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RESEARCH ARTICLE

Symbiotic relationship analysis of predominant bacteria in a lab-scale anammox UASB bioreactor

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Abstract In order to provide the comprehensive insight into the key microbial groups in anaerobic ammonium oxidation (anammox) process, high-throughput sequencing analysis has been used for the investigation of the bacterial communities of a lab-scale upflow anaerobic sludge bed (UASB) anammox bioreactor. Results revealed that 109 operational taxonomic units (OTUs; out of 14,820 reads) were identified and a domination of anammox bacteria of Candidatus *Kuenenia stuttgartiensis* (OTU474, 35.42 %), along with heterotrophs of *Limnobacter* sp. MED105 (OTU951, 14.98 %), *Anerolinea thermophila* UNI-1 (OTU465 and OTU833, 6.60 and 3.93 %), *Azoarcus* sp. B72 (OTU26, 9.47 %), and *Ignavibacterium* sp. JCM 16511 (OTU459, 8.33 %) were detected. Metabolic pathway analysis showed that Candidatus *K. stuttgartiensis* encountered gene defect in synthesizing a series of metabolic cofactors for growth, implying that *K. stuttgartiensis* is auxotrophic. Coincidentally, the other dominant species severally showed complete metabolic pathways with full set gene encoding to corresponding cofactors presented in the surrounding environment. Furthermore, it was likely that the survival of heterotrophs in the autotrophic system indicates the existence of a symbiotic and mutual relationship in anammox system.

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Introduction

Since first discovered and described in the mid-1990s, anaerobic ammonium oxidation (anammox) is now widely considered as the cost-effective and environment-friendly process in contrast to conventional nitrification and denitrification for nitrogen removal from wastewater with features like high concentration of ammonium and lack of biodegradable carbon, due to less exogenous electron accepter, less energy consumption, and no secondary pollution (Mulder et al. 1995; Van de Graaf et al. 1995, 1996; Strous et al. 1999; Kartal et al. 2010a). Anammox bacteria have been detected in many different habitats, including natural fresh water environments (Schmid et al. 2000) and marine environments (Lam et al. 2007), and engineered systems (Strous et al. 1998). So far, ten anammox species divided over five genera have been described as none of these were obtained as classical pure cultures; all have the taxonomical status of "Candidatus."

For a long time, culturing anammox bacteria has been a challenge because anammox bacteria are slow growing, strictly anoxic autotrophic microorganisms with approximate doubling time of 7–22 days (Strous et al. 1999; Tsushima et al. 2007a, b; Van der Star et al. 2008a; Oshiki et al. 2011). Different types of culture techniques have been applied to meet the demand of purer and more reactive anammox-activated sludge in many laboratories, presented sequence batch reactor (SBR) technique (Strous et al. 1998; Kartal et al. 2011a; Oshiki et al. 2011), upflow anaerobic sludge bed (UASB) reactors (Imajo et al. 2004; Schmidt et al. 2004),

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rotating biological contactors (Van de Graaf et al. 1996; Egli et al. 2001; Ni et al. 2012a), manually fed batch cultivation systems (Sànchez-Melsió et al. 2009; Suneethi and Joseph 2011; Yasuda et al. 2011), and membrane bioreactors (MBR) (Van der Star et al. 2008a; Kartal et al. 2011a) successively. Then along comes a series of integrated processes based on anammox technology such as completely autotrophic nitrogen removal over nitrite (CANON) (Third et al. 2001), simultaneous anammox and denitrification (SAD) (Ke et al. 2015), simultaneous nitrification anammox and denitrification (SNAD) (Chen et al. 2009), and single-stage nitrogen removal using anammox and partial nitrification (SNAP) (Lieu et al. 2006).

In spite of all aspects of the engineering and design of anammox systems that have been attempted, the microbial community and functional profiles of anammox have not been fully explored. Most of researches on the application of anammox process have focused on improvement of activity and abundance of anammox bacteria. However, microbial communities can be seen not only as groups of individual microbes but also as collections of biochemical functions affecting and responding to an environment or host organism. Hence, many essential aspects regarding the ecology and functional traits of the microbiome are essential for optimizing reactor design and performance (Vanwonterghem et al. 2014b). Especially, it could be particularly important to characterize anammox microbial community by different abundances of anammox bacteria, by the presence of specific anammox species or strains, alternative enzymes and pathways, and by variations of interactions between the microbes (Jetten et al. 2009; Hu et al. 2010).

Advances in 16S rRNA/rDNA-based high-throughput sequencing techniques and metagenomics have provided new tools for studying the microbiome from thousands of microbes to analysis of community structure, biochemical function, and systems-level microbial interactions. To date, more than 2500 prokaryotic genomes and about 650 metagenomes are currently published in GenBank (http://www.ncbi.nlm.nih.gov/genbank/), and particularly, the genomes of anammox bacteria Candidatus *Kuenenia stuttgartiensis* has been successfully assembled through the sequencing of anammox culture metagenomes (Strous et al. 2006).

On this basis, a series of researches have been enabled to proceed rapidly. Examples include establishment of basal metabolism model of anammox bacteria as chemolithoautotroph (Strous et al. 2006) and chemo-organotroph (Güven et al. 2005; Winkler et al. 2012), microbial community analysis of anammox-activated sludge (Ke et al. 2015; Kotay et al. 2013; Gonzalez-Martinez et al. 2014), and community-level functional profiling (Van de Vossenberg et al. 2013; Shu et al. 2015). Nevertheless, the underlying correlations between anammox bacteria and co-existing microbial community members have seldom been studied.

Based on the above arguments, the study was conducted with the following purposes by reconstructing metabolic networks in aspect of carbon, nitrogen-related metabolism from genome information: (1) to reveal the potential cooperation and interaction between anammox bacteria and co-existing abundant microorganisms in aspect of energy metabolism and (2) to find out why the anammox bacteria grow slowly and cannot be purely cultured. It would be helpful for developing start-up and operational strategies, constructing a stable and sustainable microbial community and developing other combination technologies based on anammox. Hence, in this effort, a typical lab-scale UASB anammox bioreactor has been successfully established and steadily operated by feeding with synthetic wastewater over months and the characteristics of microbial community was further investigated using Illumina-generated 16S rRNA gene amplicon sequencing.

Materials and methods

Experimental setup and operational strategy

The UASB anammox seeding sludge in this study was collected from a 22-L upflow fixed-bed reactor located at Kumamoto University Japan using a polyethylene nonwoven fabric as biomass carrier and fed with synthetic wastewater. The mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) of the seeding sludge were 1360 and 1070 mg/L, respectively. MLVSS/MLSS was 0.78.

The UASB anammox bioreactor was fed with synthetic wastewater for the purpose of anammox microorganism cultivation. The composition of the synthetic wastewater contained NH_4^+-N ($(NH_4)_2SO_4$), NO_2^--N ($NaNO_2$), $NaHCO_3$ 1000 mg/L, KH_2PO_4 68 mg/L, $FeSO_4.7H_2O$ 9 mg/L, and ethylenediamine tetraacetic acid disodium salt (EDTA-2Na) 5 mg/L. The molar ratio of influent NO_2^--N to NH_4^+-N was kept at 1.1–1.3 approximately. The synthetic wastewater was prepared with tap water that it was not pretreated to remove the dissolved oxygen (DO) or adjusted pH before being fed to the reactor for simulating the actual operation. The initial DO concentration and pH of the synthetic wastewater were measured randomly and their corresponding values fluctuated at 3–6 mg/L and 7.4–7.7, respectively. No trace element solution according to Van de Graaf et al. (1996) was added as the trace elements in the tap water can be sufficient to meet the bacteria growth demand.

As shown in Fig. 1, the performance of a 18.0-L UASB anammox bioreactor (internal diameter of 0.15 m, height of 1.0 m) has been analyzed. The influent was continuously injected into the bottom of the reactor by a peristaltic pump with a stepwise increase in flow rate. The treated effluent was purged from the gas-liquid-solid separator where the gas was

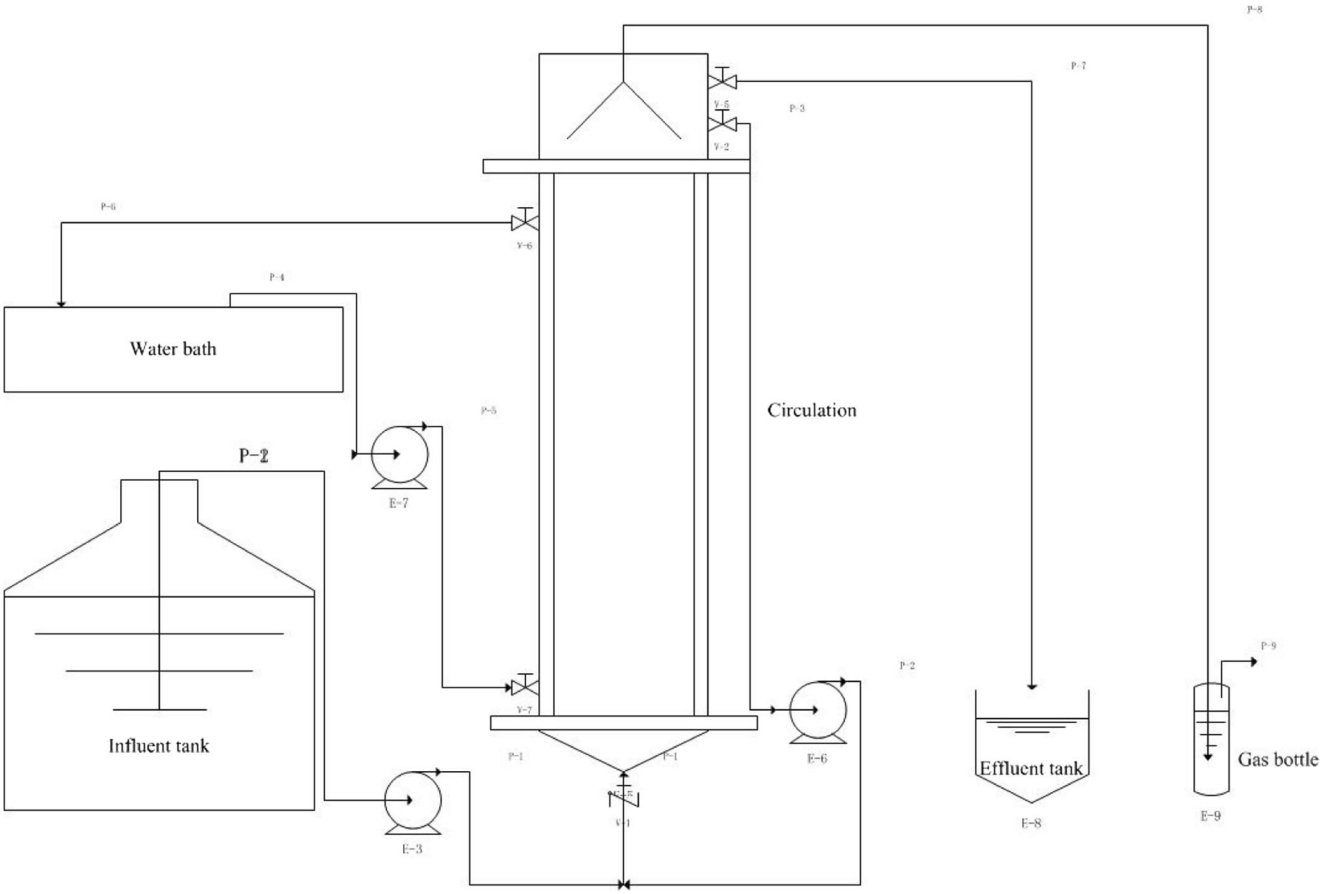

Fig. 1 Schematic representation of the UASB anammox used in this research

led off by a gas tube. The bioreactor was covered with black vinyl sheet to impede the exposure of the bioreactor to light radiation. Water jacket was applied to maintain reaction temperature at about 33 ± 1 °C. The operational parameters of the bioreactor can be seen in Table 1.

Analytical methods

The influent and effluent samples were collected on a daily basis and were analyzed immediately. The nitrogen compound concentrations (NH_4^+-N, NO_2^--N, and NO_3^--N) were colorimetrically measured using a UV1240 spectrophotometer (Shimadzu, Japan) and the MLSS/MLVSS were determined according to standard methods (APHA 2005). DO and pH were estimated using portable DO meter (WTW Oxi3210, Germany) and pH meter (HANNA HI98183, Italy).

DNA extraction, 16S rDNA Illumina Miseq sequencing and post-run analysis

Sampling was done by collection of water-sludge mixture inside the bioreactor of stable operation of the system at different heights at day 178. Samples were then eluted with

Table 1 Operational conditions of the UASB anammox

Operational conditions	
Hydraulic parameters	
Volume of the bioreactor (L)	18
HRT (day)	24–2.06
SRT (day)	–
Bioreactor parameters	
Temperature (°C)	33 ± 1
pH	No control
DO (mg/L)	No control
Influent composition	
SS influent (mg/L)	0
BOD influent (mg/L)	0
TN influent (mg/L)	77.78–840.26
NH_4^+-N influent (mg/L)	31.14–407.74
NO_2^--N influent (mg/L)	43.45–440.75
NO_3^--N influent (mg/L)	1.35–9.26
$NaHCO_3$ influent (mg/L)	1000
KH_2PO_4 influent (mg/L)	68
$FeSO_4.7H_2O$ influent (mg/L)	9
EDTA-2Na influent (mg/L)	5

saline solution 9 % NaCl and centrifuged during 10 min at 3000 rpm for biomass collection. Biomass collected was stored at −20 °C for further DNA extraction.

The DNA extraction procedure was accomplished using Powersoil DNA Isolation Kit (MoBio, Carlsbad, CA, USA) and following the instructions given by the manufacturer. The quality of the DNA extracted was examined by 1 % (*w*/*v*) agarose gel electrophoresis and concentration measured with a UV–Vis spectrophotometer (NanoDrop 2000, USA). The primers 338F (5′-ACT CCT ACG GGA GGC AGC AG-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′) were used to amplify of V3-V4 regions of 16S rRNA gene of Bacteria. PCR conditions were set up as follows: initial preheating at 95 °C for 2 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, and then final elongation for 5 min in a GeneAmp 9700 Thermo Cycler (ABI, USA). The triplicate amplicons were pooled together, electrophoresed on a 2 % (*w*/*v*) agarose gel, and recovered using an AxyPrep DNA Gel Extraction Kit (AXYGEN, China). The purified amplicon was quantified using a QuantiFluor-ST Fluorometer (Promega, USA), and then a composite sequencing library was constructed by combining equimolar ratios of amplicons from all samples. The resulting library for paired-end sequencing (2 × 250 bp) was analyzed on an Illumina Miseq platform at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Raw sequence data of this study have been deposited to the NCBI Sequence Read Archive with accession no. SRR2960312.

Low-quality reads (ambiguous nucleotides and quality value < 20) were removed from the raw sequence data as described in (Caporaso et al. 2011). The paired-end reads from each sample were overlapped to assembly V3–V4 tags of 16S rRNA gene using SeqPrep (https://github.com/jstjohn/SeqPrep), and then UCHIME was used to remove chimera sequences from the tags (http://drive5.com/usearch/manual/uchime_algo.html).

The effective sequences were clustered into OTUs at 97 % sequence identity using UPARSE (a high-accuracy method for generating clusters (OTUs) from sequencing reads of marker genes such as 16S rRNA, the fungal ITS region, and the COI gene) (version 7.1 http://drive5.com/uparse/) embedded in QIIME (QIIME is an open-source bioinformatics pipeline for performing microbiome analysis from raw DNA sequencing data) (Caporaso et al. 2010), and a representative sequence was then picked for each OTU by selecting the most abundant sequence in that OTU. These representative sequences were assigned to taxonomic classification by Ribosomal Database Project Classifier (RDP assigning sequences derived from bacterial and archaeal 16S genes and fungal 28S gene to the corresponding taxonomy model) (http://rdp.cme.msu.edu/) with a confidence threshold of 70 % (Wang et al. 2007).

Phylogenetic tree

Six representative OTU sequences of bacteria accounting for more than 3 % relative abundance within the bioreactor were compared with identified species/sequences using nucleotide Basic Local Alignment Search Tool (BLASTN) (version 2.3.0+ http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1997) to search against the reference genomic sequences databases (refseq_genomic). Megablast was used to select an appropriate model of sequence evolution for phylogenetic inference which is intended for comparing a query to closely related sequences and works best if the target percent identity is 95 % or more. For anammox bacteria, Candidatus *K. stuttgartiensis* scaffolds were used for phylogenetic analysis. The full-length 16S rRNA gene and BLAST hits were collected from NCBI. Multiple sequences alignments were performed with ClustalW in MEGA (version 5.0 www.Megasoftware.net) (Tamura et al. 2011). The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are in the units of the number of base substitutions per site.

Pathway reconstruction based on KEGG

The protein sequences of five organisms identified by BLAST from above were retrieved from NCBI. The protein sequences were then uploaded to the BlastKOALA (KEGG Orthology and Links Annotation) (http://www.kegg.jp/blastkoala/) for annotation. Prokaryotes as target taxonomy group and family_eukaryotes + genus_prokaryotes database were chosen in the scoring scheme for K number assignment. Annotation was performed automatically on the BlastKOALA server located at the Supercomputer Facility in the Institute for Chemical Research, Kyoto University (scl.kyoto-u.ac.jp). Once the annotation was completed, annotation data was downloaded. All five annotation results were integrated into one text file in the order of relative abundance of bacteria from most to least. After that, the integrated data was uploaded to KEGG Mapper to reconstruct pathway (http://www.kegg.jp/kegg/tool/map_pathway.html) that all pathway reconstruction results were displayed. The study focused on the basal metabolism of dominate bacteria so that carbon fixation pathways in prokaryotes (map00720), nitrogen metabolism (map00910), and also four metabolic pathways of cofactors and vitamins (pantothenate and CoA biosynthesis map00770; biotin

metabolism map00780; folate biosynthesis map00790; ubiquinone and other terpenoid-quinone biosynthesis map00130) were selected for further study. In the present paper, a putative inorganic nitrogen metabolic pathway of anammox was depicted instead of map00910. All pathway maps have been redrawn using Microsoft Visio software (version 2010 http://www.microsoft.com/) for better visual effect.

In Figs. 4 and 5, enzymes are shown in the form of Enzyme Commission number with five-grid shaded background representing the 5 dominant species in order from left to right (OTU474 Candidatus *K. stuttgartiensis*, OTU951 *Limnobacter* sp. *MED105*, OTU26 *Azoarcus* sp. *BH72*, OTU459 *Ignavibacterium album JCM 16511*, OTU465 and OTU833 *Anaerolinea thermophila UNI-1*). The enzyme is colored by pink if the expression of relevant gene has been identified in the genome, otherwise by grey.

Results and discussion

Reactor performance

The reactor performance was monitored for 178 days. The treatment results for continuous operation of the reactor are shown in Fig. 2. The anammox reactor was started up with a total nitrogen (TN) concentration of 77.79 mg/L and an hydraulic retention time (HRT) of 24 h. During days 1–35, total nitrogen removal efficiencies were not very stable around 50 % by increasing the influent TN concentration and decreasing the HRT. From day 36 to day 100, as the influent TN concentration remained constant, nitrogen removal rate (NRR) was steadily increased via successively shortened HRT to about 2 h. Subsequently, the anammox performance was further accelerated with increasing influent TN concentration step by step. The nitrogen removal performance finally reached a relative stationary phase as the average TN removal efficiency maintained at about 80 %.

During the whole period of operation, the influent TN concentration was progressively increased from 77.78 to 840.26 mg/L and HRT was decreased from 24 to 2.06 h. The maximum total nitrogen loading rate (NLR) of 8.25 kg N/m^3/day and corresponding NRR of 6.93 kg N/m^3/day were obtained in 178 days of operation.

The average stoichiometric coefficients of NH_4^+-N to NO_2^--N and NH_4^+-N to NO_3^--N were 1:1.31 and 1:0.25, respectively, which were closed to the results (1:1.32 and 1:0.26) proposed by Strous et al.(1999). Anammox stoichiometry may vary dependently upon inoculum source, substrate, operating conditions, and reactor configuration (Ahn 2006; Kieling et al. 2007; Ni et al. 2012b).

The start-up of a typical common bench-scale UASB anammox reactor was carried out by inoculating with acclimated activated anammox granular sludge. Consequently, after a brief adaptive phase, the anammox performance of the reactor was sharply enhanced following by a relative stationary phase. Over the course of the study, the influent DO had no impact on anammox performance. There are two possible explanations for this phenomenon. Firstly, there might exist a small number of aerobic bacteria or facultative bacteria which could eliminate DO. Another possible reason could be that the structural features of granular sludge have certain effect on resisting oxygen penetration, which ensured relatively less aerobic and more anoxic in the intra of a granule (Volcke et al. 2012). Most of the reported anammox reactors were operated without pH control at a self stabilizing pH between 7 and 8.5, which has been reported as the optimum range for anammox consortia (Strous et al. 1998). The fluctuations of pH within this range have not been considered important with respect to the stability issue (Fux et al. 2004). The rise in pH depends on the intensity of anammox reaction. In the present research, it has been explored that the pH values in the reactor remained in optimal range. Thus, we have not done any adjustment about pH value.

Normally, total NRR of 0.5 kg N/m^3/day has been considered the standard for successful anammox process induction (Jin et al. 2008). Therefore, the above results and analysis suggested that the anammox system has been quickly started-up and stably operated. Biomass at day 178 was collected for further DNA extraction.

Phylogenetic tree and relative abundance of dominant bacterial species

Species richness analysis of the UASB anammox bioreactor shows a total of 14,820 reads and a number of OTUs of 109. Hill diversity indices of first order (Shannon index) and second order (Simpson index) are of 2.601256 and 0.167969, respectively. A representation of the phylogenetic diversity of OTUs >3 % relative abundance in the UASB anammox bioreactor is shown in Fig. 3. The total percentage of 6 OTUs accounted for about 80 % of the microbial community which can be considered to express the representative characters of the whole anammox system.

The dominant OTU in the bacterial community structure was OTU 474, accounting for a 35.42 % relative abundance. OTU 474 was phylogenetically related to *Kuenenia* spp. had a 99 % similarity with Candidatus *K. stuttgartiensis*. It is also supported by many other sources that found a dominance of Candidatus *Kuenenia* in enriched ANAMMOX communities within lab-scale and full-scale bioreactors (Kuenen 2008; Speth et al. 2012), while Candidatus *Brocadia* known as another representative anammox genus was not detected in the study. In contrast to our findings, Gonzalez-Martinez et al. (2014) found that the bacterial community of a lab-scale MBR anammox bioreactor operated for more than 500 days was dominated by Candidatus *Brocadia* as detected by 16s

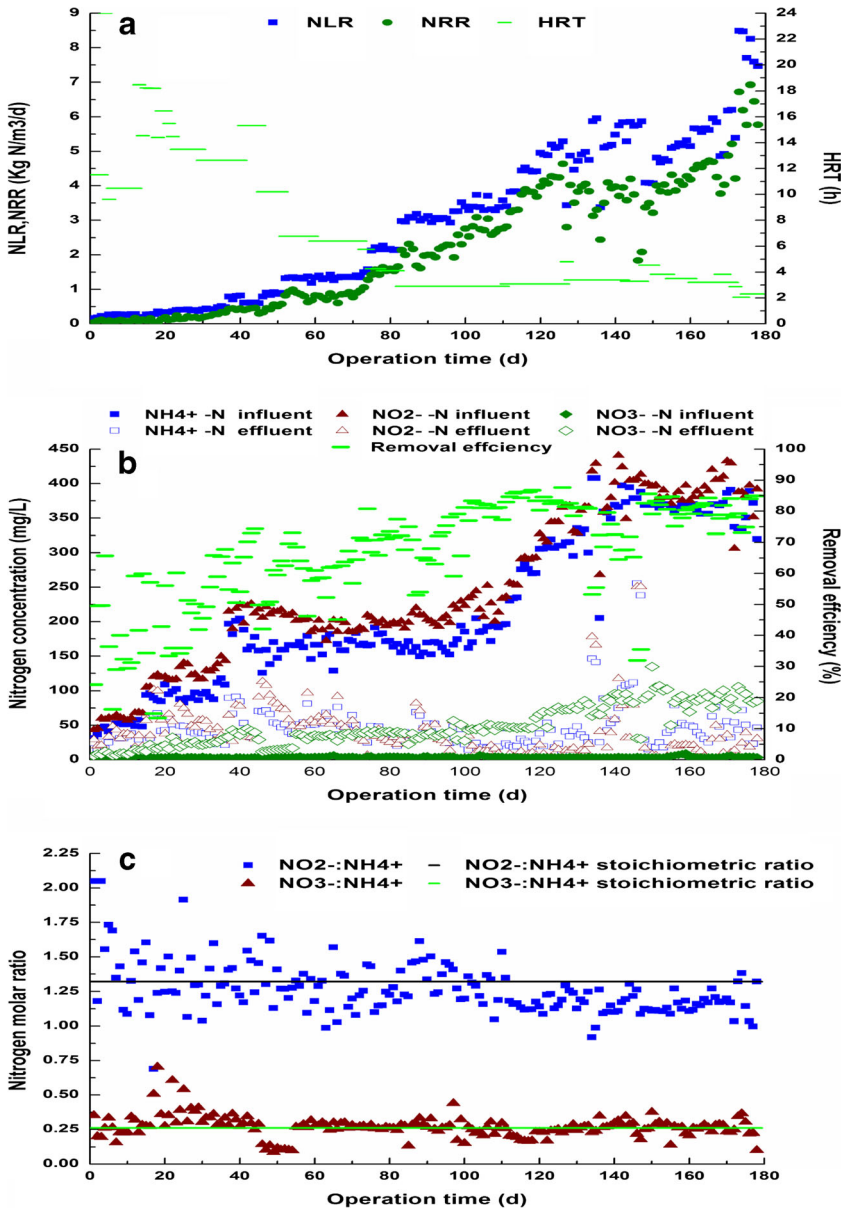

Fig. 2 Time course of nitrogen removal performance measured in the UASB anammox reactor

rDNA analysis. It seemed that the anammox bacteria tend to develop a simple community with mono species (Ni et al. 2010; Cho et al. 2010). Nevertheless, the genus *Jettenia* was also detected with low relative abundance of 0.56 %. Similar result was reported by Guo et al. (2015). Such selection was probably driven by the composition of the feed, extant substrate concentrations in the reactor, the inoculums, reactor configuration, and the length of operation period.

OTU 951 shares a 98 % similarity with *Limnobacter* sp. MED105. *Limnobacter* sp. MED105 is a Gram-negative, motile, rod-shaped mesophile first isolated from surface water of the Mediterranean Sea by J Craig Venter Insititute (USA) (NCBI BioProject Accession: PRJNA19317). The genome of strain MED105 has been clarified but there were few research reports on further explorations.

OTU 26 had a close relationship with *Azoarcus* spp. at a 97 % similarity to strain BH72 and accounted for a total

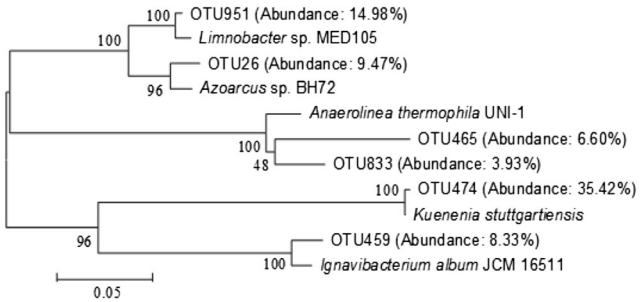

Fig. 3 Phylogenetic tree of selected OTUs (>3 %) identified in UASB constructed by Mega 5.0 on basis of the Blast results of the first six predominant sequences

abundance of about 9.47 %. Strain BH72 was first isolated from surface-sterilized roots of Kallar grass in the Punjab of Pakistan; *Azoarcus* sp. strain BH72 is found to be highly adapted to environments poor in available nitrogen sources; as an endophytic bacteria, *Azoarcus* sp. strain BH72 supplies the majority of ammonia derived from N_2 fixation to its host plant (Hurek and Reinhold-Hurek 2003). Strain BH72 has a strictly respiratory type of metabolism and does not grow on any carbohydrate tested that the major carbon sources for strain BH72 are dicarboxylic acids and ethanol (Krause et al. 2006). Although we cannot clear whether anammox bacteria could provide dicarboxylic acid or ethanol to strain BH72 directly, considering the complexity and functional diversity of microbial community, metabolites of anammox bacteria or other organisms might be converted into a series of metabolic process to carbon compounds utilized by BH72. Moreover, it was a N_2-sufficient environment inside of the UASB bioreactor that provided adequate nitrogen substrate to BH72. Ability of *Azoarcus* sp BH72 for undergoing nitrogen metabolism is thought to allow this species to gain advantage over other heterotrophs.

OTU 459 was affiliated with *I. album*, sharing a 97 % similarity with strain JCM 16511. *Ignavibacterium* sp., the only non-phototrophic member of the phylum *Chlorobi*, has been suggested to live under both oxic and anoxic conditions (Iino et al. 2010). It is proposed as a heterotrophic bacterium which is dependent on exogenous amino acids for protein and nitrogen sources because of its inability to synthesize several amino acids (Liu et al. 2012).

OTU465 and OTU 833 were both related to *Anerolinea thermophila* UNI-1 in sharing 95 and 96 % similarity, respectively. Strain UNI-1, a representative of subphylum I of *Chloroflexi*, is a strictly anaerobic organism: no growth occurred in the presence of oxygen (20 %, *v*/*v*, in the gas phase); it is not photosynthetic and grows chemo-organotrophically on a number of carbohydrates and amino acids (Sekiguchi et al. 1998& 1999). Moreover, the previous publications have proposed that *Chloroflexi* bacteria could utilize the macromolecules derived from biomass decay and reinforce the granule structure within a network of filamentous biomass (Sekiguchi et al. 2001; Miura and Okabe 2008). Molecular studies on activated sludge systems have also shown a remarkable abundance and distribution of microbes belonging to *Chloroflexi* in these systems (Björnsson et al. 2002; Juretschko et al. 2002).

Operational conditions of the bioreactor, such as oxygen concentration, and influent composition made the system a proper environment for anammox bacteria to develop OTU474 *K. stuttgartiensis* highly enriched—the only one found to be capable of performing autotrophic and anaerobic denitrification inside the system. The existence of heterotrophic bacteria suggests that some anaerobic heterotrophic bacteria capable of respiration of organic carbon sources derived from the soluble microbial products (SMP) and extracellular polymeric substances (EPS) released from surroundings could grow inside the system. For instance, Kindaichi et al.(2012)-revealed that the uncultured *Chloroflexi* gradually utilized ^{14}C-labled products from an anammox bacteria culture, indicating that these bacteria preferentially utilized the decaying anammox bacteria cell materials.

Energy metabolic pathways

Based on the above assessment, and by emphasizing once again that only inorganic carbon source and inorganic ammonium and nitrite were available in the influent, we considered that the autotrophic nitrogen removal by anammox bacteria was the primary reaction over heterotrophic reactions.

In Fig. 4a, we depicted a general overview of the nitrogen metabolism of anammox bacertia based on previous studies (Strous et al. 2006; Jetten et al. 2009; Kartal et al. 2013). A net translocation of protons across a semi-permeable membrane system is carried out by a series of soluble cytochrome c (Cyt c) proteins and quinone (Q) derivatives present in the membranes create the proton-motive force (*pmf*) in the course of ammonium-nitrite coupling redox reaction (red arrows); high-energy electrons (e) from hydrazine (N_2H_4) are transferred via ferredoxin to the acetyl-coenzyme A (acetyl-CoA) synthetase/carbon monoxide dehydrogenase and the replenishment of the hydrazine pool to compensate for the hydrazine invested in carbon fixation requires no additional enzymes except reverse electron transport; this results in adenosine triphosphate (ATP) synthesis and reduction potential lying in nicotinamide adenine dinucleotide (phosphate) hydrogen (NAD(P)H) and ferredoxin for cell anabolism (blue arrows); moreover, the scheme involves cyclic electron flow that the electron withdrawn from reduction reactions is replenished by the oxidation of nitrite to nitrate (green arrows). The catabolism with nitrogen compounds is an energy-generating reaction that energy is then used for carbon fixation into cell biomass (Strous et al. 1998).

Cell carbon fixation in anammox bacteria involves a folate-dependent one-carbon metabolic pathway as known as the Wood-Ljungdahl pathway (Fig. 4b). The two branches of the Wood-Ljungdahl pathway merge at the synthesis of acetyl-CoA by giving a methyl group and a carbonyl group separately. Acetyl-CoA is the substrate for all cell constituents starting with the glucogeogensis/glycolysis route and the tricarboxylic acid cycle as intermediary pathways (Jetten et al. 2009). The genome of Candidatus *K. stuttgartiensis* contains all relevant genes expressed of the Wood-Ljungdahl pathway for CO_2 fixation (Strous et al. 2006; Kartal et al. 2011a, b). While a series of genes coded to enzymes for synthesis acetyl-CoA from CO_2 are either missing or incomplete in the other four detected strains (Fig. 4b). These are further evidences that Candidatus *K. stuttgartiensis* is the only one conducting autotrophic carbon fixation. Further carboxylation related

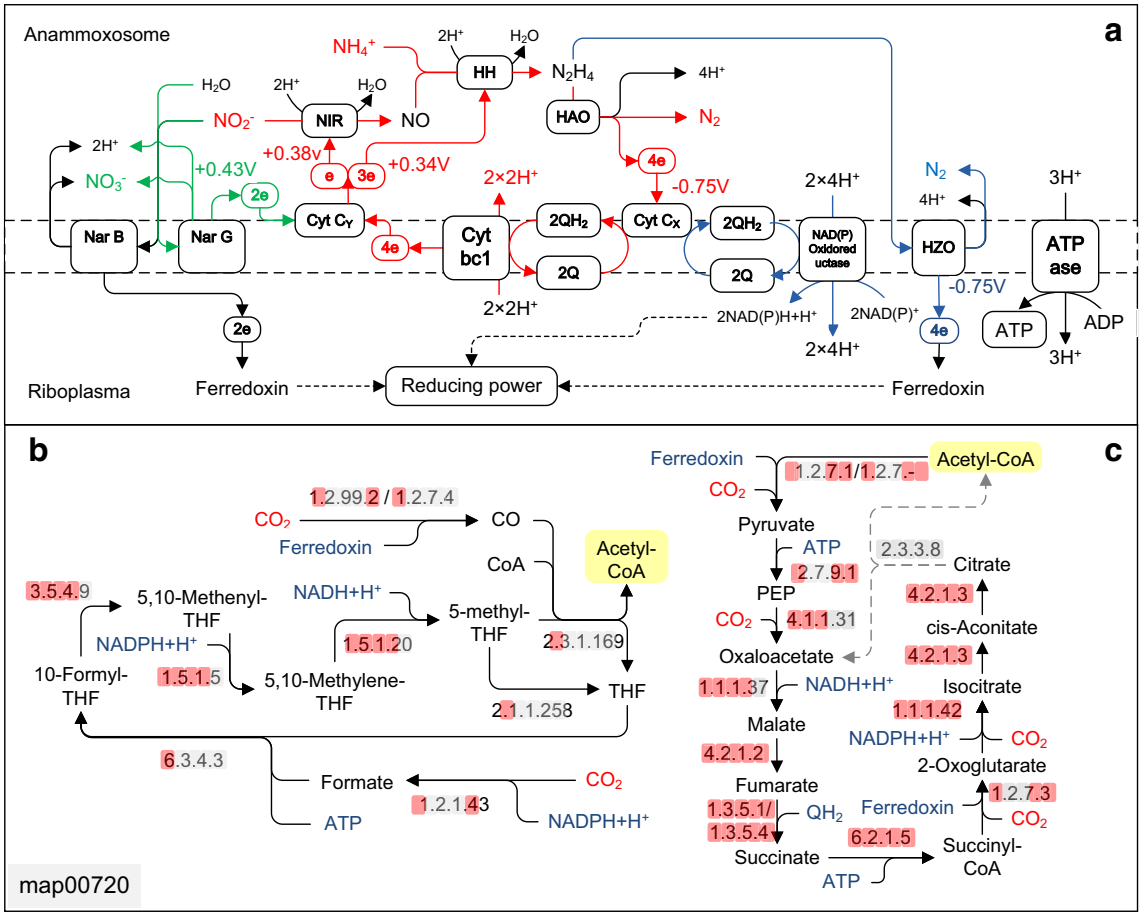

Fig. 4 Energy related metabolic pathway of the first five dominant bacteria reconstructed by KEGG Mapper

metabolism starting from acetyl-CoA follows the reductive carboxylic acid cycle as shown in Fig. 4c. Candidatus *K. stuttgartiensis* shows all genes coding for the reverse tricarboxylicacidcycle acid cycle (TCA) except for the one coding for EC 2.3.3.8 citrate synthase/citrate lyase. This would suggest that the reverse TCA cycle is not fully closed and it is a one-way reaction for carbon accumulation from 2 carbon to 6 carbon unit. The genes coding for EC 1.2.7.1 pyruvate synthase and EC 2.7.9.1 pyruvate orthophosphate dikinase are missing in *Limnobacter* sp. MED105 and *Azoarcus* sp. BH72, resulting in inability of pyruvate synthesis from acetyl-CoA. Likewise, EC 4.1.1.31 phosphoenolpyruvate carboxylase is missing in *I. album* JCM 16511 and *A. thermophila* UNI-1 which caused incapacity of oxaloacetate replenishment.

In genetics, gene expression is the most fundamental level at which the genotype gives rise to the phenotype. Such phenotypes are often expressed by the synthesis of proteins that act as enzymes catalyzing specific metabolic pathways characterizing the organism. It means that organisms do not have certain functions without the expression of related functional genes. Giving the above, Candidatus *K. stuttgartiensis* was the only one with efficacy of energy-producing via nitrogen denitrification and then utilizing the energy on biosynthesis of organic compounds from inorganic HCO_3^-/CO_2. The metabolites or intermediates of anammox reaction could be utilized by other bacteria. It is considered that the energy flow of the anammox biological system originated from the catabolic and anabolic process of anammox bacteria.

Cofactor deficiency

As mentioned above, CO_2 assimilation in Candidatus *K. stuttgartiensis* is performed in a folate-dependent pattern. Tetrahydrofolate (THF) and its derivatives, a family of related molecules named indistinctly under the generic term "folate," play essential roles in major cellular processes where the reactions involved in one carbon metabolism rely on the availability of THF cofactors (Gambonnet et al. 2001). Nevertheless, a key gene coding for EC 1.5.1.3 named dihydrofolate reductase or tetrahydrofolate dehydrogenase is missing in Candidatus *K. stuttgartiensis* (shown in Fig. 5a) which would imply an occurrence of THF deficiency, and hence having severe repercussions on carbon fixation. Likewise, for coenzyme Q (CoQ), a series of UbI protein genes are missing in Candidatus *K. stuttgartiensis* (in

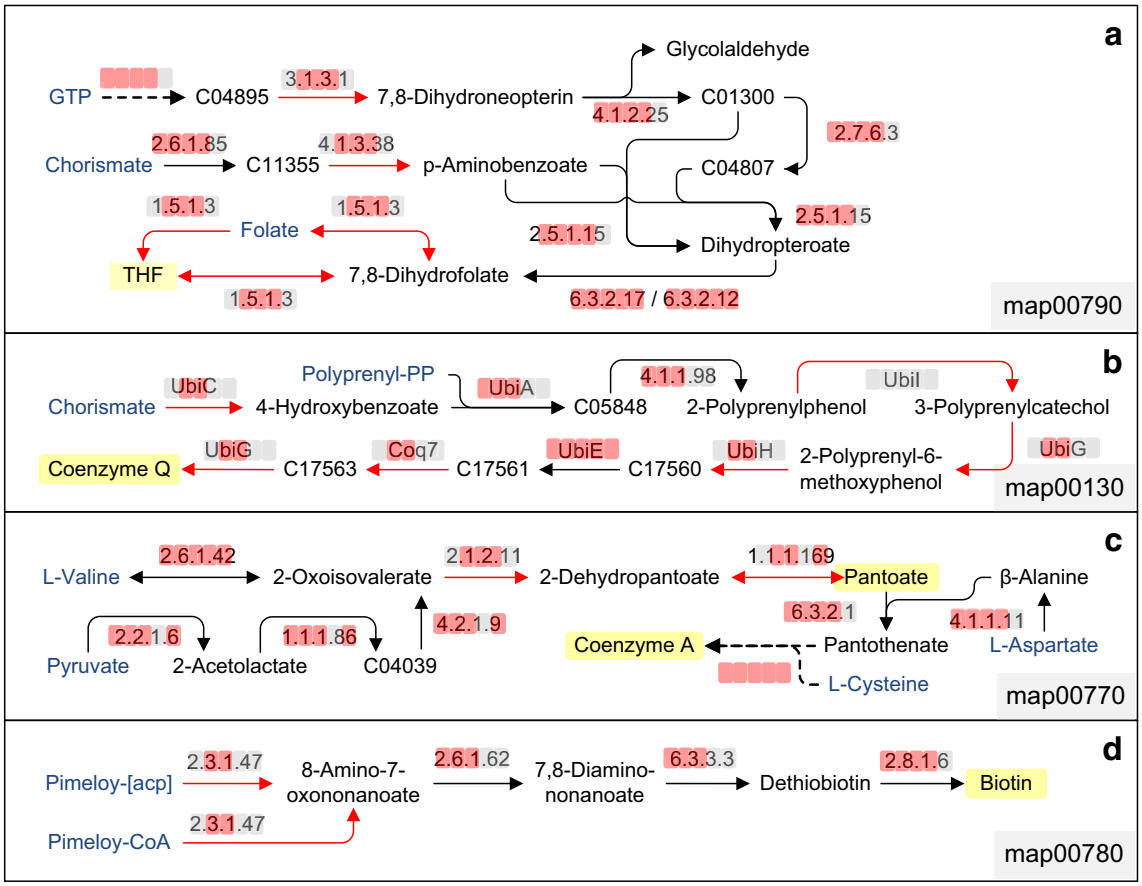

Fig. 5 Synthetic pathway of key cofactors and essential vitamins in the first five predominant bacteria reconstructed by KEGG Mapper

Fig. 5b). CoQ coupled with cytochrome C proteins plays a vital role in proton translocation across anammoxosome membrane during nitrite oxidation (Kartal et al. 2013). Coenzyme A is an indispensable cofactor in all living organisms, where it functions as an acyl group carrier and carbonyl-activating group in a number of central biochemical transformations, including the tricarboxylic acid cycle and fatty acid metabolism (Leonardi et al. 2005). In Fig. 5c, in CoA biosynthetic pathway, a gene deletion of enzyme EC1.1.1.169 2-dehydropantoate 2-reductase in Candidatus *K. stuttgartiensis* genome might result in synthesis obstruction of CoA precursor Pantoate. Due to lack of EC 2.3.1.47 8-amino-7-oxononanoate synthase, there is a trouble in catalyzing the decarboxylative condensation of L-alanine and pimeloyl-[acyl-carrier protein] (in Fig. 5d), a key step in the pathway for biotin biosynthesis. Biotin (also known as coenzyme R) is a coenzyme for carboxylase enzymes. It involves in the synthesis of fatty acids, branched-chain amino acid catabolism, and gluconeogenesis (Zempleni et al. 2009).

Although these typical cofactors are required in minute quantities, they play indispensable roles in metabolic process. With similar finding, significant progress has been made in growth promotion of beneficial bacteria through cofactor regulation in the field of biological pharmacy. For example, *Ketogulonigenium vulgare* LMP P-20356, a 2-keto-L-gulonic acid-producing strain for the manufacture of vitamin C was greatly promoted by adding folate derivatives into fermentative medium as the strain showed a folate-dependant biosynthesis pathways and the folate metabolism was not efficient (Leduc et al. 2004).

It is generally known that anammox bacteria are extremely hard to grow in pure culture; presumably, they are dependent on co-occurring microbes for lost metabolic functions in an auxotrophic lifestyle. Many free-living bacteria with gene loss are dependent on co-occurring microbes for lost metabolic functions (Jeffrey et al. 2012) and such life strategy provides prokaryotes with competitive advantage in nutrient-poor environmental niches (Giovannoni et al. 2014). The study provided not only insight into metabolic processes, interactions, and nutrient cycling but also into diversity–productivity relationships.

Conclusion

High throughput sequencing analysis of the predominant bacteria of a typical UASB anammox bioreactor has been done. Anammox bacteria Candidatus *K. stuttgartiensis* showed

clear domination along with heterotrophs *Limonbacter*, *Azoarus*, *Ignavibacterium*, and *Anaerolinea* in the microbial community. Metabolic pathway analysis, based on functional genomics information proposed, anammox bacteria was the primary energy producer and might benefit from surroundings for nutrients as genes implicated in typical functional cofactors synthesis invalidated in Candidatus *K. stuttgartiensis* genome. Hints of metabolic roles of these strains within the bioreactor have been given, suggesting a symbiotic relationship in between. Although the genomic analysis predicted a mutualistic symbiosis relationship among the predominant bacteria, it still needs to be verified by further researches. In addition, our study did not provide information on how the system may act under changing environmental conditions. Following environmental responses, the community structure may vary in their community members and relative abundance. Even so, our study provided not only insight into the metabolic potential, microbial cooperation, and interaction of key abundant bacteria in anammox process but also into diversity–productivity relationships. This study could be useful for establishing better operating strategy and development of combination wastewater treatment process based on anammox technique.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

References

Ahn YH (2006) Sustainable nitrogen elimination biotechnologies: a review. Process Biochem 41(8):1709–1721

American Public Health Association (APHA) (2005) Standard methods for the examination of water and wastewater, 21st ed. American Public Health Association, Washington, DC, USA.

Altschul FS, Madden LT, Schäffer AA, Zhang J, Zhang Z, Miller M, Lipman JD (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402

Björnsson L, Hugenholtz P, Tyson GW, Blackall LL (2002) Filamentous Chloroflexi (green non-sulfur bacteria) are abundant in wastewater treatment processes with biological nutrient removal. Microbiology 148:2309–2318

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335–336

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc Natl Acad Sci U S A 108:4516–4522

Chen H, Liu S, Yang F, Xue Y, Wang T (2009) The development of simultaneous partial nitrification, ANAMMOX and denitrification (SNAD) process in a single reactor for nitrogen removal. Bioresour Technol 100:1548–1554

Cho S, Takahashi Y, Fujii N, Yamada Y, Satoh H, Okabe S (2010) Nitrogen removal performance and microbial community analysis of an anaerobic up-flow granular bed anammox reactor. Chemosphere 78:1129–1135

Egli K, Fanger U, Alvarez PJ, Siegrist H, van der Meer JR, Zehnder AJ (2001) Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium-rich leachate. Arch Microbiol 175:198–207

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Fux C, Marchesi V, Brunner I, Siegrist H (2004) Anaerobic ammonium oxidation of ammonium-rich waste streams in fixed-bed reactors. Water Sci Technol 49(11–12):77–82

Gambonnet B, Jabrin S, Ravanel S, Karan M, Douce R, Rébeillé F (2001) Folate distribution during higher plant development. J Sci Food Agric 81:835–841

Giovannoni SJ, Cameron Thrash J, Temperton B (2014) Implications of streamlining theory for microbial ecology. ISME J 8:1553–1565

Gonzalez-Martinez A, Osorio F, Rodriguez-Sanchez A, Martinez-Toledo MV, Gonzalez-Lopez J, Lotti T, van Loosdrecht MCM (2014) Bacterial community structure of a lab-scale anammox membrane bioreactor. Biotechnol Progress 31:186–193

Guo J, Peng Y, Fan L, Zhang L, Ni BJ, Katal B, Feng X, Jetten MSM, Yuan Z (2015) Metagenomic analysis of anammox communities in three different microbial aggregates. Environ Microbiol. doi:10.1111/1462-2920.13132

Güven D, Dapena A, Kartal B, Schmid MC, Maas B, van de Pas-Schoonen K, Sozen S, Mendez R, Op den Camp HJM, Jetten MSM, Strous M, Schmidt I (2005) Propionate oxidation by and methanol inhibition of anaerobic ammonium-oxidizing bacteria. Appl Environ Microbiol 7(12):1066–1071

Hu BL, Zheng P, Tang CJ, Chen J, van der Biezen E, Zhang L, Ni BJ, Jetten MSM, Yan J, Yu HQ, Kartal B (2010) Identification and quantification of anammox bacteria in eight nitrogen removal reactors. Water Res 44:5014–5020

Hurek T, Reinhold-Hurek B (2003) Azoarcus sp. strain BH72 as a model for nitrogen-fixing grass endophytes. J Biotechnol 106:169–178

Iino T, Mori K, Uchino Y, Nakagawa T, Harayama S, Suzuki K (2010) Ignavibacterium album gen. nov., sp. nov., a moderately thermophilic anaerobic bacterium isolated from microbial mats at a terrestrial hot spring and proposal of Ignavibacteria classis nov., for a novel lineage at the periphery of green sulfur bacteria. Int J Syst Evol Microbiol 60:1376–1382

Imajo U, Tokutomi T, Furukawa K (2004) Granulation of Anammox microorganisms in up-flow reactors. Water Sci Technol 49:155–163

Jeffrey MJ, Lenski RE, Zinser ER (2012) The Black Queen hypothesis: evolution of dependencies through adaptive gene loss. MBiology 3(2):203–216

Jetten MSM, Niftrik L, Strous M, Kartal B, Keltjens JT, Op den Camp HJ (2009) Biochemistry and molecular biology of anammox bacteria. Crit Rev Biochem Mol 44:65–84

Jin RC, Zheng P, Hu AH, Mahmood Q, Hu BL, Jilani G (2008) Performance comparison of two anammox reactors: SBR and UBF. Chem Eng J 138(1–3):224–230

Juretschko S, Loy A, Lehner A, Wagner M (2002) The microbial community composition of a nitrifying-denitrifying activated sludge from an industrial sewage treatment plant analyzed by the full-cycle rRNA approach. Syst Appl Microbiol 25(1):84–99

Kartal B, Kuenen JG, van Loosdrecht MC (2010) Sewage treatment with anammox. Science 328:702–703

Kartal B, Geerts W, Jetten MSM (2011) Cultivation, detection, and ecophysiology of anaerobic ammonium-oxidizing bacteria. Methods Enzymol 486:89–108

Kartal B, de Almeida NM, Maalcke WJ, Op den Camp HJ, Jetten MSM, Keltjens JT (2013) How to make a living from anaerobic ammonium oxidation. FEMS Microbiol Rev 37:428–461

Ke Y, Azari M, Han P, Görtz I, Gu JD, Denecke M (2015) Microbial community of nitrogen-converting bacteria in anammox granular sludge. Int Biodeterior Biodegrad 103:105–115

Kieling DD, Reginatto V, Schmidell W, Travers D, Menes RJ, Soares HM (2007) Sludge wash-out as strategy for Anammox process start-up. Process Biochem 42(12):1579–1585

Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Kindaichi T, Yuri S, Ozaki N, Ohashi A (2012) Ecophysiological role and function of uncultured Chloroflexi in an anammox reactor. Water Sci Technol 66:2556–2561

Kotay SM, Mansell BL, Hogsett M, Pei H, Goel R (2013) Anaerobic ammonia oxidation (ANAMMOX) for side-stream treatment of anaerobic digester filtrate process performance and microbiology. Biotechnol Bioeng 110:1180–1192

Krause A, Ramakumar A, Bartels D et al (2006) Complete genome of the mutualistic, N(2)-fixing grass endophyte Azoarcus sp. strain BH72. Nat Biotechnol 24(11):1385–1391

Kuenen JG (2008) Anammox bacteria: from discovery to application. Nat Rev Microbiol 6:320–326

Lam P, Jensen MM, Lavik G, McGinnis DF, Müller B, Schubert CJ, Amann R, Thamdrup B, Kuypers MMM (2007) Linking crenarchaeal and bacterial nitrification to anammox in the Black Sea. Proc Natl Acad Sci U S A 104(17):7104–7109

Leduc S, de Troostembergh JC, Lebeault JM (2004) Folate requirements of the 2-keto-L-gulonic acid-producing strain Ketogulonigenium vulgare LMP P-20356 in L-sorbose/CSL medium. Appl Microbiol Biotechnol 65:163–167

Leonardi R, Zhang YM, Rock C, Jackowski S (2005) Coenzyme A: back in action. Prog Lipid Res 44(2–3):125–153

Lieu PK, Homan H, Kurogi A, Kawagoshi Y, Fujii T, Furukawa K (2006) Characterization of sludge from single-stage nitrogen removal using anammox and partial nitrification (SNAP). Jpn J Water Treat Biol 42(2):53–64

Liu Z, Frigaard NU, Vogl K, Iino T, Ohkuma M et al (2012) Complete genome of Ignavibacterium album, a metabolically versatile, flagellated, facultative anaerobe from the phylum Chlorobi. Front Microbiol 3:185

Miura Y, Okabe S (2008) Quantification of cell specific uptake activity of microbial products by uncultured chloroflexi by microautoradiography combined with fluorescence in situ dybridization. Environ Sci Technol 42:7380–7386

Mulder A, van de Graaf AA, Robertson LA, Kuenen JG (1995) Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. FEMS Microbiol Ecol 16:177–183

Ni BJ, Hu BL, Fang F, Xie WM, Kartal B, Liu XW, Sheng G, Jetten M, Zheng P, Yu HQ (2010) Microbial and physicochemical characteristics of compact anaerobic ammonium-oxidizing granules in an upflow anaerobic sludge blanket reactor. Appl Environ Microbiol 76:2652–2656

Ni SQ, Ni JY, Hu DL, Sung S (2012a) Effect of organic matter on the performance of granular anammox process. Bioresour Technol 110: 701–705

Ni SQ, Sung S, Yue QY, Gao BY (2012b) Substrate removal evaluation of granular anammox process in a pilot-scale upflow anaerobic sludge blanket reactor. Ecol Eng 38:30–36

Oshiki M, Shimokawa M, Fujii N, Satoh H, Okabe S (2011) Physiological characteristics of the anaerobic ammonium oxidizing bacterium Candidatus 'Brocadia sinica'. Microbiology 157:1706–1713

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sànchez-Melsió A, Cáliz J, Balaguer MD, Colprim J, Vila X (2009) Development of batch-culture enrichment coupled to molecular detection for screening of natural and man-made environments in search of anammox bacteria for N-removal bioreactors systems. Chemosphere 75:169–179

Schmid M, Twachtmann U, Klein M, Strous M, Juretschko S, Jetten MSM, Metzger JW, Schleifer KH, Wagner M (2000) Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. Syst Appl Microbiol 23:93–106

Schmidt JE, Batstone DJ, Angelidaki I (2004) Improved nitrogen removal in upflow anaerobic sludge blanket (UASB) reactors by incorporation of Anammox bacteria into the granular sludge. Water Sci Technol 49:69–76

Sekiguchi Y, Kamagata Y, Syutsubo K, Ohashi A, Harada H, Nakamura K (1998) Phylogenetic diversity of mesophilic and thermophilic granular sludges determined by 16S rRNA gene analysis. Microbiology 144:2655–2665

Sekiguchi Y, Kamagata Y, Nakamura K, Ohashi A, Harada H (1999) Fluorescence in situ hybridization using 16S rRNA-targeted oligonucleotides reveals localization of methanogens and selected uncultured bacteria in methophilic and thermophilic sludge granules. Appl Environ Microbiol 65:1280–1288

Sekiguchi Y, Takahashi H, Kamagata Y, Ohashi A, Harada H (2001) In situ detection, isolation, and physiological properties of a thin filamentous microorganism abundant in methanogenic granular sludges: a novel isolate affiliated with a clone cluster, the green non-sulfur bacteria, subdivision I. Appl Environ Microbiol 67: 5740–5749

Shu D, He Y, Yue H, Zhu L, Wang Q (2015) Metagenomic insights into the effects of volatile fatty acids on microbial community structures and functional genes in organotrophic anammox process. Bioresour Technol 196:621–633

Speth DR, Hu B, Bosch N, Keltjens JT, Stunnenberg HG, Jetten MS (2012) Comparative genomics of two independently enriched "candidatus kuenenia stuttgartiensis" anammox bacteria. Front Microbiol 3:307

Strous M, Heijnen JJ, Kuenen JG, Jetten M (1998) The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. Appl Microbiol Biotechnol 50:589–596

Strous M, Heijnen JJ, Kuenen JG, Jetten MSM (1999) Key physiology of anaerobic ammonium oxidation. Appl Environ Microbiol 65:3248–3250

Strous M, Pelletier E et al (2006) Deciphering the evolution and metabolism of an anammox bacterium from a community genome. Nature 440:790–794

Suneethi S, Joseph K (2011) Batch culture enrichment of ANAMMOX populations from anaerobic and aerobic seed cultures. Bioresour Technol 102(2):585–591

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Third KA, Sliekers AO, Kuenen JG, Jetten MSM (2001) The CANON system (completely autotrophic nitrogen-removal over nitrite) under ammonium limitation: interaction and competition between three groups of bacteria. Sys. Appl Microbiol 24:588–596

Tsushima I, Ogasawara Y, Kindaichi T, Satoh H, Okabe S (2007a) Development of high-rate anaerobic ammonium oxidizing (anammox) biofilm reactors. Water Res 41:1623–1634

Tsushima I, Ogasawara Y, Kindaichi T, Satoh H, Okabe S (2007b) Development of a super high-rate Anammox reactor and in

situ analysis of biofilm structure and function. Water Sci Technol 55:9–17

Van de Graaf AA, Mulder A, de Bruijn P, Jetten MSM, Robertson LA, Kuenen JG (1995) Anaerobic oxidation of ammonium is a biologically mediated process. Appl Environ Microbiol 61:1246–1251

Van de Graaf AA, de Bruijn P, Robertson LA, Jetten MSM, Kuenen JG (1996) Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor. Microbiology 142:2187–2196

Van de Vossenberg J, Woebken D, Maalcke WJ, Wessels HJ, Dutilh BE, Kartal B, Janssen-Megens EM, Roeselers G, Yan J, Speth D, Gloerich J, Geerts W, van der Biezen E, Pluk W, Francoijs KJ, Russ L, Lam P, Malfatti SA, Tringe SG, Haaijer SC, Op den Camp HJ, Stunnenberg HG, Amann R, Kuypers MM, Jetten MS (2013) The metagenome of the marine anammox bacterium 'Candidatus Scalindua profunda' illustrates the versatility of this globally important nitrogen cycle bacterium. Environ Microbiol 15(5):1275–1289

Van der Star WR, Miclea AI, van Dongen UG, Muyzer G, Picioreanu C, van Loosdrecht MC (2008) The membrane bioreactor: a novel tool to grow anammox bacteria as free cells. Biotechnol Bioeng 101: 286–294

Vanwonterghem I, Jensen PD, Dennis PG, Hugenholtz P, Rabaey K, Tyson GW (2014) Deterministic processes guide long-term synchronised population dynamics in replicate anaerobic digesters. ISME J 8:2015–2028

Volcke EIP, Picioreanu C, De Baets B, van Loosdrecht MCM (2012) The granule size distribution in an anammox-based granular sludge reactor affects the conversion—implications for modeling. Biotechnol Bioeng 109:1629–1636

Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73:5261–5267

Winkler MK, Yang J, Kleerebezem R, Plaza E, Trela J, Hultman B, van Loosdrecht MC (2012) Nitrate reduction by organotrophic Anammox bacteria in a nitritation/anammox granular sludge and a moving bed biofilm reactor. Bioresour Technol 114:217–223

Yasuda T, Waki M, Yoshinaga I, Amano T, Suzuki K, Tanaka Y, Yamagishi T, Suwa Y (2011) Evidence of exponential growth of an anammox population in an anaerobic batch culture. Microbes Environ 26:266–269

Zempleni J, Wijeratne SS, Hassan YI (2009) Biotin. BioFactors 35:36–46