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Whole-Community Metagenomics in Two Different Anammox Configurations: Process Performance and Community Structure

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Abstract

Anaerobic ammonia oxidation (anammox) combined with partial nitrification (PN) is an innovative treatment process for energy-efficient nitrogen removal from wastewater. In this study, we used genome-based metagenomics to investigate the overall community structure and anammox species enriched in suspended growth (SGR) and attached growth packed-bed (AGR) anammox reactors after 220 days of operation. Both reactors removed more than 85% of the total inorganic nitrogen. Metagenomic binning and phylogenetic analysis revealed that two anammox population genomes, affiliated with the genus *Candidatus* Brocadia, were differentially abundant between the SGR and AGR. Both of the genomes shared an average nucleotide identity of 83%, suggesting the presence of two different species enriched in both of the reactors. Metabolic reconstruction of both population genomes revealed key aspects of their metabolism in comparison to known anammox species. The community composition of both the reactors was also investigated to identify the presence of flanking community members. Metagenomics and 16S rRNA gene amplicon sequencing revealed the dominant flanking community members in both reactors were affiliated with the phyla *Anaerolinea*, *Ignavibacteria*, and *Proteobacteria*. Findings from this research adds two new species, *Ca. Brocadia* sp. 1 and *Ca. Brocadia* sp. 2, to the genus *Ca. Brocadia* and sheds light on their metabolism in engineered ecosystems.

Graphical Abstract

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1 Introduction

There is a need for energy neutrality and resource positivity in municipal wastewater treatment plants (WWTPs). Subsequently, the wastewater community is being proactive as it embraces new treatment technologies that rely on minimum aeration and smaller carbon footprints. The anaerobic ammonium oxidation (anammox) process has evolved as a powerful process to address energy autarky and carbon footprints. The anammox process is the oxidation of ammonia to nitrogen gas with nitrite as an electron acceptor.(1, 2) When applied together with partial nitrification, it reduces oxygen demand and organic carbon requirements.(3) Anammox provides a more-sustainable solution to the need for energy autarky and smaller carbon footprints of modern WWTPs than conventional aerobic nitrification and heterotrophic denitrification.(4) Within the past decade, wastewater practitioners have accelerated the adoption of anammox systems. Many full-scale anammox plants are already running successfully in Europe and Asia, while several are being constructed in the United States.

Engineering applications of the anammox process rely on strategies where ammonium ($\text{NH}_4^+ - \text{N}$) is partially oxidized to nitrite ($\text{NO}_2^- - \text{N}$) (nitrification) by ammonia oxidizing bacteria (AOB). Subsequently, anammox (AMX) bacteria oxidize the remaining $\text{NH}_4^+ - \text{N}$ to nitrogen gas, using the formed $\text{NO}_2^- - \text{N}$ as an electron acceptor.(5) When combined, nitrification and anammox are known as partial nitrification anammox (PN/A) systems. The success of PN/A systems depends on the synergy between AMX and AOB. Several PN/A configurations have been developed to achieve this synergy, including two-stage PN/A systems where nitrification and anammox occur in separate reactors(6, 7) or single-stage PN/A systems in which nitrification and anammox processes occur in a single reactor.(8) Additionally, different strategies have been used for biomass cultivation that encourage the community to either form highly organized granular structures (suspended growth) or to adhere to the plastic carrier media (attached growth).(9–14)

Appropriate microbial community assembly is a necessary step in successfully implementing PN/A systems. Consequently, understanding the microbial ecology of organisms living in PN/A systems has been the focus of many studies.(14–16) These studies have focused on analyzing the diversity of the microbial communities using phylogenetic markers such as the 16S rRNA gene(15) and the diversity of anammox organisms using sequence analyses of a primary anammox functional genes, hydroxylamine oxidoreductase (HAO) and hydrazine synthase (HZS).(17–21) However, efforts have seldom focused on

applying whole community metagenomics to study the function and diversity of microbial communities present in PN/A reactors.

Direct random shotgun sequencing of total DNA extracted from environmental samples (i.e., metagenomics), followed by functional and taxonomic analysis, can provide a higher resolution of microbial and anammox diversity in comparison to a phylogenetic marker gene-based approach.(22–28) To date, only two studies have used metagenomic approaches to investigate the microbial community structure of anammox reactors.(14, 16) This research provided information on key flanking communities and as well as on anammox bacteria in PN/A systems. The use of metagenomics helped to elucidate the differences in the overall microbial ecology in attached growth and suspended growth anammox systems as well as to study the overall functional gene repertoire in two laboratory scale anammox reactors operated for over 220 days. Possible metabolic pathways were constructed on the basis of the extracted anammox genomes from metagenomic sequencing data.

2 Material and Methods

2.1 Reactor Setup, Operational Strategy, and Sampling Suspended Growth with Partially Nitrifying and Anammox Reactors

A suspended growth anammox reactor (SGR) has been in operation in Goel's laboratory at the University of Utah for the past 6 years. The start-up seed for the reactor was received from an ongoing anammox reactor stationed at the City College of New York, which was reported to be enriched with *Candidatus* Brocadia fulgida.(29) The SGR has been fed with partially nitrified real reject water from an ongoing suspended growth nitrification reactor (S-PN). Details of both reactors (i.e., nitrification reactor and SGR) have been provided elsewhere.(5) Briefly, the SGR has a working volume of 4L with a hydraulic retention time (HRT) of 2 days, and a pH was maintained at 7.8 ± 0.3 .

Attached Growth with Partially Nitrifying and Anammox Reactor—An attached growth packed-bed anammox reactor (AGR) was initiated at the University of Utah with seed from the ongoing SGR and fed with the partially nitrified effluent from an attached growth packed-bed nitrification reactor (A-PN). The A-PN reactor was seeded from the S-PN reactor. The AGR was filled with Bio-Balls (diameter of 2.54 cm and surface area of 88.9 cm²; Coralife, WI) as the biofilm support media. It had an effective pore volume of 4 L. Initially, diluted feed was used for the reactor system to establish healthy nitrification and anammox biofilm communities. The amount of nitrogen loading was gradually increased as the biofilm developed and the reactor performance improved. Anaerobic conditions in both anammox reactors (i.e., SGR and AGR) were maintained by continuously purging with a mixture of 95% nitrogen gas and 5% carbon dioxide. A schematic of the SGR and AGR setups, as well as their corresponding nitrification reactors, are included in Supplementary Figure 1. Influent and effluent samples were routinely collected, filtered (0.45 μm). These were analyzed for NH₄⁺-N, NO₂⁻-N and NO₃⁻-N concentrations using HACH methods 10031 (salicylate method), 10020 (chromotropic acid method), and 8153 (ferrous sulfate method), respectively. Schematics of both suspended and attached growth PN/A systems are provided in Supplementary Figure 1.

2.2 DNA Extraction

Biomass sampling for genomic DNA extraction from SGR and AGR was performed when the total inorganic nitrogen (TIN) removal efficiency was consistently greater than 80%, and the reactors appeared to be at steady state. Approximately 2 g of biomass from each anammox reactor were collected aseptically and suspended in 500 μ L of 1 $\times$ TE buffer (Fisher Scientific). For the AGR, five Bioballs were taken out from the mid-depth of the reactor, and the biomass was aseptically scraped off using a sterile 1 mL pipet tip. The biomass collection procedure was repeated three times. DNA from each biomass sample was extracted using the PowerMax soil DNA isolation kit (MoBio Laboratories), according to the manufacturer's instructions. The extracted triplicate DNA samples were mixed to create one homogenized single DNA sample. The concentration of DNA was measured with a Nanodrop 1000 spectrophotometer (Thermo Scientific), and the quality was verified by running the genomic DNA on a 1% agarose gel electrophoresis.

2.3 Microbial Community Analysis Using 16S rRNA Gene-Based Sequencing

The overall bacterial community was obtained by sequencing 16S rRNA gene fragments through high-throughput amplicon sequencing as described by Kapoor et al.,(30) with some modifications. Specifically, barcoded PCR primers 515F/806R were used to partially amplify the 16S rRNA gene.(31) PCR conditions were adopted from Kapoor et al.(30) PCR products were visualized on an agarose gel and pooled in an equimolar ratio prior to sequencing on an Illumina MiSeq sequencer (Illumina, San Diego, CA), using pair-end 250 bp kits at the Cincinnati Children's Hospital DNA Core facility.

A total of 101192 and 38537 of 16S rRNA partial gene sequences were obtained from both the SGR and AGR, respectively. Reads obtained were processed using the Quantitative Insights into Microbial Ecology (QIIME) version 1.8.0.(32) Paired-end MiSeq reads were first assembled using the FLASH version 1.2.11 with default parameters.(33) Sequences with a quality score less than 20 and ambiguous N bases were removed before chimera detection. Chimeric sequences were identified with QIIME via USEARCH and removed prior to taxonomic assignment.(34) Nonchimeric 16S rRNA gene sequences were clustered into operational taxonomic units (OTUs) using UCLUST(35) with a 97% nucleotide similarity. Representative sequences from each cluster were queried against the SILVA 119 rRNA database,(36) using UCLUST(35) to assign taxonomy. Singleton OTUs (represented by one read only) were omitted from downstream analysis to reduce over prediction of rare OTUs.(37) Prior to analysis, data sets were normalized to the total number of reads per sample.

2.4 Whole-Community Metagenomics, Assembly, and Analysis

A total of 1 μ g of genomic DNA from both the SGR and AGR reactors were submitted to the high-throughput sequencing core facility, Huntsman Cancer Institute (HCI) at the University of Utah. Genomic DNA was sequenced on a MiSeq sequencer (Illumina) to generate 300 nt paired-end reads. The raw reads obtained were quality filtered using Sickle version 1.33.(38) Reads were filtered using a minimum quality score of 20 with a minimum length of 100 bp. The quality filtered reads were interleaved using the shuffleSequences_fasta.pl Perl script from MetaVelvet.(39) A phylogenetic analysis of the metagenomes for high-resolution

microbial community assemblages was generated using PhyloSift.(40) A set of 37 elite marker gene families, along with 16S, 18S rRNA genes, mitochondrial, eukaryotic-specific, and viral gene families, were used for classification. Assembly of interleaved reads for the AGR and SGR were performed using omega with an overlap length of 150 bp.(42) The contigs generated were binned into population genomes based on (a) differential coverage, (b) tetranucleotide frequency, and (c) single-copy marker genes analysis with MaxBin.(42) A pair of anammox genomes were recovered from both the SGR and AGR reactors. CheckM was used to assess the completeness and quality of each binned genome using collocated sets of marker genes that are a single copy but ubiquitous within a phylogenetic lineage.(43) Scaffolding of anammox genome contigs was performed by multidraft-based scaffolder (MeDuSa).(44) To identify the correct order and orientation of the contigs, five published draft anammox genomes available in the NCBI database were used as templates; (a) *Candidatus* Brocadia fulgida (PRJNA263557), (b) *Candidatus* Jettenia caeni (PRJDB68), (c) *Candidatus* Brocadia sinica (PRJDB103), (d) *Candidatus* Kuenenia stuttgartiensis (PRJNA16685), and (e) *Candidatus* Scalindua brodae (PRJNA262561). The rRNA and tRNA prediction for the anammox genomes were recovered from both reactors was performed using webMGA(45) and ARAGORN,(46) respectively. Annotation of the scaffolded genome bins was performed using Metapathways v2.0.(47)

To appraise the bins depicting anammox lineages enriched in the SGR and AGR reactors, phylogenetic classification was performed on the basis of hydrazine synthase among the five known genera of anammox bacteria; *Ca. Brocadia*, *Ca. Kuenenia*, *Ca. Scalindua*, *Ca. Anammoxoglobus*, and *Ca. Jettenia*. A maximum likelihood phylogenetic tree of the *hzsA* gene was constructed using MEGA 5 along with reference sequences of anammox bacteria. (48) Genome-wide average nucleotide identity (gANI), along with the alignment frequency (AF) between the anammox draft genomes recovered from SGR, AGR, and other available anammox genomes, were calculated using the microbial species identified (MiSI) method. (49)

The raw reads were deposited in the MG-RAST repository with ID 4537331.3 and 4537777.3 for the SGR and AGR metagenome, respectively (Supplementary Table 1). Additionally, DNA sequences from the SGR and AGR have been submitted to the National Center for Biotechnology Information (NCBI) repository with BioProject PRJNA343219.

3 Results and Discussion

3.1 Reactor Performance

The performances of suspended growth and attached growth partial nitrification reactors are shown in supplementary Figure 2 (panels a and b respectively). The average $\text{NH}_4^+\text{-N}$ concentration in the influent of the suspended growth partial nitrification (S-PN) reactor was 1260 ± 120 mg/L (supplementary Figure 2a). The reactor was able to produce an influent for SGR with 570 ± 60 mg/L of $\text{NH}_4^+\text{-N}$ and 690 ± 70 mg/L of $\text{NO}_2^-\text{-N}$, yielding an NO_2^- -to- NH_4^+ ratio of 1.24 ± 0.09 . On the other hand, $\text{NH}_4^+\text{-N}$ in the influent was gradually increased from an initial concentration of 120 mg/L to a final concentration of 920 mg/L in the attached growth partial nitrification (A-PN) reactor (supplementary Figure 2b). At the highest $\text{NH}_4^+\text{-N}$ influent concentration, the resulting $\text{NO}_2^-\text{-N}/\text{NH}_4^+\text{-N}$ ratio in the effluent

of the A-PN reactor was 1.07. Effluent from each partial nitrification reactor was fed to respective anammox reactor counterpart. Hence, under the steady-state conditions, both S-PN and A-PN reactors enabled the required ratio of nitrite and ammonium needed for anammox bacteria in SGR and AGR reactors.

During the 220-day operation, the SGR consistently removed more than 85% total inorganic nitrogen (TIN), as shown in Figure 1a. The average $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$ and $\text{NO}_3^-\text{-N}$ in the effluent were 93.7 ± 45.8 , 0.38 ± 0.30 , and 51.3 ± 13.3 mg/L, resulting in an average $88 \pm 2\%$ TIN removal. The AGR, fed with the effluent from the attached growth partial nitrification (A-PN) reactor, did not initially have high biomass quantities so a step-feeding technique was adopted to ensure stable reactor operation. Figure 1b shows changes in $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$, and $\text{NO}_3^-\text{-N}$ concentrations in the influent and effluent of the AGR. When the loading was increased, the N removal rate also increased steadily. The total inorganic nitrogen (TIN) removal efficiency was close to 80% on day 163. After day 169, the influent $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentrations to the AGR were increased to 430 ± 30 and 460 ± 50 mg/L, respectively. A TIN removal of $86 \pm 2\%$ was observed during this phase. The nitrogen removal efficiencies observed in this study were like efficiencies observed by other researchers using anammox enrichment in sequencing batch reactors and membrane bioreactors. (1, 13, 50)

3.2 Microbial Community in SGR and AGR Anammox Reactors Revealed by 16S rRNA Gene Amplicon Sequencing

The purpose of 16S rRNA gene amplicon sequencing was to get a snapshot of the overall microbial community in both anammox reactors. 16S rRNA gene amplicon sequencing was performed to identify the microbial phylotypes in the SGR and AGR reactors. 16S rRNA gene being an excellent phylogenetic marker provides insight into the microbial taxa present in the sample. (31) When the 16S rRNA gene sequencing data were analyzed at the phylum level from both reactors (Figure 2a), Planctomycetes was the dominant phylum, with an abundance of 80.6% in SGR and 41.3% in AGR. Chlorobi accounted for 7.4% of total reads in SGR and 26.3% in AGR. Moreover, Proteobacteria in AGR was as high as 19.2% compared to 3.8% in SGR. The phyla Bacteroidetes and Chlorolipid were responsible for 1.4% and 4.3% of total reads for SGR and 1.7% and 10.0% for AGR, respectively. Because both reactors were highly enriched with anammox bacteria affiliated with the genus *Brocadia*, the genera level for the microbial communities was studied. The SGR was dominated by two genera: *Ignavibacteria* (Chlorobi) and *Candidatus Brocadia* (Planctomycetes), while in the AGR, an unclassified organism within the order Rhodocyclales (Proteobacteria), represented by *Dok59* in the database, was detected with a relative abundance of 15.7%. The genus *Ignavibacteria* is classified within the phylum Chlorobi in Silva SSU r119. To compare the community diversity between both reactors (Table 1), Shannon, Chao1, and Simpson indices were calculated. On the basis of the results obtained, it could be concluded that the AGR harbored a more-diverse microbial community than the SGR.

3.3 Microbial Community Analysis in Anammox Reactors Using Whole-Community Metagenomics

After obtaining information on the overall community structure using 16S rRNA gene amplicon sequencing, “deep” metagenomic sequencing using representative DNA samples from both reactors was employed to obtain a genome-scale view of the microbial community. This allowed for the examination of the functional potential of each reactor community and extract nearly complete genomes of key anammox lineages present in both SGR and AGR reactors. The metagenomes of SGR and AGR provides insight into all the genes present in the representative community. (31)

The taxonomic compositions of quality filtered metagenomic reads from each reactor were analyzed using PhyloSift. (40) The analysis indicated the presence of anammox-associated Planctomycetes as the dominant phylum in both the SGR (54%) and AGR (35%) metagenomes (Figure 2b). These results were consistent with those obtained using 16S rRNA gene-based amplicon analysis (Figure 2a). The second-most-dominant phylum in both SGR and AGR was found to be the phylum Ignavibacteriae (11 and 25%, respectively). Taxonomic classification published by NCBI classifies Ignavibacteriae and Chlorobi as separate phyla, both belonging to the Bacterioidetes and Chlorobi group. The third-most-abundant phylum was Chloroflexi composed of unclassified Chloroflexi and Anaerolineae (7 and 10%). Class Beta-proteobacteria (6 and 10%) (Figure 2b) belonging to phylum Proteobacteria was the fourth most abundant in the reactors.

Bacteria belonging to the phylum Ignavibacteriae and class Anaerolineae are not yet completely understood, but they are thought to be polymer-degrading, fermentative anaerobes. (15) Ignavibacteriae was found to encode the respiratory nitrate reductase (Nar) gene involved in denitrification. Therefore, presence of these bacteria as dominant taxa among the flanking community indicates that nitrate respiration coupled to organic carbon oxidation may be occurring in both reactors. Biomass degradation is most likely important for the overall reactor operation because hydrolysis of larger biomass components into smaller organic molecules may support denitrification activity of heterotrophic bacteria abundant in the system while recycling dead biomass and reducing sludge yields.(15, 51) In summary, 16S rRNA gene amplicon results obtained independently (Figure 2a) of the taxonomic information gathered from the whole community metagenomics showed a similar microbial composition (Figure 2b and Supplementary Figures 3 and 4).

Recovery of Anammox Draft Genomes—The ability to assemble metagenomic sequences into draft population-genomes allowed the investigation of the role that individual microbial lineages play in natural and engineered ecosystems. Anammox bacteria are currently classified with an interim taxonomic status of *Candidatus* and remain a challenge to get into pure culture. Therefore, it becomes imperative to recover more genome sequences of uncultivated prokaryotes to begin to understand their ecology and metabolism.

Metagenomic sequencing, assembly, (41) and binning (42) of assembled contigs yielded two high-quality draft anammox bacterial genomes (BROC1 and BROC2) with differential coverage in the SGR and AGR metagenomes. BROC1 and BROC2 were found to be 94.4% and 97.2% complete (Table 2), determined based on the number of single-copy marker genes

present in each genome.(42) Contig scaffolding and genome reorganization yielded 75 and 13 contigs for BROCC1 and BROCC2, respectively, comparable to other anammox draft genomes.(16) The relative abundance for both anammox genomes present in each reactor was calculated using coverage data generated using MaxBin.(42) The relative abundance (%) of BROCC1 was 56.1 and 15.8% for SGR and AGR, respectively; BROCC2 had a relative abundance of 23.5% and 7.1% for AGR and SGR, respectively. This suggests that anammox bacteria are a significant fraction of the microbial community present in the SGR and AGR systems, consistent with 16S and raw metagenomic sequencing results (Figure 2a, b).

Phylogeny of Anammox Draft Genomes—To assess the phylogenetic distance between all previously identified and characterized anammox species as well as the recovered anammox genomes from SGR and AGR, a functional gene-based marker approach was employed. Hydrazine synthase enzyme has been used to investigate the diversity of anammox organisms.(21) The central metabolic pathways of anammox organisms have been proposed by Katal et al.,(13) where nitrite is first reduced to nitric oxide (NO) by a cd1-type nitric oxide–nitrite oxidoreductase (*nirS*) in *Kuenenia*(24) and *Scalindua*.(23) Other anammox bacteria, such as *Jettenia* KSU-1, may employ a copper-based *nirK*-type nitrite reductase.(52, 53) Oshiki et al.(54, 55) suggested that the anammox species belonging to genus *Ca. Brocadia* (*Ca. B. sinica* and *Ca. B. fulgida*) may employ an unidentified nitrite reductase for NO₂[−] reduction. This is due to the absence of both *nirS* and *nirK* genes in their genome. (55, 56) Hydrazine synthase enzyme is the most suitable biomarker to investigate the diversity of anammox organisms. (21, 28) Therefore, *HzsA* encoding genes were used to assess the phylogenetic relationship of the recovered anammox draft genomes by alignment with known *hzsA* genes (Figure 3a). A total of four copies of the *hzsA* gene from the BROCC1 lineage aligned with 89, 89, 87, and 87% nucleotide identities to *Ca. J. caenis*, respectively. For the BROCC2 lineage, both *hzsA* gene copies aligned with 86% identity to *Ca. J. caeni*.

Phylogenetic analysis revealed two copies each of the *hzsA* gene encoded by BROCC1 and BROCC2 population genomes clustered within the genus *Ca. Brocadia* genus. The third and fourth copies of *hzsA* gene from the BROCC1 draft genome formed a separate cluster with *Ca. Anammoxoglobus propionicus* (JN703712.1) (Figure 3). This analysis suggests that both enriched BROCC1 and BROCC2 from SGR and AGR belong to the genus *Ca. Brocadia*. The organization and arrangement of the *hzsCBA* gene cluster was investigated to determine unifying themes among recovered and published anammox genomes. The synteny of the gene cluster neighborhood within each genome (57) was examined to reveal gene organization in genomes. Figure 3b represents the *hzsCBA* gene clusters of five anammox species (a) *Ca. B. sinica*, (b) *Ca. B. fulgida*, (c) *Ca. K. stuttgartiensis*, (d) *Ca. J. caeni*, (e) BROCC2 (*Ca. Brocadia* sp. 2), and (f) BROCC1 (*Ca. Brocadia* sp. 1) as well as anammox lineages recovered from the SGR and AGR metagenomes. For *Ca. K. stuttgartiensis* HZS, a heterotrimeric protein was encoded by kuste2859–2861 as *hzsC*, *hzsB*, and *hzsA*. (13, 24, 58) A similar organization of the *hzsCBA* gene cluster was also observed in *Ca. J. caeni* (two copies: forward and reverse), as well as in the *Ca. Brocadia* sp. 1 (two copies: forward and reverse). In the case of *Ca. B. fulgida*, the *hzsCBA* genes were not co-localized; *hzsC* and *hzsB* genes are on separate operons compared to the *hzsA* gene identified on the

antisense strand (Figure 3b). We also observed that the *Ca. B. sinica* genome did not have syntenic regions for the gene cluster compared to other anammox genomes. Notably, in the *Ca. Brocadia* sp. 2 genome, only *hzsA* and *hzsC* genes were recovered, which may be a result of gaps in the draft genome.

Taxonomic Classification of the Anammox Draft Genomes—Average nucleotide identity (gANI) of the whole genome has been proposed as an accurate means of comparing genetic relatedness among strains of interest.(59) A gANI of >~94% among strains corresponds to 70% DNA–DNA hybridization standard for species definition. Therefore, gANI combined with genome alignment fraction (AF) is useful for tentative taxonomic classification of *Candidatus* organisms,(49) including genomes recovered from metagenomes. For determining the taxonomic positions of the recovered anammox draft genomes from the SGR and AGR metagenomes, gANI with AF was performed against five published anammox genomes (Figure 4). gANI was calculated using BLASTn with a minimum of 30% sequence identity match over an alignment region of at least 70% for each genome length. The gANI and AF result reflects the relatedness of BROCI and BROCI2 genomes to *Ca. B. fulgida* followed by *Ca. B. sinica*. Based on the analysis performed, it is concluded that the BROCI and BROCI2 population genomes from the SGR and AGR are two different species belonging to the genus *Ca. Brocadia*. The relatedness between anammox lineage genomes from SGR and AGR were observed to be lower than 94%, suggesting an enrichment of two distinct species of anammox bacteria in different reactor configurations. Therefore, the results from the *hzsA* gene phylogenetic analysis, synteny, and gANI indicated that the anammox genomes (BROCI and BROCI2) recovered from SGR and AGR belong to the genus *Ca. Brocadia*, providing an increase in the diversity of anammox species known to date.

Anammox Catabolism—To judge the performance of anammox bioreactors, it is vital to understand the pathways involved in nitrogen removal by the anammox bacteria. Therefore, catabolic pathways of anammox lineages BROCI and BROCI2 were investigated. Anammox bacteria are known to take up inorganic nitrogen substrates (NH_4^+ and NO_2^-) and fix CO_2 . To date, several genes coding for nitrite, nitrate, and ammonium transporters have been reported in genomes for all known anammox lineages.(60, 61) Anammox genomes recovered from both of these metagenomes were found to contain the formate and nitrite transporter *focA* (7 for BROCI and 1 for BROCI2), the nitrate and nitrite antiporter *narK* (1 for BROCI and 1 for BROCI2), and the ammonium transporter *amtB* (5 for BROCI and 5 for BROCI2) (Figure 4b and Supplementary Table 2a,b). Multiple copies of *focA* genes in our SGR-anammox lineage suggest that it has the potential to survive in oligotrophic environments where the concentrations of inorganic nitrogenous compounds are low.(28, 61, 62) To facilitate transport, anammox bacteria carry genes that encode for two nitrate channel proteins *narK* (a) high-affinity nitrate/ H^+ symporters and (b) low-affinity nitrate/ H^+ antiporters. The BROCI and BROCI2 genomes contained only a single copy of low-affinity nitrate and H^+ antiporter (*narK*) each (Supplementary Table 2a,b).

Nitrite (inorganic nitrogen substrate) is reduced to NO by cytochrome *cd1*-type nitrite reductase *NIRS*.(24) No genes encoding *NIRS* were recovered from the BROCI and

BROC2. This finding was similar to earlier research in which *Ca. B. fulgida* and *Ca. J. caeni* genomes seemed to lack *NIRS* encoding genes.(56) Instead, a copper-containing nitrite reductase, *NIRK* was identified in the *Ca. J. caeni* genome. Much like *Ca. J. caeni*, two copies of the gene encoding for copper-containing nitrite reductase, *NIRK*, were observed in the BROC1, whereas none was recovered for the BROC2 genome. To date, anammox genomes affiliated with the genus *Ca. Brocadia* have been reported to miss both (copper) *nirK* and *nirS* encoding for cd1-type nitric oxide and nitrite oxidoreductase.(54–56) This finding suggests that the *Ca. Brocadia* sp. 1 abundant in the SGR have developed the metabolic flexibility to generate the essential intermediate NO. The nitrate reductase complex including *narG* and *narH* is required to reduce nitrate to nitrite. Here, four and two copies of *narG* and *narH* encoding genes, respectively, were identified in the BROC1. However, the BROC2 only contained two identifiable copies each of *narG* and *narH*.

Anammox bacteria have developed an intrinsic mechanism to take advantage of the oxidative potential of highly reactive free radical NO. Bacteria employ *NorVW* flavorubredoxin for detoxification of this free radical molecule.(63) Genes encoding a protein similar to flavoproteins, *norVW* and *fprA*, were also identified in the SGR and AGR anammox genomes (Supplementary Table 2a,b).

The ability to synthesize hydrazine (N_2H_4) is arguably the most intriguing property of anammox bacteria. The direct precursor of hydrazine production is NO, produced through reduction of nitrite and ammonium. HZS is the second known enzyme, after nitric oxide reductase, that has the ability to forge the N–N bond.(13, 28, 56) Multiple copies of genes encoding *hzsA* (four copies for BROC1 and two copies for BROC2) were identified. However, in the case of *hzsC*, only one and two copies were identified for BROC1 and BROC2, respectively (Supplementary Table 2a,b). Such observations of variable numbers for components of *hzs* gene cluster could be due to gaps in draft genomes. The final step of converting hydrazine to dinitrogen gas is catalyzed by hydrazine oxidoreductase (*hzo*) or hydroxylamine oxidoreductase (*hao*) (Figure 4b). The anammox lineage genomes recovered from both metagenomes showed the presence of multiple copies of these genes (Supplementary Table 2a,b).

CO₂ fixation performed by anammox bacteria is accomplished through acetyl-coenzyme A pathway or the Wood–Ljungdahl pathway.(58, 64) Both *acsA* and *acsB* genes encoding synthase were recovered from the BROC1 and BROC2 genomes (Supplementary Table 2a,b), much like the ones present in *Ca. K. stuttgartiensis*.

The practical question that arises is “How do observations from reactor performance and metagenomics help to investigate the importance in the implementation of lower energy treatment technology, such as anammox, for real-world applications?” Microbial communities play important roles in the nutrient cycles in natural and engineered ecosystems. Understanding the microbial diversity has become an integral part of reactor operation and a way to gain more insight into reactor performance. Based on these metagenomic data, measured reactor performances, and chemical concentrations, a conceptual nitrogen-cycling network for anammox reactors can be constructed.

Suspended (SGR) and attached (AGR) growth reactor configurations achieved comparable rates in total inorganic nitrogen (TIN) removal of $88 \pm 4\%$ and $86 \pm 2\%$, respectively, in a period of 220 days. A major factor influencing start-up of the anammox process is the slow growth rate of anammox organisms (0.072 per day when grown at 32 °C). (1, 65) Even seemingly insignificant amounts of biomass loss via effluent could become an impediment to a quick start-up process. Therefore, reducing chances of biomass loss via a suitable reactor configuration is being considered as a possible solution. Attached growth anammox reactors (AGR) immobilize the biomass on the surface of media (K3, and K5; Veolia Municipal Solutions) or on Bio-Balls (for this study).

Our metagenomics analysis revealed enrichment of two anammox-affiliated prokaryotes, BROCI and BROCI2, in both of the reactors. The dominant flanking community in SGR and AGR reactors consisted of prokaryotes affiliated with the phyla Anaerolinea, Ignavibacteria, and Proteobacteria. The dominant flanking community remains similar, even for reactors operated with different configurations. However, the relative abundances of specific microbial groups were different in both reactors despite similar reactor performances. For example, Ignavibacteria and Anaerolinea relative abundances in the AGR were relatively more than in the SGR, whereas the relative abundance of BROCI and BROCI2, two different anammox species, were higher in the SGR. However, the total nitrogen removal percentages in both reactors were nearly identical. This suggests that engineering reactors need not to be highly enriched in anammox biomass to accomplish efficient total inorganic N removal.

Degradation of biomass by Ignavibacteria and Anaerolinea may support community interactions in the system, as hydrolysis processes could provide short-chain volatile fatty acids (VFAs) and alcohols to Proteobacteria. The abundance of Ignavibacteria and Anaerolinea was higher in the AGR, which could be explained by the fact that AGR has theoretically infinite solid retention time (SRT) (no biomass wastage). High SRT can lead to a greater degree of bacterial decay in the system. Therefore, higher Ignavibacteria and Anaerolinea abundances in the AGR may improve the degradation of organics generated via cell decay. (15, 51) Detailed genome analysis of BROCI and BROCI2 genomes extracted from the SGR and AGR metagenomes revealed both species could be placed within the genus *Ca. Brocadia*. gANI and AF analysis concluded that the *Ca. Brocadia* sp. 1 and *Ca. Brocadia* sp. 2 are two different species as their relatedness is $> \sim 94\%$. Additionally, through the investigation of the anammox catabolism process inferred via genome annotations, it was observed that *Ca. Brocadia* sp. 1 and *Ca. Brocadia* sp. 2 were metabolically well-suited to grow in inorganic nitrogen environments.

In conclusion, the findings from this research offer much-needed insight into the microbial functions occurring in anammox bioreactors and should guide future efforts aimed at developing strategies for optimizing and controlling process performance. This research provided a detailed understanding of the microbial community structure and reactor performance. The system-based approach detailed in this manuscript provided a better understanding of the molecular responses of molecular community in two different anammox reactors to complex bioreactor conditions. The metabolic pathway depicted in this manuscript, based on whole-community metagenomics, illustrated the presence of the dominant flanking community in enriched anammox reactor. The high abundance of

heterotrophic organisms in the SGR and AGR systems investigated here suggests that they play an important role in community function, which should be investigated in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Nitrogen concentration (NH_4^+-N , NO_2^--N , and NO_3^--N) in influent and effluent and the removal efficiency for anammox in (a) suspended growth and (b) attached growth reactor.

The dotted line represents the day of biomass sampling, and the dotted line depicts the time (130th day) of biomass sampling for DNA extraction from SGR and AGR reactors.

Figure 2. Taxonomic classification of QC filtered reads using (a) 16S itag sequencing and (b) PhyloSift (phylum and class).

Figure 3.

(A) Neighbor-joining trees indicating the phylogenetic relationships within the Brocadiales based on hydrazine synthase subunit A (H₂S_A) nucleotide sequences. ORFs from the BROC1 and BROC2 recovered from the SGR and AGR metagenome are highlighted in bold. Numbers of the nodes are percentages of bootstrap values based on 1000 replicates. NCBI accession numbers for the reference sequences have been mentioned in parentheses. (B) Gene diagram representing the syntenies of *hzsCBA* gene cluster encoding for H₂S enzyme among five anammox species (a) *Ca. B. sinica*, (b) *Ca. B. fulgida*, (c) *Ca. K. stuttgartiensis*, (d) *Ca. J. caeni*, (e) *Ca. Brocadia* sp 2, and (f) *Ca. Brocadia* sp 1 recovered from SGR and SGR metagenome. Color represents each gene among the *hzsCBA* gene cluster (red, *hzsA*; orange, *hzsB*; gray, *hzsC* gene). A single asterisk represents the start and end position of the genes. Double asterisks represent the orientation (+) and (−) as (1) and (−1), respectively.

Figure 4.

(A) gANI, AF, and percent protein identification between all anammox genomes from public databases and *Ca. Brocadia* sp 1 and *Ca. Brocadia* sp 2 extracted from SGR and AGR metagenomes. (B) Overview of anammox metabolism of *Ca. Brocadia* sp. 1 and *Ca. Brocadia* sp. 2.

Table 1.

Summary of 16S Sequencing Processing and Results

sample name	total sequences	OTU counts	Shannon index	Chao1	Simpson index
SGR-Amplicon	101192	2008	1.72	2501.8	0.595
AGR-Amplicon	15549	1301	2.83	2011.4	0.174

Table 2.

Quality of Anammox Bacterial Bins

bin name	abundance		relative abundance (%)		total marker	unique Marker	completeness	genome size (bps)	features (23S/16S/5SrRNA/tRNA)	GC content (%)
	SGR	AGR	SGR	AGR						
<i>Cz. Brocadia</i> sp. 1	263.51	82.05	56.1	15.8	113	101	94.40%	4476597	2*/2*/1/55	44.9
<i>Cz. Brocadia</i> sp. 2	33.18	122.45	7.1	23.43	109	104	97.20%	3334435	1/1/1/48	42.2