for treating wastewater containing high NH_4^+ concentrations.

FISH analysis clearly showed that AAOB and anammox bacteria coexisted mainly in the surface biofilm. AAOB probably consumed a trace amount of oxygen (< 0.5 mg L^{-1}) and oxidized NH_4^+ to NO_2^-, providing anammox bacteria with suitable microenvironments. While anammox bacteria converted the toxic NO_2^- and remaining NH_4^+ to N_2 gas. A similar distribution of AAOB and anammox bacteria was also found in microbial granules in the oxygen-limited sequence batch reactors (SBRs) (Sliekers et al., 2002; Nielsen et al., 2005). The spatial distribution and types of AAOB could be governed by O_2 concentration in the bulk liquid. FISH and phylogenetic analyses also revealed the presence of other bacteria that were not affiliated with anammox bacteria and AAOB in the deeper part of the biofilm. Since

no organic compounds except EDTA was supplied to the reactor, these bacteria (probably heterotrophs based on the 16S rRNA gene sequence analysis) could utilize the organic compounds derived from dead cells or produced by anammox bacteria as the electron donor for denitrification. This was speculated from the decrease in NO_3^- concentration in the deeper part of the biofilm (**Fig. 7**). Otherwise, anammox bacterial activity might be self-inhibited by accumulation of organic compounds. Further study is necessary to study the interaction between anammox bacteria and other heterotrophic bacteria in the biofilm. We are presently investigating if the coexisting heterotrophic bacteria can utilize the metabolites of anammox bacteria by using microautoradiography-combined with FISH (MAR-FISH) technique (Kindaichi *et al.*, 2004, Okabe *et al.*, 2005).

Application of anammox process

Our findings will provide fundamental understanding on how we could promptly develop and efficiently operate high-rate anammox biofilm reactors. Application of anammox process as the major nitrogen removal process in wastewater treatment systems is advantageous in terms of reduced O_2 (i.e., aeration) and organic carbon demands. In future, energy-saving anammox reactors with small footprint will attain a sustainable nitrogen removal process.

Furthermore, the results of the present study suggested that the up-flow fixed-bed biofilm column reactor used in this study is suited for the combined aerobic ammonia oxidation (partial oxidation to NO_2^-) and anammox process within a single biofilm reactor because of its high biomass retention capacity. Since the solubility of oxygen in water is relatively low, the penetration of oxygen in the biofilm will also be limited. Thus it is likely that when introducing oxygen partial nitrification will occur in the outer layers of the biofilm and anammox will occur in the inner layers. It is, therefore, relatively easy to maintain high anammox activity in such biofilm reactors. This combined process is the so-called completely

autotrophic nitrogen removal over nitrite (CANON) process (Third *et al*., 2001; Sliekers *et al*., 2002) and has been suggested to be a promising nitrogen removal process for wastewater characterized by a low content of organic materials (Schmidt *et al*., 2003). The microbiology, feasibility, and optimization of the single-reactor CANON process have been investigated in SBRs (Nielsen *et al*., 2005; Third *et al*., 2001, 2005; Sliekers *et al*., 2002) and gas-lift reactors (Sliekers *et al*., 2003). Similarly, oxygen limited autotrophic nitrification/denitrification (OLAND) processes have been reported in moving-bed reactors (Szatkowska et al., 2006, Rosenwinkel et al. 2005) and rotating disk contactors (Pynaert et al., 2004). However, these processes have never been applied to fixed-bed biofilm column reactors. Further study on the feasibility and optimization of the CANON process in the fixed-bed biofilm reactors is definitely needed.

Conclusions

In conclusion, we could successfully develop high-rate anammox biofilm reactors using the up-flow fixed-bed biofilm column reactor by inoculating seeding sludge with high abundance of anammox bacteria for short periods, operate steadily and achieve a high nitrogen removal rate of 26.0 kg-N m^{-3} day^{-1} within 250 days. Community structure, spatial distribution and *in situ* metabolic activity of anammox bacteria in the biofilm were also analyzed. The results will provide profound insights into optimal design and operation of anammox biofilm reactors.

Acknowledgement

This research was partly supported by Grant-in Aid (K1740) for Research and Technology Development on Waste Management from the Ministry of the Environment of Japan. Ikuo Tsushima was financially supported by the 21st Century Center Of Excellence (COE)

program “Sustainable Metabolic System of Water and Waste for Area-Based Society” Hokkaido University, Sapporo Japan.

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List of Figures

Figure 1 Schematic drawing of an up-flow fixed-bed column reactor. The nutrient medium was purged with N_2 gas from line A for at least 1 h to achieve the concentration of dissolved oxygen below 0.5 mg L^{-1}. A N_2-filled gas bag was connected with line B to prevent O_2 penetration during the operation.

Figure 2 Rates of influent nitrogen (NH_4^+, NO_2^-, and NO_3^-)-loading and effluent nitrogen removal in the column reactor I (**A**) and reactor II (**B**). The filled triangles represents the nitrogen (NH_4^+, NO_2^-, and NO_3^-)-loading rate into the reactor; the empty circles represents the nitrogen removal rate; and the gray area represents the nitrogen removal efficiency in the reactors. Solid line represents HRT in the reactor. The effluent of the reactor I after 3-mounth operation was inoculated into the reactor II.

Figure 3 The nitrogen removal rates at different influent nitrogen (NH_4^+-N + NO_2^--N) concentrations in the reactor II between the day 259 and 362 when HRT was fixed at 1 h. Error bars indicate the standard deviations (n = 3-6). The whole bars represent total

nitrogen-loading rates and white parts represent nitrogen removal rates. Line plots (●) represent residual nitrite concentrations. Nitrogen removal efficiencies were 57% (400 mg-N L^{-1}), 61% (500 mg-N L^{-1}), 63% (600 mg-N L^{-1}), 64% (700 mg-N L^{-1}), 62% (800 mg-N L^{-1}), 58% (900 mg-N L^{-1}), and 42% (1000 mg-N L^{-1}), respectively.

Figure 4 The nitrogen removal rates and produced DOC concentrations in the reactor II at different HRTs (1 h, 4 h and 8 h). The whole bars represent loading rates and the white parts represent the nitrogen removal rates. Line plots (●) represent the produced DOC concentrations in the reactor. Error bars indicate the standard deviations (n = 10). The produced DOC concentration was obtained by subtracting the effluent DOC concentration from the influent DOC concentration. The nitrogen removal efficiencies and the residual nitrite concentrations were 118 mg-N L^{-1} and 57% at HRT 1.0 h, 53 mg-N L^{-1} and 69% at HRT 4.0 h, 129 mg-N L^{-1} and 60% at HRT 8.0 h, respectively.

Figure 5 The nitrogen concentrations in influent and effluent at different influent NH_4^+/NO_2^- molar ratios in the reactor II when HRT was fixed at 8 h and the influent total nitrogen concentration was fixed at 800 mg-N L^{-1} (black; ammonium, gray; nitrite, white; nitrate concentrations, respectively). Error bars indicate the standard deviations (n = 8). The nitrogen removal rates and nitrogen removal efficiencies were 1.2 kg-N m^{-3} day^{-1} and 50% (NH_4^+/NO_2^-; 0.75-0.85), 1.3 kg-N m^{-3} day^{-1} and 55% (0.95-1.05), 1.5 kg-N m^{-3} day^{-1} and 63% (1.15-1.25), respectively.

Figure 6 Phylogenetic tree of anammox bacteria showing the positions of the clones obtained from the biofilm in the reactor I after 392-day operation. The tree was generated by using 1429 bp of the 16S rRNA and neighbor-joining method. The scale bar represents 2%

sequence divergence. The filled and empty circles at the nodes represent bootstrap values higher than 95% and 80%, respectively (100 times resampling analysis). The *Aquifex aeolicus* sequence served as the outgroup for rooting the tree. Numbers in parentheses indicate the frequency of appearance of the identical clones in the total clones analyzed with specific primer set.

Figure 7 Steady-state concentration profiles of O_2, NH_4^+, NO_2^-, NO_3^- and pH in the anammox biofilm. Surface of the biofilm is at a depth of 0 mm.

Figure 8 Phase contrast image (A) and CLSM images (B and D) of vertical sections (30-μm thick) of the anammox biofilm. (B) FISH with TRITC-labeled EUB338 probe mixture (green) and FITC-labeled Amx820 probe (red) (specific to anammox bacteria; Candidatus *Brocadia anammoxidans* and Candidatus *Kuenenia stuttgartiensis*). Yellow signals result from binding both probes to one cell. (C) FISH with TRITC-labeled Amx820 probe (green) and FITC-labeled Nse1472 probe (red) (specific to *Nitrosomonas eutropha*, *N. europaea* and *N. Halophila*). (D) FISH with TRITC-labeled EUB338 probe mixture (green) and FITC-labeled Amx820 probe. The biofilm surfaces are at the top of all images.

Table 1. Summary of quantification of anammox bacterial 16S rDNA copy numbers and influent C/N ratios in sludges taken from different wastewater treatment plants.

Sludge	Sampling point	Location[a]	Wastewater	copies/mg-dry sludge	C/N (g/g)	Day[b]	MNRR[c] (kg-N m^{-3} d^{-1})
A	Pilot-scale, membrane bioreactor	Soseigawa WWTP	Domestic	2.7×10^{7}	0.9	N.D.[d]	N.D.
B	Full-scale, denitrifying basin	Hakodate-bay WWTP	Domestic	3.1×10^{7}	5.1	223	0.032
C	Full-scale, nitrifying RBC[e]	Teine landfill leachate TP	Landfill leachate	1.7×10^{6}	2.0	171	0.004
D	Full-scale, oxidation ditch	Kuriyama WWTP	Domestic	1.1×10^{8}	1.1	64	0.054
E	Full-scale, denitrifying basin	Hakodate-nanbu WWTP	Domestic	3.0×10^{7}	4.0	143	0.044
F	Full-scale, oxidation ditch	Kiritappu WWTP	Domestic	5.0×10^{7}	0.9	203	0.026
G	Full-scale, anaerobic digester	Ebetsu WWTP	Domestic	1.9×10^{7}	1.5	107	0.039
H	Full-scale, aeration tank	Kitasorachi night soil TP	Night soil	2.4×10^{7}	3.0	54	0.058
I	Full-scale, denitrifying basin	Esan night soil TP	Night soil	1.1×10^{7}	12.4	69	0.048
J	Full-scale, RBC[e]	Chino WWTP	Domestic	9.7×10^{6}	6.8	N.D.	N.D.
K	Full-scale, denitrifying basin	Higashihiroshima WWTP	Domestic	1.6×10^{8}	0.9	37	0.083

[a] All treatment plants were located in Japan. WWTP, wastewater treatment plant; TP, treatment plant.
[b] The day when complete consumptions of 30 mg L^{-1} of NH_4^+-N and NO_2^--N were observed.
[c] Maximum nitrogen removal rates determined in anammox activity tests under anoxic conditions.
[d] Not detected.
[e] Rotating biological contactor.

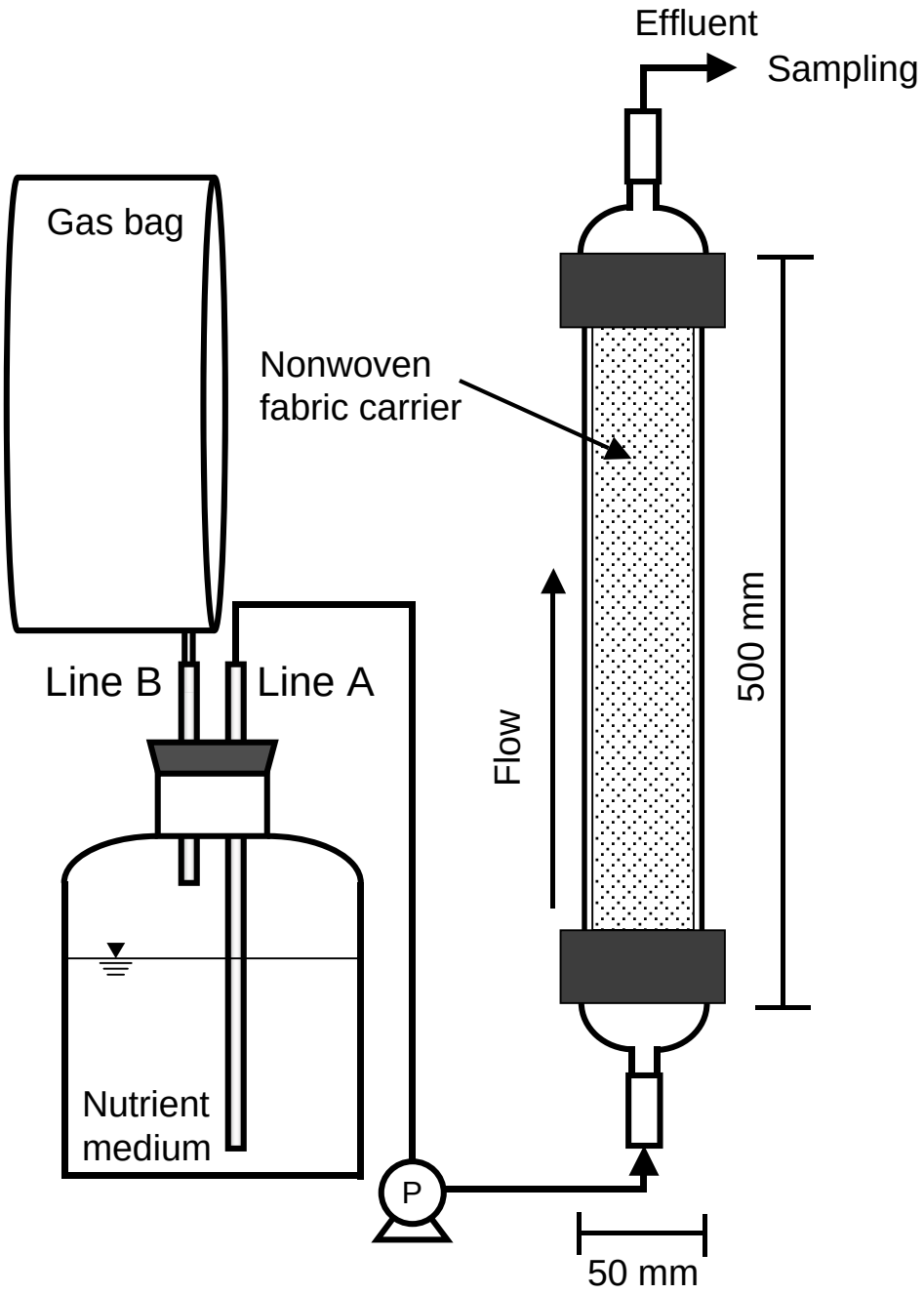

Fig.1 Tsushima et al.

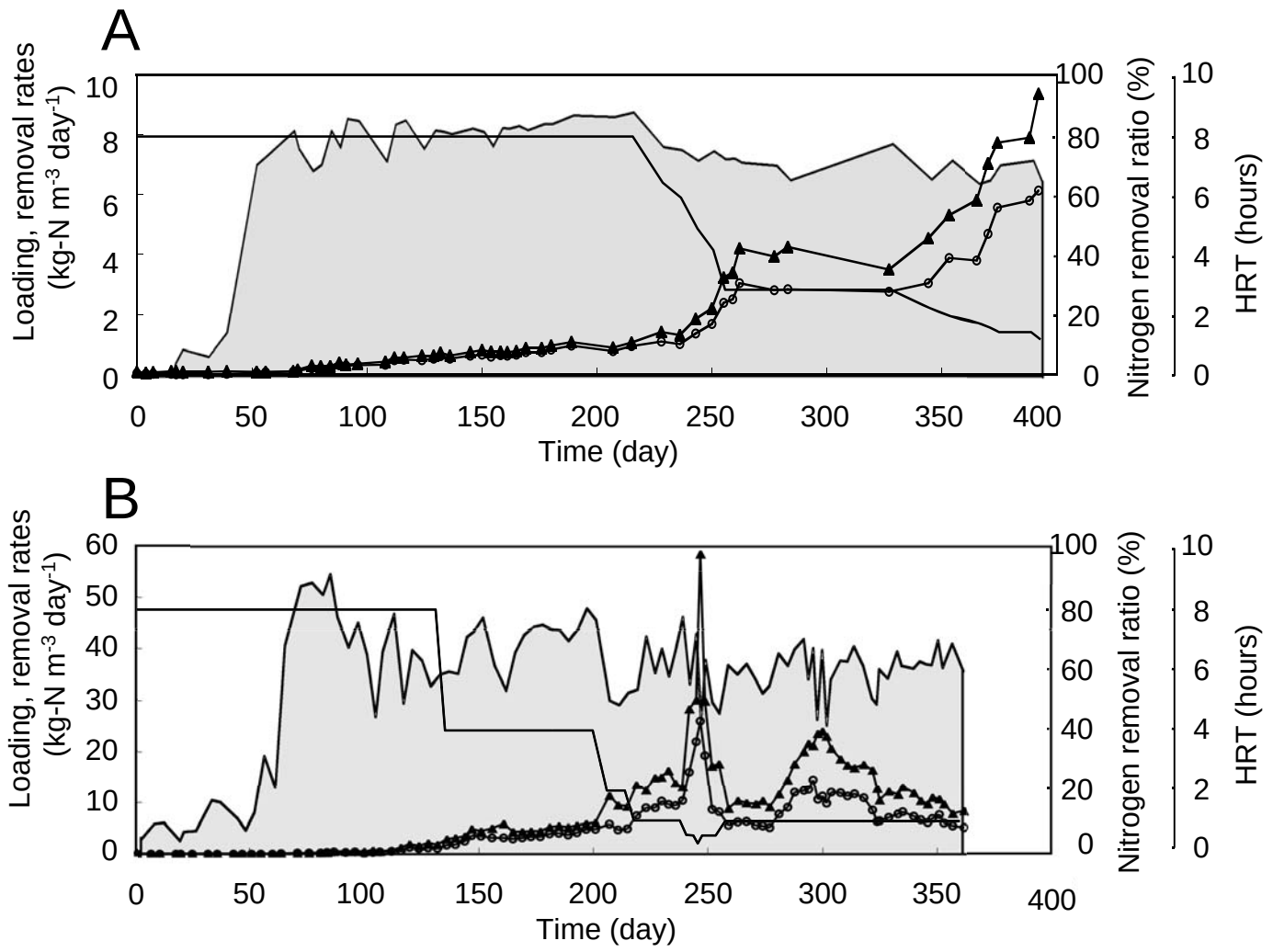

Fig.2 Tsushima et al.

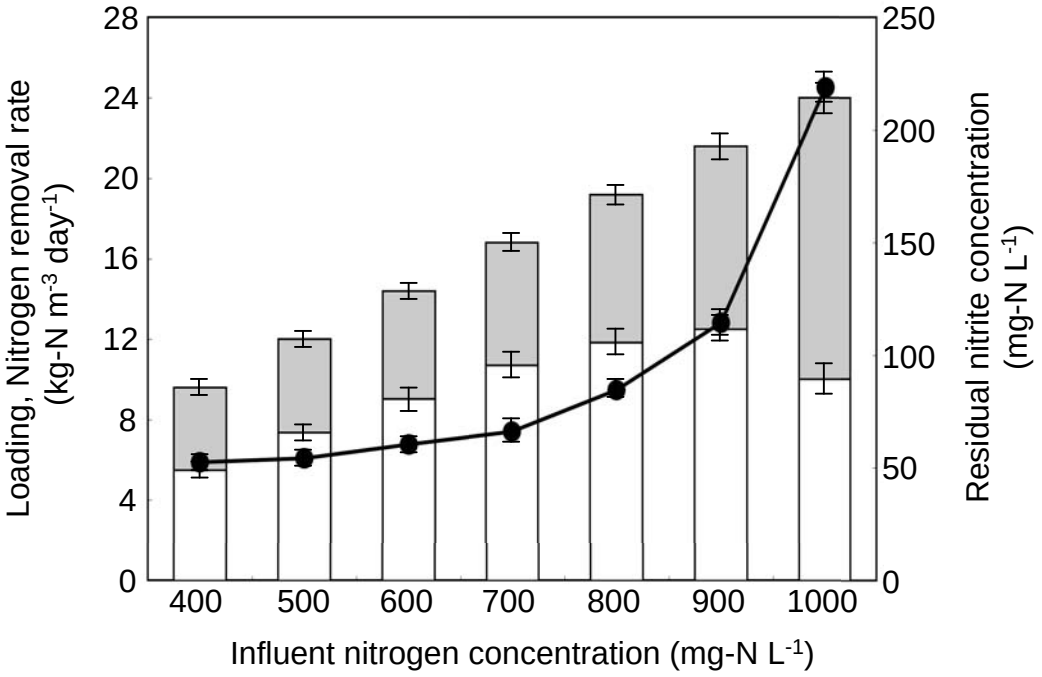

Fig.3 Tsushima et al.

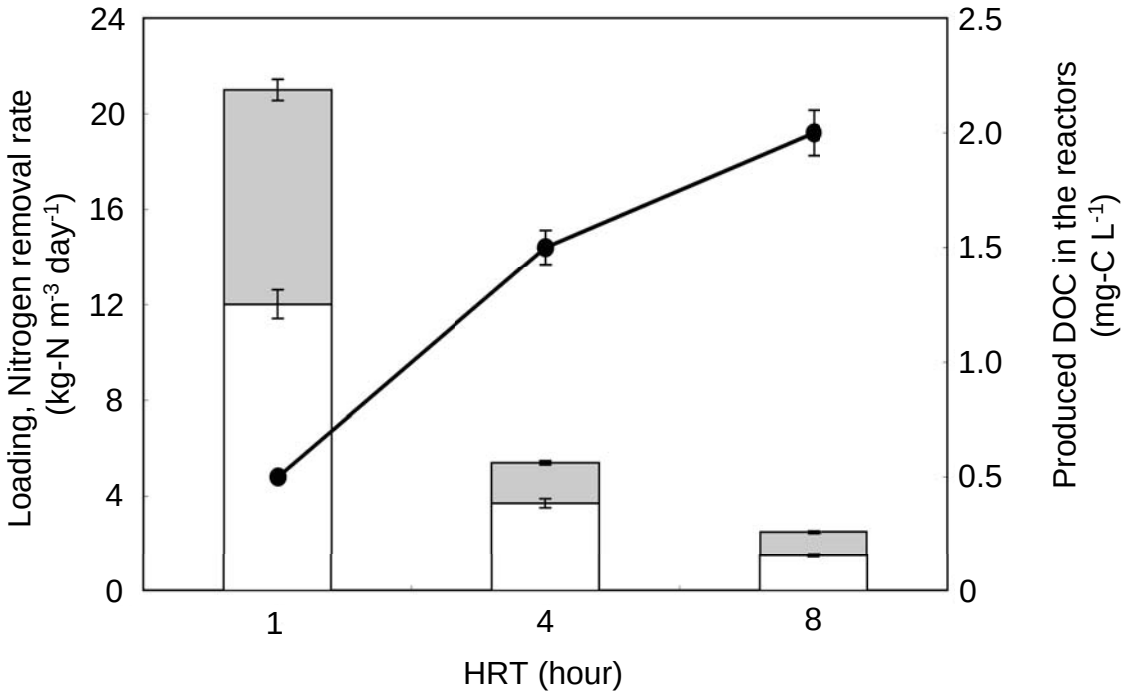

Fig.4 Tsushima et al.

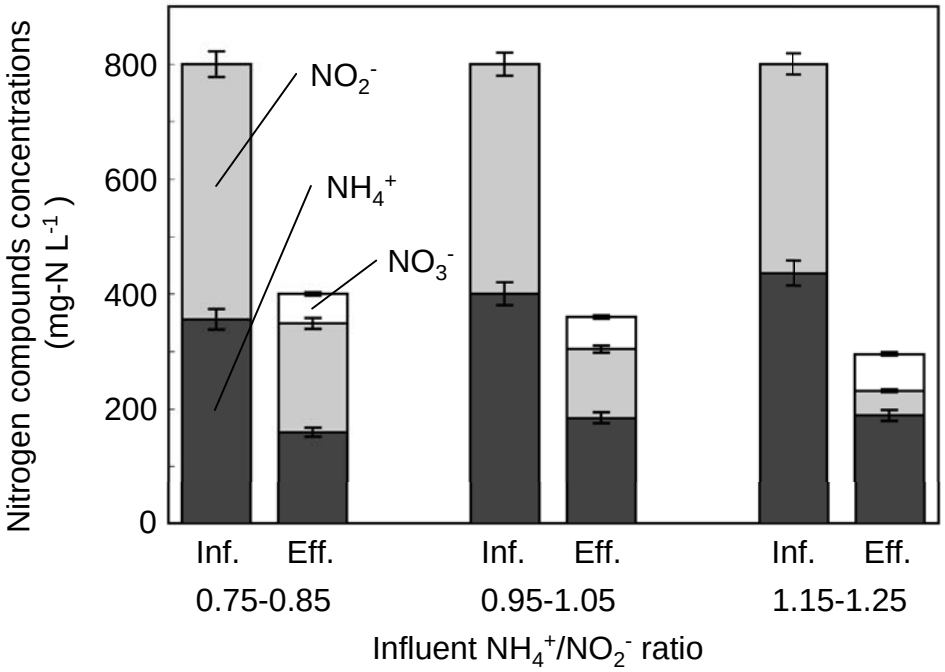

Fig.5 Tsushima et al.

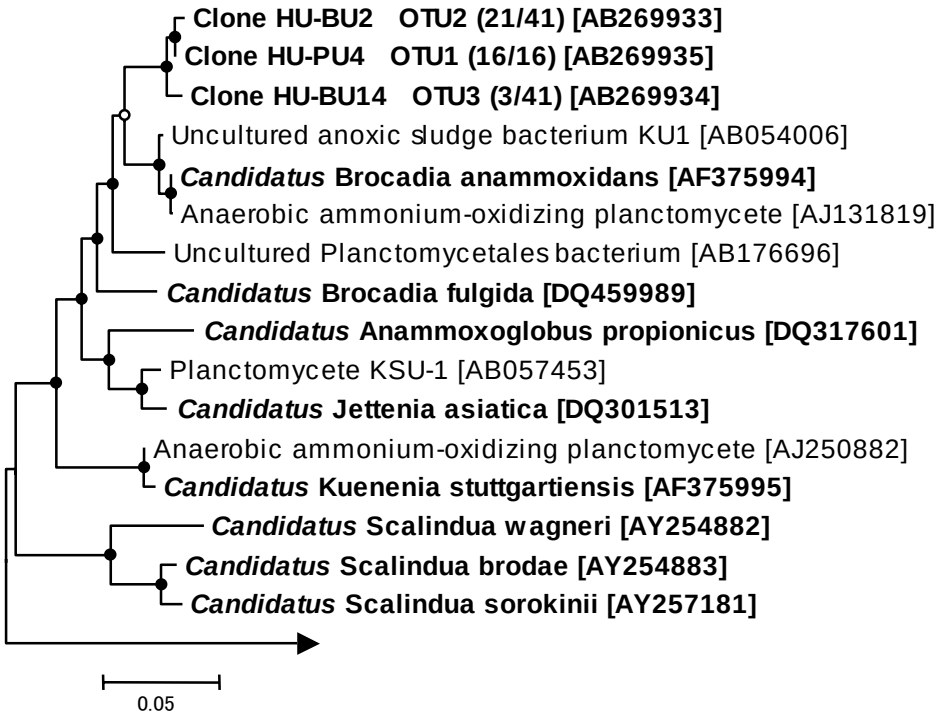

Fig.6 Tsushima et al.

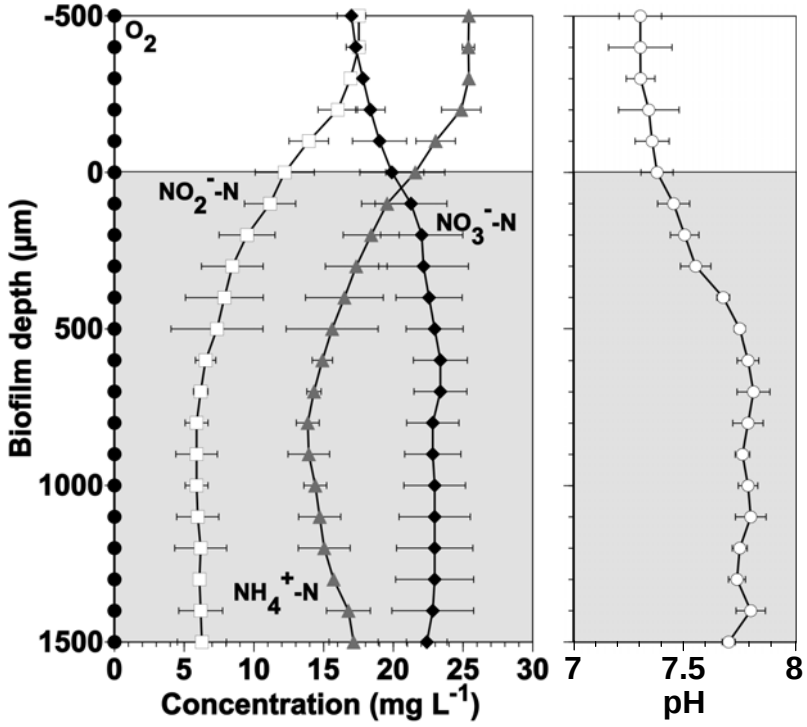

Fig.7 Tsushima et al.

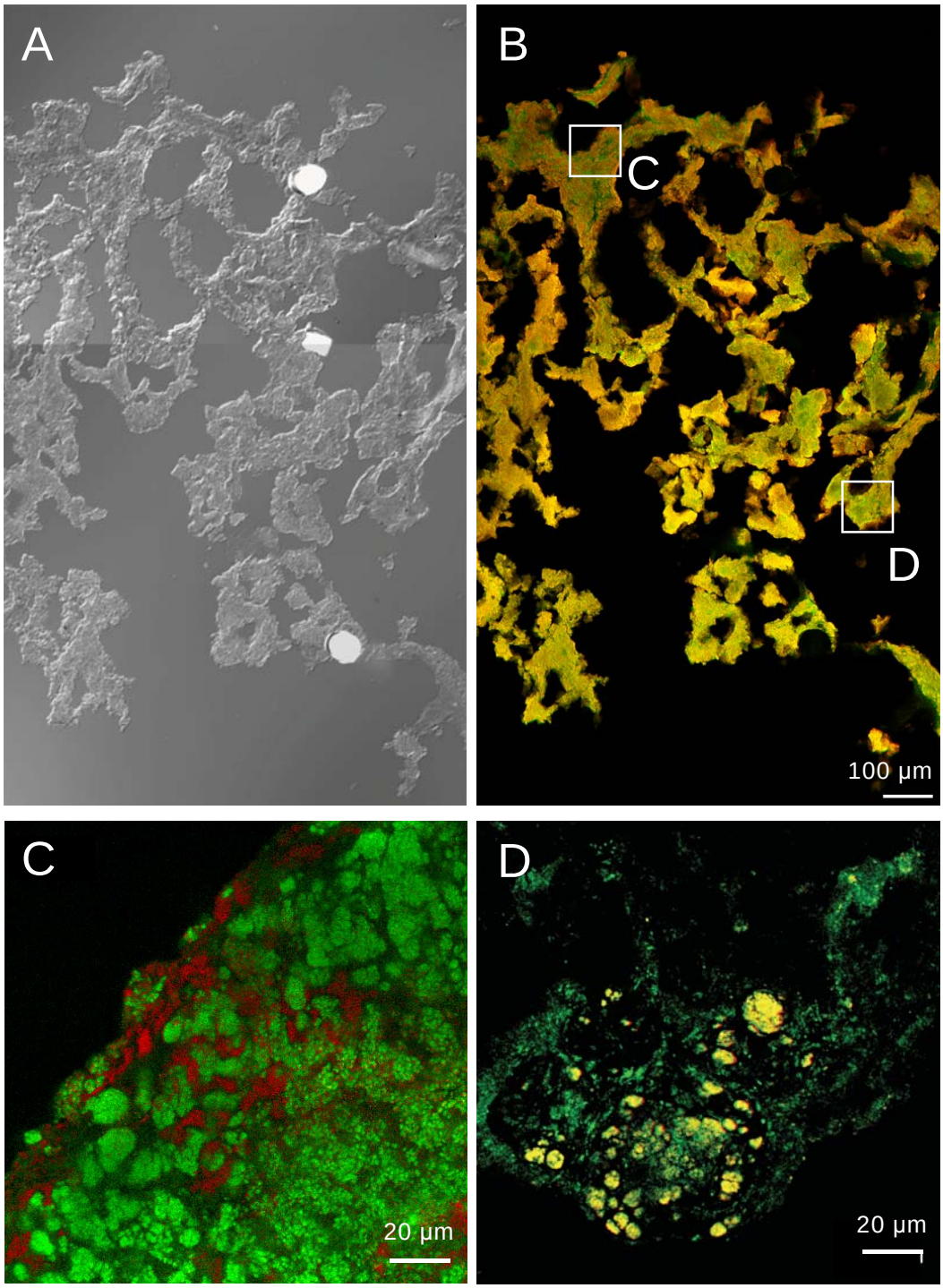

Fig.8 Tsushima et al.