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Discovery and metagenomic analysis of an anammox bacterial enrichment related to *Candidatus* "Brocadia caroliniensis" in a full-scale glycerol-fed nitritation-denitritation separate centrate treatment process

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ABSTRACT

A distinctive red biofilm was observed in a glycerol-fed digester liquid effluent treatment process coupling partial nitrification (nitritation) and partial denitrification (denitritation) processes. Based on initial phylogenetic screening using 16S rRNA clone libraries and quantitative polymerase chain reaction, the biofilm was enriched in novel anaerobic ammonium oxidizing bacteria (AMX/anammox) closely related to *Candidatus* "Brocadia caroliniensis". The metabolic functionality of the *C.* "Brocadia caroliniensis" enrichment was further explored using high-throughput sequencing and *de novo* metagenome assembly. The population anammox genome that was binned from the metagenome consisted of 209 contigs with a total of 3.73 Mbp consensus sequences having 43.3% GC content, and 27.4 average coverage depth. The assembled metagenome bin was comprised of 3582 open reading frames (ORFs). Based on 16S rRNA similarity the binned metagenome was closely related with *Candidatus* "Brocadia caroliniensis", *Candidatus* "Brocadia fulgida", planctomycete KSU-1, and *Candidatus* "Kuenenia stuttgartiensis" with 99%, 96%, 92% and 93% similarity, respectively. Essential genes in anammox metabolic functions including ammonium and nitrite transport, hydrazine synthesis, electron transfer for catabolism, and inorganic carbon fixation, among several other anabolic pathways, were also observed in the population genome of the *C.* "Brocadia caroliniensis" related enrichment. Our results demonstrate the wider profusion of anammox bacteria in engineered nitrogen removal systems than expected. The utility of metagenomics approaches to deciphering such novel functionality in these systems is also highlighted.

1. Introduction

Anaerobic ammonium oxidation (anammox) is a cost and energy efficient alternative to conventional biological nitrogen removal (BNR) processes, in which ammonium and nitrite are concurrently employed as electron donor and acceptor, respectively, generating nitrogen gas (Strous et al., 2006). The advantages of anammox based BNR strategies include reduced oxygen demand and alkalinity and no requirement of organic carbon addition (Kartal et al., 2011; Mulder, 2003). In the 1990s, nitrogen removal through anammox was reported for the first time to occur in a wastewater treatment system (Mulder et al., 1995), whereas it had been theoretically predicted to exist before (Broda, 1977).

Five anammox bacterial genera have been established within the order of Brocadiales including *Candidatus* "Brocadia", *Candidatus* "Kuenenia", *Candidatus* "Scalindua", *Candidatus* "Jettenia" and *Candidatus* "Anammoxoglobus". With the exception of *Candidatus* "Scalindua", the remaining anammox bacteria have been discovered or enriched in lab- or full-scale bioreactors in the presence of high influent ammonium concentrations in the range 5×10^2 mg-N/L or higher (Park et al., 2010b; van der Star et al., 2007). *Candidatus* "Scalindua", on the other hand, have been often found in marine habitats where limited oxygen, ammonium and nitrite are present (Schmid et al., 2003). Pure culture cultivation of anammox bacteria has been a challenge possibly due to their slow growth and

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susceptibility to factors such as oxygen and nitrite (Strous et al., 1999). Accordingly, *sensu stricto,* no complete anammox bacterial genomes are currently available for systems biology based studies. Instead, *de novo* assembly approaches have been applied to obtain metagenomes from highly enriched anammox bacterial bioreactors via high throughput next generation sequencing (NGS) technology. To date, the population genomes binned from metagenome sequences of three wastewater anammox bacterial consortia from lab-scale reactors (*Candidatus* "Kuenenia stuttgartiensis", *Candidatus* "Brocadia fulgida" and *planctomycetes* KSU-1) and one marine anammox bacterium *Candidatus* "Scalindua profunda" have been published (Gori et al., 2011; Hu et al., 2012; Strous et al., 2006; van de Vossenberg et al., 2013).

Here, we report the shotgun metagenomic analysis and *de novo* assembly of an anammox bacteria enrichment containing *Candidatus* "Brocadia caroliniensis" from a full-scale process treating centrifuged anaerobic digester liquid effluent (hereafter referred to as centrate). The full-scale separate centrate treatment (SCT) system is part of the overall BNR program of New York City and is operated in Aeration Tank 3 (AT3) at the 26th Ward wastewater treatment plant in Brooklyn, NY. The process involves coupled partial ammonia oxidation to nitrite (nitritation) and partial denitrification (denitritation) of anaerobic digestion centrate with the addition of glycerol as an external carbon and electron source (Fig. 1). During routine maintenance of the SCT process, red biofilm aggregates were discovered from one of the anoxic mixer stands in AT-3 (Fig. 1B). Based on visual inspection and the fact that the anoxic zone was continuously subjected to high ammonium and nitrite concentrations (Table S1), it was hypothesized that the red biofilm could be enriched in anammox bacteria. One basis for this hypothesis is that the large fraction of multiheme proteins within the anammoxosome, a unique intracellular membrane-bounded anammox bacterial organelle (Jetten et al., 2009; Kartal et al., 2013; Shimamura et al., 2007), could be a visual (but not conclusive) indicator of anammox bacteria presence.

The discovery of anammox bacteria in such an environment would be somewhat unexpected since the system is not designed or operated for autotrophic nitrogen removal but for heterotrophic denitrification using glycerol. The exploration of anammox functionality using metagenomics could greatly help understating the functional (and possibly structural) diversity within intentional and unintentional anammox bacteria-enriched microbial systems. Further, the increased profusion of anammox population genomes could contribute to the ever increasing reliability in anammox metagenomics through expanded reference metagenome databases. It must be emphasized that the focus herein was not on the isolation in pure culture of novel anammox bacterial organisms. Rather, the focus was to decipher anammox functionality in a BNR process not intentionally engineered for anammox activity, using metagenomics techniques.

2. Materials and methods

2.1. Sample collection, DNA extraction, and anammox bacteria quantification and identification

Triplicate biofilm samples were scraped from the surface of the anoxic mixer stand and immediately transferred with snap-freezing on dry ice and stored at −80 °C for subsequent DNA extraction. The characteristics of influent and mixed liquor wastewater at the time of biomass sampling are depicted in Table S1 in *Supplementary information*. The biomass was uniformly used without any specific bias or selection. Once thawed, 0.1 g (wet weight) of each biomass sample was transferred into 1.5 mL microcentrifuge tubes with sterile forceps. Pellet pestle grinder (Fisher Scientific, MA) for 30 s with 0.5 mL TE buffer (Fisher Scientific, MA) was applied for homogenization. DNeasy mini kit with QIAcube (Qiagen, CA) was used for DNA extraction from the homogenate.

For anammox bacteria quantification, quantitative PCR (qPCR) was conducted targeting overall anammox bacterial genera (pla46F/amx667R) as previously described (van der Star et al., 2007) and species-specific qPCR targeting hydrazine synthase subunit A (*hzsA*) of *C.* "Brocadia fulgida" & *C.* "Kuenenia stuttgartiensis" (*hzsA*-BR, *hszA*-KU) (Supplementary information). In parallel, clone libraries with *Planctomycetes* 16S rRNA gene-specific primers (Pla46F/1392R) were constructed to identify potential anammox bacteria present in the biofilm samples. Briefly, end-point PCR with template genomic DNA was conducted as previously described (Tal et al., 2005) and PCR amplicons were inserted into TOPO vector using TOPO TA cloning kit for sequencing (Life Technologies, NY) followed by the transformation of competent *E. coli* cells. The inserts were further purified using QIAprep Spin Miniprep kit (Qiagen, CA) and sequenced using ABI3730XL DNA analyzer (Applied Biosystems). Phylogenetic trees were generated using MEGA6 (Tamura et al., 2013) by neighbor-joining method with bootstrap of 500 replications and Jukes-Cantor computational model (Jukes and Cantor, 1969).

2.2. Library preparation, template preparation, ISP (Ion Sphere Particle) enrichment, and PGM™ (Personal Genome Machine) sequencing

Three replicate samples of the biofilm were individually prepared to generate independent libraries. DNA extracts of the individual replicates were purified using QIAquick DNA Cleanup kit with QIAcube (Qiagen, CA). The quality and quantity of DNA were checked via NanoDrop Lite Spectrophotometer (Thermofisher, MA). DNA libraries with 500 ng and >1.8 A_{260}/A_{280} ratio for each extract were constructed using NEBnext Fast DNA Fragmentation and Library Prep Set for Ion Torrent (New England Biolabs, MA). Ion Xpress barcode kit (Life Technologies, NY) was applied in each sample for replication experiment. The size selection of the DNA libraries was conducted targeting 400 bp with E-Gel® SizeSelect™ (Life Technologies, NY). Prior to template preparation, library quantification was performed with KAPA Library Quantification kit for Ion Torrent (KAPA biosystems, MA) to avoid polyclonality of ISP. Template preparation with DNA library followed by ISP enrichment was performed using the Ion OneTouch2 system following manufacturer's instructions (Ion OT2 400 kit, Product No. 4479878). The enriched ISP was loaded into Ion Torrent 316 chip and sequenced according to manufacturer's instructions (Ion PGM™ Sequencing 400 Kit, Product No. 4482002). Ion Torrent Suite software ver. 4.0.2 was used for base calling, signal processing and quality filtering (>Phred score of 15) of the raw sequences.

2.3. Post-run bioinformatics analyses

After pooling the fastq datasets of the three replicates, *de novo* assembly was conducted using four assembler packages (MIRA Assembler (ver. 3.9.9) (Chevreux et al., 2004), SPAdes Genome Assembler (ver. 3.5.0) (Nurk et al., 2013), Ray assembler (ver. 2.3.1) (Boisvert et al., 2012) and String Graph Assembler (ver. 0.10.10) (Simpson and Durbin, 2010)). The quality assessment of the assembled metagenomes were compared using QUAST (Gurevich et al., 2013) (Table S2). Binning of the assembled results with the highest N50 length was conducted in order to exclude contigs with genes that are not homologous to the anammox bacteria (for example, aerobic ammonia-oxidizing bacteria (AOB) and

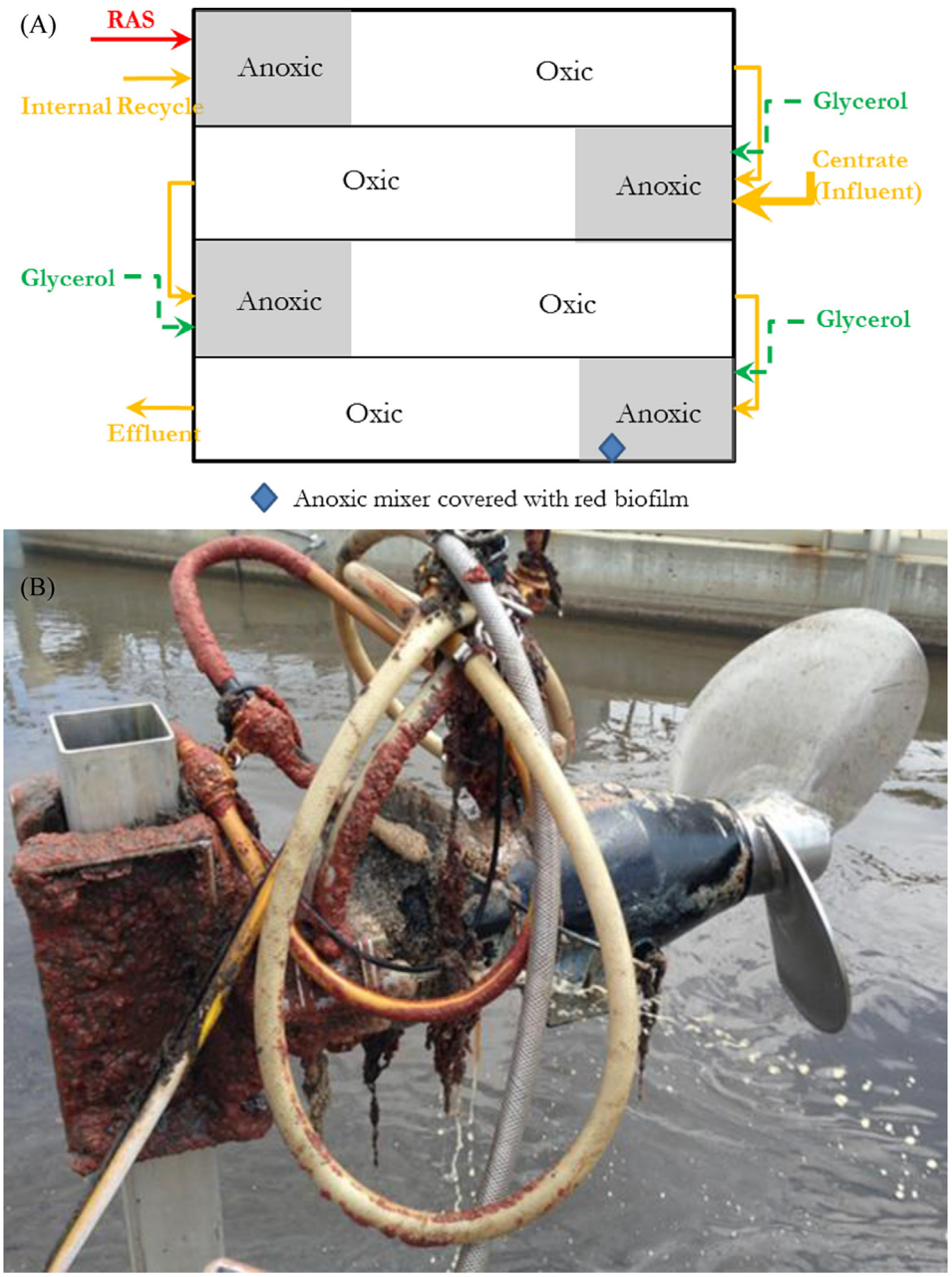

Fig. 1. (A) Schematic diagram of the full-scale nitritation-denitritation process treating separate centrate with glycerol addition. (B) Red biofilm on the surface of anoxic mixer.

heterotrophs, which can also be present in the AT-3 reactor). MaxBin was used as the binning tool as this package has been applied to the environmental microbial metagenome even with novel species. (Wu et al., 2014). Using MaxBin, the metagenome sequences were organized into bins using tetranucleotide frequencies and contigs coverages. Binned contigs were further verified with CheckM, which assesses the quality and completeness of the genome (Parks et al., 2014). Prokka package (Prokaryotic Genome Annotation System - http://vicbioinformatics.com/) were used in genome annotation. Prokka runs several dependent packages including Aragorn for predicting tRNAs and tmRNAs (Laslett and Canback, 2004), RNAmmer for predicting ribosomal RNAs (Lagesen et al., 2007), Prodigal for predicting protein-coding sequences (Hyatt et al., 2010), BLASTP against anammox bacteria protein database for protein-coding sequence assignment (Camacho et al., 2009), and HMMER3 against HAMAP and Pfam for protein similarity search (Finn et al., 2011). Artemis was used for annotated population genome overview and detailed search of sequence features (Rutherford et al., 2000). BRIG (BLAST Ring Image Generator, v 0.95) were used for comparative genome analysis (Alikhan et al., 2011). 16S rRNA gene assignment of the metagenomic dataset was conducted in BLASTN against 16SMicrobial database (NCBI BioProjects PRJNA33175 and PRJNA33317). The workflow of the *de novo* assembly study is described in Fig. 2 and raw sequence data can be accessed in MG-RAST (4541396.3) and NCBI SRA (SRR1745247). All command line parameters used in the post analysis and annotated sequences are provided in supplementary information.

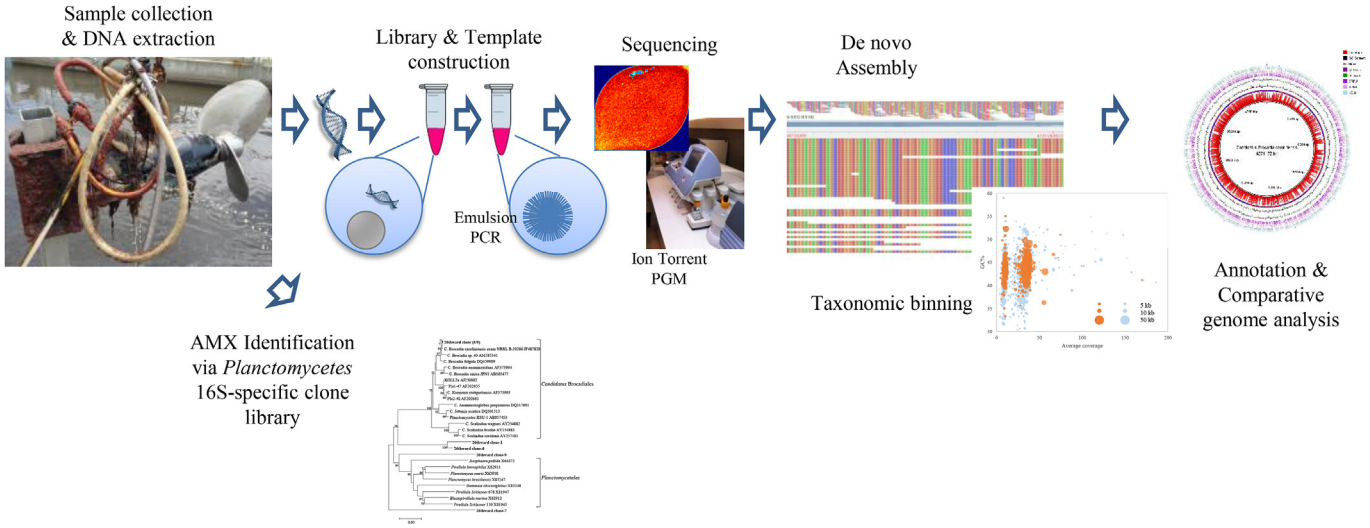

Fig. 2. Workflow of the *de novo* assembly study for anammox enrichment related to *C.* "Brocadia caroliniensis".

3. Results and discussion

3.1. Characterization, quantification, and physiological confirmation of anammox bacterial enrichment

High anammox bacterial 16S rRNA gene concentrations were measured in the biofilm homogenate whereas anammox bacteria specifically related to *C.* "Brocadia fulgida" & *C.* "Kuenenia stuttgartiensis" (based on *hzsA* gene sequences) were only minimally detected (Fig. S1). This was a difference from our past studies, where the dominant anammox bacteria enriched on anaerobic digestion reject water (centrate) were mostly closely related to *C.* "Brocadia fulgida" and *C.* "Brocadia sp. 40" (Park et al., 2010a, 2010b). However, these earlier studies and reactor systems did not involve glycerol or other substrate addition for enhanced nitrogen removal. Clone libraries based on 16S rRNA genes associated with the phylum *Planctomycetes* revealed that the dominant anammox bacteria from the biofilm were closely related to *Candidatus* "Brocadia caroliniensis" NRRL B-50286 strain with 99% sequence similarity (Fig. S2). *Candidatus* "Brocadia caroliniensis" related anammox bacteria have been previously observed in BNR reactors treating swine wastewater (Rothrock et al., 2011). In addition to the genomic evidence, the concomitant consumption of ammonium and nitrite as well as consumption of hydrazine (a unique anammox intermediate) by the enriched biomass samples was also observed (Fig. S3), thereby providing physiological support for functional anammox presence.

Potential reasons behind the unexpected proliferation of anammox bacteria in the nitritation-denitritation process may include operation conditions that favored localized accumulation of nitrite from incomplete nitritation and denitritation as well as ammonia in the final stage of the process (Fig. 1A, Table S1: 36.4 mg NH_4^+–N/L and 17.7 mg NO_2^-–N/L)). Such localized accumulation could have been a result of poor mixing strategies, as evidenced by the failed mixer ultimately. In addition, a low shear stress environment at the back of the mixer stand could have promoted formation of the anammox biofilm therein (Arrojo et al., 2008).

Notwithstanding the discovery of *C.* "Brocadia caroliniensis" related anammox bacteria in the BNR reactor, the contribution of anammox bacteria to overall nitrogen removal in the reactor could not be inferred. Also, in general, the propensity of SCT systems supplemented with glycerol for enhanced denitrification to support anammox bacteria remains to be determined.

3.2. High-throughput sequencing and de novo assembly

Semiconductor-based high-throughput sequencing yielded a total of 482 Mbp of nucleotides with 2,113,425 total reads and 228 bp average read length from the biofilm samples after quality filtering. Quality assessment of the assembled metagenome through the different packages performed using QUAST indicated that the Ray and SGA assemblers generated larger number of contigs and lower N50 length compared to MIRA and SPAdes (Table S2). Particularly, the SPAdes assembler generated the highest N50, the longest size of the largest contig, and the lowest number of large contigs (Table S2), which rendered it suitable for *de novo* assembly of the metagenome sequencing data in this study (Xavier et al., 2014). The assembly was binned into two metagenome bins (Bin 1 and Bin 2) after implementing MaxBin (to exclude the contribution of non-anammox bacteria). Bin 1 contained higher coverage and longer average contig lengths than Bin 2, which possessed a total of 209 contigs with 1.0 kbp and 137.2 kbp of the minimum and maximum contig size, respectively (Fig. S4). Thus, Bin 1 was selected for downstream annotation analysis. CheckM confirmed the completeness of Bin 1 as high as 89.7% (Fig. S5). The average GC content of the Bin 1 sequences was 43.3%, which is similar to the GC content of other anammox bacteria, which have been reported close to 40% (Strous et al., 2006; van de Vossenberg et al., 2013). The annotation results from Prokka revealed that Bin 1 consisted of 3 rRNA, 43 tRNA, 3582 open reading frames (ORFs) and a total sequence of 3,728,197 bp (Fig. 3). Complete sequences of 5S rRNA (109 bp, 26THWARD_01422), 16S rRNA (1580 bp, 26THWARD_01424), and 23S rRNA (3001 bp, 26THWARD_01423) were successfully recovered from the assembly. A phylogenetic tree using the 26THWARD_01424, 16S rRNA sequences confirmed that dominant anammox bacteria from the biofilm samples were closely related with *Candidatus* "Brocadia caroliniensis" NRRL B-50286 strain with 99% sequence similarity (Fig. S6A).

Among the 3582 ORFs, 989 and 1334 ORFs were closely mapped against the protein sequences of *C.* "Brocadia fulgida", and KSU-1, respectively. However, only 283 ORFs were close to *C.* "Kuenenia stuttgartiensis" proteins suggesting that *C.* "Brocadia caroliniensis" is likely evolutionarily more distant from *C.* "Kuenenia stuttgartiensis" than from *C.* "Brocadia fulgida", and KSU-1. 976 out of 3582

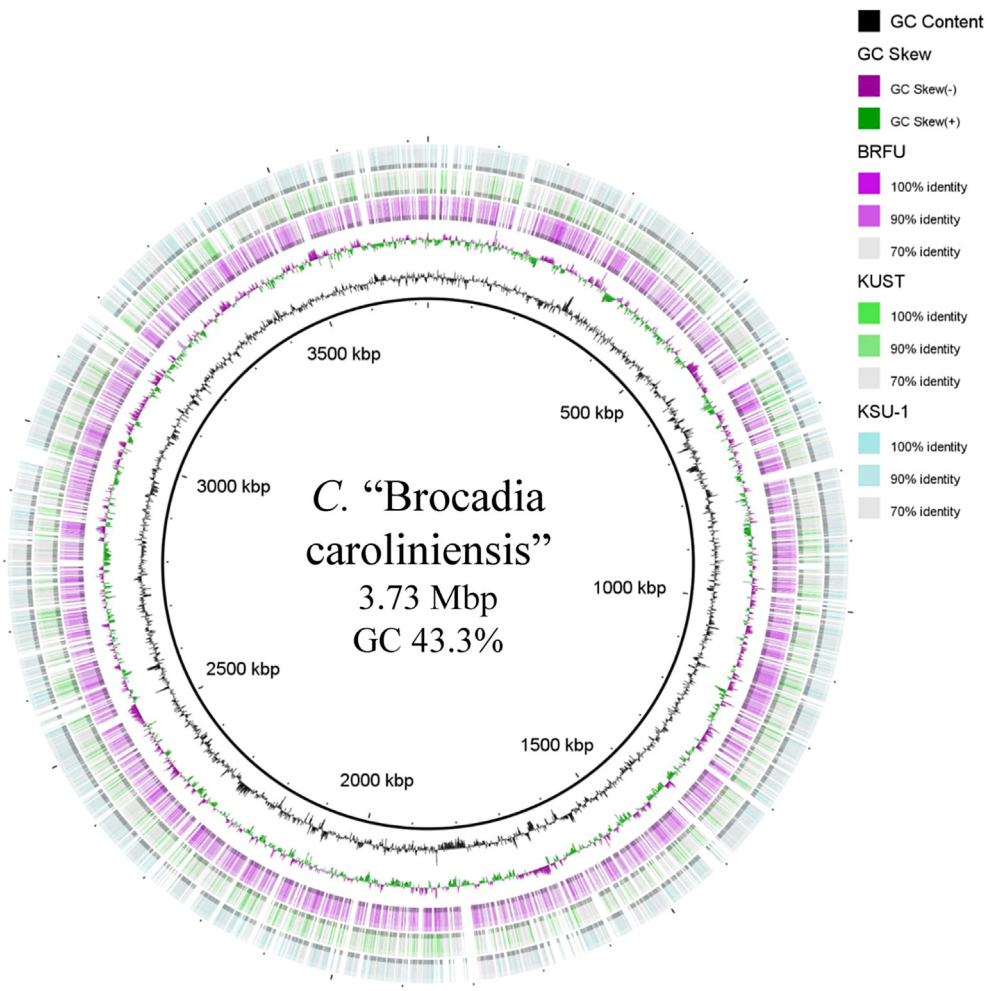

Fig. 3. Comparative population genome analysis of *C.* "Brocadia caroliniensis" based on BLAST search against *C.* "Kuenenia stuttgartiensis" (KUST), *C.* "Brocadia fulgida" (BRFU), and KSU-1 genomes. The three innermost rings show GC content and GC skew. The remaining rings showed BLAST comparison with three available anammox bacterial population genomes (BRFU, KUST, and KSU-1).

ORFs were not assigned to any of the anammox bacterial metagenomes and 32% of these ORFs were annotated as hypothetical proteins, revealing the yet to be discovered functionality in this enrichment. BLASTP analysis of all 3582 ORFs showed that the genes encoding key proteins in nitrogen and carbon metabolism were found to be have on average 95% of mapping coverage with a minimum identity of 68% (Table 1). No possible best hit of these key proteins against *C.* "Kuenenia" was found in the BLASTP results.

Taxonomic assignment was inferred via a BLASTN search of 16S rRNA genes in the metagenome against the NCBI 16S Microbial database. A total of 936 16S rRNA genes in the 2,113,425 raw sequencing reads were retrieved via BLASTN search. 42% of the 936 16S rRNA genes were assigned to *C.* "Brocadia caroliniensis" (Fig. S7). The assignment of the 16S rRNA gene result is corresponding with qPCR and clone library results, and show that anammox bacteria other than *C.* "Brocadia fulgida" and *C.* "Kuenenia" were indeed dominant in the enriched biofilm samples.

3.3. Pathways for ammonium and nitrite transport in C. "Brocadia caroliniensis"

In anammox bacteria, ammonium and nitrite are processed in the anammoxosome, where nitrite is reduced to nitric oxide (NO) and followed by hydrazine (N_2H_4) synthesis and dinitrogen gas production (Strous et al., 2006). Multiple copies of formate/nitrite transporter (*focA*) were identified in the *C.* "Kuenenia", *C.* "Scalindua", *C.* "Jettenia", and KSU-1 genomes (six, four, three, and three *focA* copies, respectively). As previously suggested, the possession of multiple nitrite/formate transporters could be due to the adaptive selection in the nitrite limited environments, which could physiologically be manifested in low half-saturation coefficients for nitrite (Hu et al., 2012; Kindaichi et al., 2006; Lotti et al., 2014) and the potential to scavenge nitrite to low concentrations. Four copies of *focA* were detected in the present anammox population genome, similar to *C.* "Scalindua". In addition to *focA*, *C.* "Brocadia caroliniensis" might rely upon additional nitrite delivery mechanisms, possibly through dissimilatory nitrate reduction using organic electron donors (Kartal et al., 2013). Indeed, the *C.* "Brocadia caroliniensis" population genome contained 19 copies of ABC ATP-binding component, which may play a role in transporting organic compounds (such as glycerol in this case). It is also possible that glycerol upon fermentation to short chain volatile fatty acids by heterotrophic bacteria, (Grabińska-ńoniewska et al., 1985; Temudo et al., 2008), is used for denitrification by *C.* "Brocadia caroliniensis" related anammox bacteria. Indeed, evidence of the glycerol utilization (G3P dehydrogenase: 26THWARD_03572, and 03573) was obtained in *C.* "Brocadia caroliniensis", suggesting at least partial glycerol transformation capability. Nevertheless, the

Table 1
BLASTP results of *C.* "Brocadia caroliniensis" select proteins in nitrogen and carbon metabolism.

Annotation	Gene	Gene id	Protein length (aa)	Hit protein accession[a]	Hit length (aa)	Bit score	% ID	E-value	Mapped length (aa)	Mapped coverage (%)[b]
NO_3^-/NO_2^- antiporter	*narK*	26THWARD_02781	401	2228662094, BRFU	404	530.0	70.4	0	395	99%
		26THWARD_03403	408	2228662094, BRFU	404	604.0	88.1	0	404	99%
Nitrate reductase	*narG*	26THWARD_00886	1150	gi|494420714	1149	2221.0	93.4	0	1149	100%
		26THWARD_02408	850	2228661873, BRFU	696	1040.0	77.3	0	622	73%
		26THWARD_02948	1150	gi|494425975	1149	2239.0	93.0	0	1149	100%
	narH	26THWARD_00889	404	gi|494420717	392	790.0	94.9	0	392	97%
		26THWARD_02407	336	2228662377, BRFU	344	588.0	80.1	0	336	100%
	narI	26THWARD_00890	325	gi|494420718	324	576.0	81.2	0	324	100%
Copper-type NO_2^- reductase	*nirK*	26THWARD_01606	374	gi|494426684	337	481.0	68.0	1E-166	350	94%
Hydroxylamine oxydoreductase	*hao*	26THWARD_00348	555	gi|494424926	502	728.0	70.8	0	462	83%
		26THWARD_01176	551	gi|494425838	537	1072.0	93.7	0	536	97%
		26THWARD_01499	537	gi|494422835	537	1010.0	90.5	0	536	100%
		26THWARD_01772	582	gi|494426007	581	1043.0	89.0	0	580	100%
		26THWARD_02143	512	2228661087, BRFU	314	521.0	84.0	2E-180	313	61%
		26THWARD_02579	534	gi|494425297	530	933.0	82.4	0	534	100%
		26THWARD_03368	863	gi|494426648	836	1379.0	75.2	0	840	97%
		26THWARD_03657	557	gi|494421125	579	929.0	77.7	0	579	104%
Hydrazine oxydoreductase	*hzo*	26THWARD_00236	465	gi|494421481	568	887.0	91.6	0	453	97%
		26THWARD_00690	488	gi|494425835	588	927.0	93.9	0	460	94%
NO_2^- transport protein	*focA*	26THWARD_01002	83	2228660084, BRFU	179	152.0	87.8	2E-44	82	99%
		26THWARD_01779	305	gi|494421139	306	433.0	71.1	2E-149	304	100%
		26THWARD_02903	301	gi|494421141	299	463.0	86.3	2E-161	299	99%
		26THWARD_02904	307	gi|494421139	306	463.0	80.1	3E-161	306	100%
NH_4^+ transport protein	*amtB*	26THWARD_00419	426	2228661332, BRFU	660	676.0	85.4	0	403	95%
		26THWARD_00420	224	2228661332, BRFU	660	344.0	88.5	4E-111	200	89%
		26THWARD_01133	447	gi|494420486	446	620.0	81.1	0	445	100%
		26THWARD_01135	448	gi|494420482	454	709.0	79.6	0	445	99%
		26THWARD_01137	85	2228657191, BRFU	212	139.0	89.3	3E-39	84	99%
		26THWARD_01138	382	gi|494420478	517	544.0	80.3	0	375	98%
		26THWARD_01139	64	2228657189, BRFU	155	83.6	75.5	2E-18	53	83%
		26THWARD_01912	439	gi|494421163	467	735.0	87.9	0	413	94%
		26THWARD_02812	272	gi|494425861	590	318.0	72.7	2E-101	216	79%
		26THWARD_02813	206	gi|494425861	590	390.0	92.1	3E-130	202	98%
Hydrazine synthase	*hzs*	26THWARD_01811	311	2228660736, BRFU	375	633.0	96.8	0	310	100%
		26THWARD_01812	809	gi|364505647	773	1548.0	95.3	0	771	95%
		26THWARD_02515	346	2228660737, BRFU	384	664.0	91.6	0	345	100%
CO dehydrogenase/acetyl-coA synthase	*acs*	26THWARD_02115	320	gi|494425622	319	553.0	86.2	0	319	100%
		26THWARD_02118	447	2228663431, BRFU	446	844.0	95.3	0	446	100%
		26THWARD_02120	728	2228663433, BRFU	727	1459.0	97.8	0	727	100%
		26THWARD_02121	654	2228663434, BRFU	642	1194.0	96.7	0	642	98%

[a] Accessions with BRFU are *C.* "Brocadia fulgida" protein accessions. All the others are NCBI gi protein accessions.
[b] Mapped coverge=(mapped length)/(annotated protein length)*100.

potential for glycerol metabolism by anammox bacteria remains to be investigated by dedicated physiological studies. Multiple homologues of ammonium transporters (*amtB*) (26THWARD_01133, and 26THWARD_01135) along with PII type nitrogen regulators (26THWARD_01132, and 26THWARD_01136) were observed in *C.* "Brocadia caroliniensis", consistent with the need for the transport of the preferred electron donor (Kartal et al., 2013).

3.4. Nitrite conversion, hydrazine synthesis and N_2 production in *C.* "Brocadia caroliniensis"

Based on the *C.* "Kuenenia" and *C.* "Scalindua" genomes, nitrite is reduced to nitric oxide in the anammoxosome by cytochrome cd-1 nitrite reductase (NirS) (Strous et al., 2006; van de Vossenberg et al., 2013). In contrast, the same reaction is encoded for by the copper-type nitrite reductase (NirK) in *C.* "Jettenia", KSU-1 and *C.* "Brocadia fulgida" (Gori et al., 2011; Hira et al., 2012; Hu et al., 2012). In the present study, only the NirK homologue (26THWARD_01606) was detected, suggesting a similar nitrite reduction pathway to *C.* "Jettenia", KSU-1 and *C.* "Brocadia fulgida". An alternate pathway for NO production through the oxidation of hydroxylamine (NH_2OH) by a hydroxylamine oxidoreductase (HAO)-like protein (26THWARD_01176) was also detected in this study, as reported for other anammox bacteria previously (Irisa et al., 2014). This alternate pathway for NO productioncould potentially be activated under nitrite limitation. Similar HAO protein coding sequences have been previously also found in *C.* "Kuenenia", *C.* "Scalindua", and KSU-1 (Shimamura et al., 2008; Strous et al., 2006; van de Vossenberg et al., 2013). HAO-like proteins were also among the most abundant in a previously studied *C.* "Kuenenia" enrichment (Kartal et al., 2011). In the mixed culture environment such as the SCT system of this study, it is possible that hydroxylamine produced by AOB and released extracellularly under transient conditions (Jiang et al., 2015; Ma et al., 2015) could be used by anammox bacteria. However, the prevalence of such interactions among the mixed microbiota cannot be inferred merely using genomic information.

Hydrazine (N_2H_4), which is the final intermediate for anammox reaction prior to conversion to N_2, is synthesized by coupling ammonium and NO (Strous et al., 2006). Two enzymes present in the anammoxosome, hydrazine synthase (HZS) and octahaem HAO-like protein (i.e., hydrazine oxidoreductase (HZO)), are involved in the production and oxidation of N_2H_4. *hzsCBA* gene clusters have expectedly been detected in all anammox genomes to date, including *C.* "Kuenenia" (kuste2859-2861), *C.* "Jettenia" (contig297–contig270), and *C.* "Scalindua" (scal01317-01318). In addition, these genes were reportedly the most highly expressed based on transcriptome and proteome studies of *C.* "Kuenenia", and

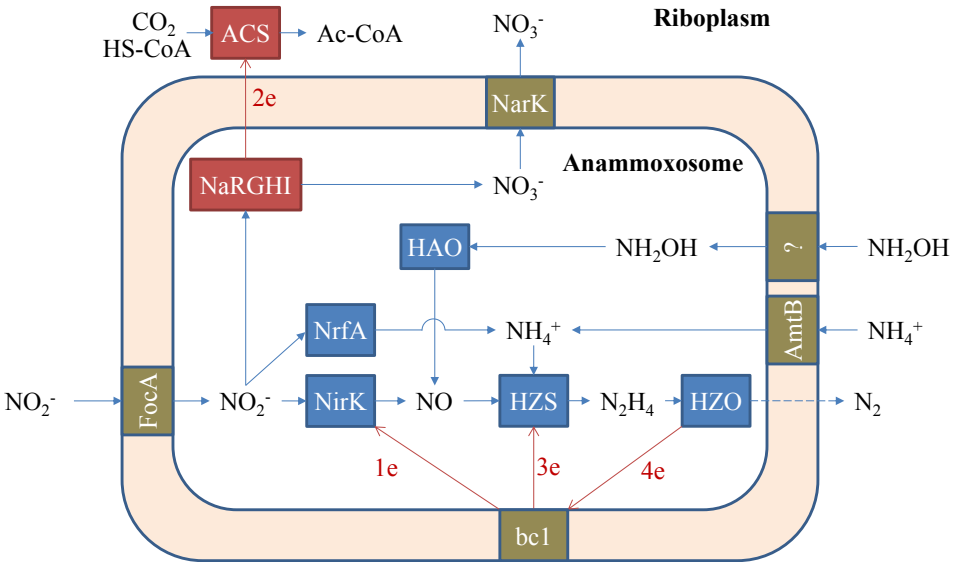

Fig. 4. Potential metabolic pathways for *C.* "Brocadia caroliniensis". FocA: nitrite/formate transporter, AmtB: ammonium transporter, NirK: nitrite reductase, HZO: hydrazine oxidoreductase, HZS: hydrazine synthase, bc1: electron transport complex, NarGHI: nitrate oxidoreductase complex, NarK: nitrate transporter, NrfA: cytochrome c nitrite reductase, HAO: hydroxylamine oxidoreductase, ACS: CO-dehydrogenase/acetyl-CoA synthase.

C. "Scalindua" (Kartal et al., 2011; van de Vossenberg et al., 2013). Indeed, multiple homologues of each HZS subunit were found in *C.* "Brocadia caroliniensis" with 92–97% similarities against *C.* "Brocadia fulgida" (Table 1).

Genes coding for ten HAO-like proteins including two almost identical HAO-like HZO proteins (26THWARD_00236, 26WARD_00690) with 92% and 94% protein similarities against KSU-1 were found in this study, whereas *C.* "Kuenenia", *C.* "Scalindua" and KSU-1 encoded for 10, 9, and 8 HAO-like proteins, respectively. As some HAO-like proteins (26THWARD_01499 and 01772) are directly linked to diheme cyt c proteins (26THWARD_01498, and 01771) as their potential redox partners, the function of these proteins may largely include electron transfer. The potential metabolic pathways of the *C.* "Brocadia caroliniensis" enrichment as inferred from the population genome are presented in Fig. 4.

3.5. Carbon fixation in *C.* "Brocadia caroliniensis"

Carbon fixation in anammox bacteria is carried out via the reductive acetyl-CoA (Wood–Ljungdahl) pathway (Gori et al., 2011; Strous et al., 2006; van de Vossenberg et al., 2013). The key enzyme for this pathway is CO-dehydrogenase/acetyl-CoA synthase (ACS). Indeed the successful retrieval of gene clusters encoding *acsAB* (26THWARD_02121, and 02120) and *acsCD* (26THWARD_02118, and 02115) with high similarities to *C.* "Brocadia fulgida" and KSU-1 (Table 1), suggests that the *C.* "Brocadia caroliniensis" enrichment shared similar inorganic carbon fixation pathways with other anammox bacteria. In addition, other steps in the same overall carbon fixation pathways including formate dehydrogenase (FDH; 26THWARD_03668) and NADH:quinone oxidoreductase (NQO; 26THWARD_00818) were also detected in this study, as previously reported in *C.* "Kuenenia" (Strous et al., 2006). Genes encoding the nitrate oxidoreductase (NarGHI; 26THWARD_00886, 26THWARD_00889, and 00890) protein complex and the nitrite/nitrate antiporter (NarK; 26THWARD_02781, and 03403), which are responsible for providing the reductant for CO_2 fixation and export of nitrate from the cytoplasm, respectively, were also detected in the population genome bin of the *C.* "Brocadia caroliniensis" (Table 1).

3.6. Metabolic versatility of *C.* "Brocadia caroliniensis"

As indicated above, anammox bacteria possess high metabolic versatility such as the usage of organic electron donors for CO_2 fixation, and catalyzing the oxidation of hydroxylamine to nitric oxide. Another versatile trait of anammox bacteria includes denitrification using selected organic compounds, nitrate, and DNRA (Kartal et al., 2007). The DNRA pathway enables anammox bacteria to utilize organic compounds and nitrate in the absence of ammonium with three combination reactions; (1) nitrate reduction to nitrite (2) dissimilatory reduction of nitrite to ammonium (3) anammox reaction with ammonium and nitrite to dinitrogen gas. The gene encoding pentaheme c nitrite reductase (*nrfA*; GAB60912) for DNRA has been annotated in KSU-1 (Hira et al., 2012). A homologous gene cluster of tetraheme and pentaheme nitrite reductase, *nrfHA* (26THWARD_02534, and 02535), was also detected in this study, suggesting also the potential capability of DNRA in the *C.* "Brocadia caroliniensis" enrichment.

3.7. Engineering implications

From a broader perspective, the presence of anammox bacteria within localized 'hot-spots', characterized by accumulation of ammonia and nitrite along with limiting DO concentrations is not entirely unexpected. However, the intense enrichment of a hitherto rarely described anammox population urges a possible re-evaluation of the limited view of BNR processes and wastewater treatment plants as a whole, fostering very defined microbial populations and transformations. It is likely that in some wastewater treatment systems not intentionally engineered for autotrophic biological nitrogen removal, anammox might still be contributing to overall nitrogen removal, perhaps to varying extents, depending upon the process design and operating conditions. If this is the case, especially in treatment processes where sidestream and mainstream reactors are connected, the overall contribution of autotrophic nitrogen removal in the mainstream could also be positively impacted. Such benefits could be amplified quite substantially, when coupled with dedicated anammox retention strategies, such as hydrocyclones, sieves or fixed-film media. Within this framework, the role of partial denitrification

(conversion to nitrate to nitrite) engendered by kinetic (for instance, through inadequate anoxic zone sizing) or stoichiometric (for instance, inadequate electron donor supply) limitation also needs to be evaluated further and perhaps even optimized in a directed fashion.

4. Conclusions

The enrichment of anammox bacteria related to *C.* "Brocadia caroliniensis" was discovered in a full-scale centrate treatment system engineering originally for coupling nitritation and denitritation with glycerol addition in New York City. The functionality of this enrichment was deciphered using next-generation sequencing and metagenomics analysis. The population genome of this enrichment revealed among others, essential ORFs coding for substrate transport, energy metabolism and carbon fixation relating to autotrophic anammox bacteria metabolism. It is expected that this draft anammox population genome of the *C.* "Brocadia caroliniensis" enrichment will in general expand our insight into the expected and unexpected microbial structure and function in engineered BNR processes and also contribute to further resolution of the *C.* "Brocadia" reference genome for continued studies of anammox systems.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2017.01.011.

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