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Comammox Functionality Identified in Diverse Engineered Biological Wastewater Treatment Systems

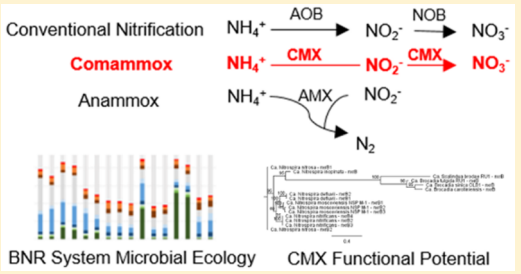
Medini K. Annavajhala,[†,#] Vikram Kapoor,[‡,§] Jorge Santo-Domingo,[‡] and Kartik Chandran*[,†]

[†]Department of Earth and Environmental Engineering, Columbia University, New York, New York 10027, United States

[‡]Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268, United States

Supporting Information

ABSTRACT: Complete ammonia oxidation (comammox) to nitrate by certain *Nitrospira*-lineage bacteria (CMX) could contribute to overall nitrogen cycling in engineered biological nitrogen removal (BNR) processes in addition to the more well-documented nitrogen transformations by ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), and anaerobic ammonia-oxidizing (anammox) bacteria (AMX). A metagenomic survey was conducted to quantify the presence and elucidate the potential functionality of CMX in 16 full-scale BNR configurations treating mainstream or sidestream wastewater. CMX proposed to date were combined with previously published AOB, NOB, and AMX genomes to create an expanded database for alignment of metagenomic reads. CMX-assigned metagenomic reads accounted for between 0.28 and 0.64% of total coding DNA sequences in all BNR configurations. Phylogenetic analysis of key nitrification functional genes *amoA*, encoding the α-subunit of ammonia monooxygenase, *haoB*, encoding the β-subunit of hydroxylamine oxidoreductase, and *nxrB*, encoding the β-subunit of nitrite oxidoreductase, confirmed that each BNR system contained coding regions for production of these enzymes by CMX specifically. Ultimately, the ubiquitous presence of CMX bacteria and metabolic functionality in such diverse system configurations emphasizes the need to translate novel bacterial transformations to engineered biological process interrogation, operation, and design.



INTRODUCTION

Biological nitrogen removal (BNR) is traditionally accomplished through the concerted action of nitrifying and denitrifying bacteria. During nitrification, the oxidation of ammonia to nitrate is mediated by two distinct groups of chemolithoautotrophs, ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB).[1] Nitrate is typically reduced by chemoorganoheterotrophic denitrifying bacteria to dinitrogen gas (N_2) (Figure 1).[2] However, alternate "shortcut" nitrogen removal pathways allow for potential cost savings of aeration energy for nitrification and external carbon for denitrification and have therefore been applied to mainstream and sidestream wastewater treatment processes in recent years.[3−7] The coupling of partial nitritation (fractional aerobic oxidation of influent ammonia to nitrite by AOB) with anaerobic ammonia oxidation (anammox) represents one such shortcut process. In such partial nitritation−anammox processes, the selective enrichment of AOB and anammox bacteria (AMX) with the concomitant out-selection of NOB can result in a significant reduction (≤62.5%) of aeration requirements relative to those of conventional BNR approaches, while eliminating the need to supply external organic carbon (Figure 1).[8]

In an exciting expansion of our current knowledge of the microbial nitrogen cycle, recent studies have uncovered the potential for complete ammonia oxidation (comammox) to nitrate by a single organism (CMX) rather than by distinct AOB and NOB (Figure 1).[9,10] Three CMX organisms related to *Nitrospira* spp., *Candidatus* "Nitrospira inopinata", *Candidatus* "Nitrospira nitrificans", and *Candidatus* "Nitrospira nitrosa", have been proposed to date.[9,10] However, understanding CMX metabolic activity and their interactions with other bacterial groups is still rather limited in natural and engineered systems, including BNR processes.

In conventional BNR processes, CMX may be a beneficial or equivalent alternative to AOB-mediated nitritation and NOB-mediated nitratation depending on the relative kinetics and substrate utilization of CMX-mediated nitritation and nitratation. Additionally, the coupling of ammonia and nitrite oxidation within CMX could potentially decrease production of the greenhouse gas nitrous oxide (N_2O), as N_2O production by classical AOB has been linked to an imbalance in electron flow due to transient dissolved oxygen (DO) concentrations, accumulation of ammonia or nitrite, or limited inorganic carbon supply.[11−14] On the other hand, energy- and cost-effective alternates to conventional BNR processes such as those targeting full or partial nitritation (e.g., for coupling with denitrification or anammox) would be less effective as a result of direct conversion of ammonia to nitrate by CMX. Therefore,

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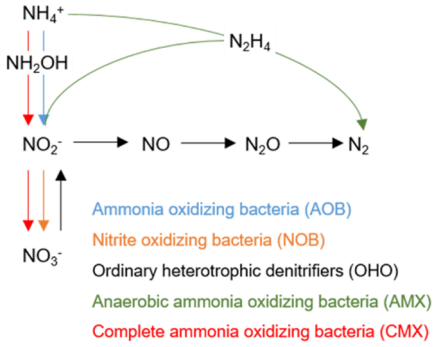

Figure 1. Nitrogen cycling in engineered biological nitrogen removal (BNR) systems. In conventional BNR, autotrophic ammonia-oxidizing bacteria (AOB) convert ammonium ($NH4^+$) to nitrite (NO_2^-) through hydroxylamine (NH_2OH), and nitrite-oxidizing bacteria (NOB) convert nitrite to nitrate (NO_3^-). Ordinary heterotrophic denitrifiers (OHO) then convert nitrate to dinitrogen gas (N_2) through nitrite, nitric oxide (NO), and nitrous oxide (N_2O). Alternatively, anaerobic ammonia-oxidizing bacteria (AMX) convert ammonium and nitrite directly to dinitrogen gas through hydrazine (N_2H_4). Complete ammonia-oxidizing bacteria (CMX) have only recently been described and studied, and are capable of converting ammonium to nitrate through hydroxylamine and nitrite in a single organism.

the abundance of CMX and their contribution to nitrogen turnover across different BNR process configurations need to be characterized to better understand and guide process design and operation. More broadly, with few publications describing the presence of CMX in drinking water[15,16] and limited-scale discussion of CMX in wastewater treatment systems,[17−20] much work remains to improve our understanding of the potential for CMX capabilities across different natural and engineered systems.

In this study, shotgun metagenomic analyses were conducted on 16 samples from six full-scale wastewater treatment plants in the United States, Denmark, and Singapore. These plants employ conventional and shortcut BNR in mainstream and/or sidestream wastewater using varying reactor configurations (Table 1). This work aimed to identify and quantify the presence of CMX in each of these systems and to explore possible CMX functionality through phylogenetic analysis of metagenomic reads assigned to key nitrogen metabolism enzymes.

MATERIALS AND METHODS

Microbial Sampling and Sequencing. Sixteen biomass samples from six full-scale wastewater treatment plants (WWTPs) were collected for analysis (Table 1). The following reactor configurations were covered: conventional BNR, moving bed biofilm reactor (MBBR), DEMON deammonification, ClearGreen cyclic low-energy ammonium removal (ClearGreen), and a hydrocyclone. These systems were operated in the United States (California and Virginia), Denmark, and Singapore. Approximately 50 mL was sampled directly from reactor granules, mixed liquor, or biofilm within each BNR process (Table 1) and stored at −80 °C after shipment on dry ice. DNA was extracted from the biomass samples with the MoBio PowerLyzer PowerSoil DNA Isolation Kit per the manufacturer's instructions (Qiagen) and quantified using Qubit dsDNA HS assay kits and the Qubit 2.0 Fluorometer (Thermo Fisher). Extracted DNA was shipped on dry ice from Columbia University to the Cincinnati Children's Hospital DNA Core Facility for next-generation sequencing. Shotgun metagenomic libraries were prepared using the Nextera XT Library Preparation Kit (Illumina) with the recommended input of DNA normalized to 5 ng/μL and the default protocol for barcoded whole-genome libraries. An Illumina MiSeq sequencer and pair-ended 2 × 250 bp Illumina MiSeq version 2 sequencing kit were used (Illumina).

Taxonomic Classification. Pair-ended reads were assembled into contigs and filtered for quality assurance (maximum homopolymeric region length of 10 bp, minimum length of 250 bp, maximum length of 450 bp, maximum number of ambiguous bases of zero) using mothur version 1.36.1.[21] The existing NCBI nonredundant protein *nr* database (version 123) was manually expanded as part of this work to include protein-coding sequences from the three currently

Table 1. Full-Scale Wastewater Treatment Plants Surveyed

sample	location	reactor type	biomass type	feed type	% CMX[g] CDS/total CDS
EB DEMON[a]	California, USA	DEMON	granule	SS[b]	0.46
EB MBBR[c]	California, USA	MBBR	biofilm	SS	0.46
SF DEMON	California, USA	DEMON	granule	SS	0.49
SF MBBR	California, USA	MBBR	biofilm	SS	0.47
PDR 1	California, USA	ClearGreen[d]	mixed liquor	MS[e]	0.38
PDR 2	California, USA	ClearGreen	mixed liquor	MS	0.45
DK overflow	Denmark	hydrocyclone	overflow	MS	0.49
DK underflow	Denmark	hydrocyclone	underflow	MS	0.54
DK ALT	Denmark	hydrocyclone	ALT (mixed liquor)	MS	0.32
DK inoculum	Denmark	–	inoculum	–	0.64
SG biofilm	Singapore	BNR[f]	biofilm	MS	0.46
SG AS	Singapore	BNR	activated sludge	MS	0.57
VA MBBR 1	Virginia, USA	MBBR	biofilm	MS	0.28
VA MBBR 2	Virginia, USA	MBBR	biofilm	MS	0.45
VA BNR 1	Virginia, USA	BNR	mixed liquor	MS	0.57
VA BNR 2	Virginia, USA	BNR	mixed liquor	MS	0.36

[a]DEMON, DEamMONification. [b]SS, sidestream wastewater. [c]MBBR, moving bed biofilm reactor. [d]ClearGreen, cyclic low-energy ammonium removal. [e]MS, mainstream wastewater. [f]BNR, (conventional) biological nitrogen removal. [g]CMX, complete ammonia-oxidizing bacteria; total CDS found in Table S1.

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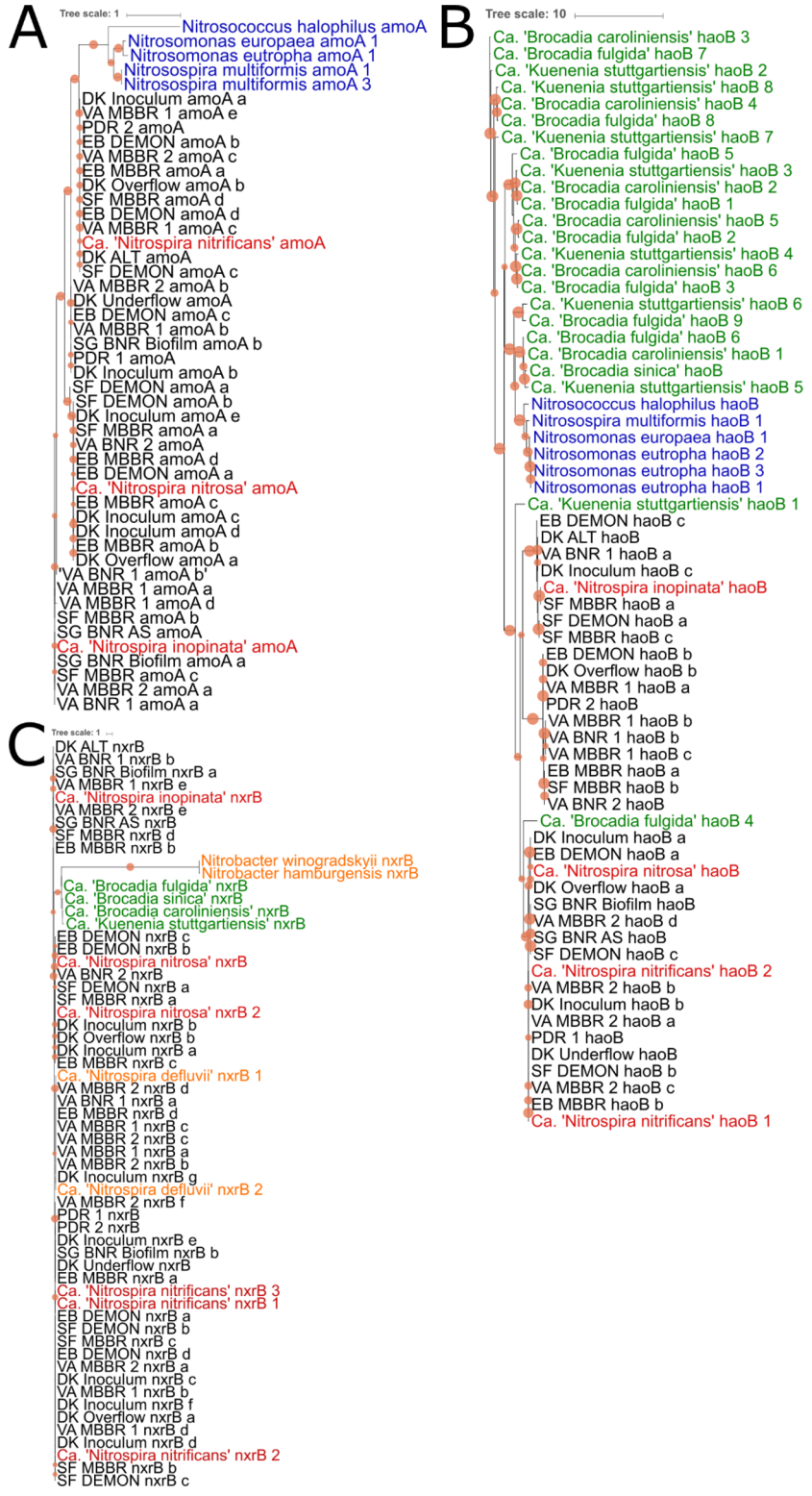

Figure 2. Phylogenetic relationships between published *amoA*, *haoB*, and *nxrB* sequences and CMX-assigned sequences from each BNR process. Unique merged contigs from each BNR process that were assigned with ≥30× coverage as CMX *amoA*, *haoB*, and *nxrB* were included in the phylogenetic analysis, along with *amoA*, *haoB*, and *nxrB* sequences from representative published sequences (see Table S2 for a complete list of accession numbers). (A) CMX (red) and AOB (blue) reference *amoA* sequences compared through phylogenetics to CMX-assigned *amoA* in each BNR process. (B) CMX (red), AOB (blue), and AMX (green) reference *haoB* sequences compared to CMX-assigned *haoB* from each BNR system. (C) CMX (red), NOB (orange), and AMX (green) reference *nxrB* sequences compared to CMX-assigned *nxrB* from each BNR system. Tree-scale units are average amino acid substitutions per site, and circles at each node are scaled to reflect *P* values at nodes with ≥50% support. Each BNR process contained at least one *amoA*, *haoB*, and *nxrB* sequence that clustered with CMX rather than AOB, NOB, or AMX, providing evidence of potential CMX functionality in each system.

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published CMX metagenome-derived genomes: *Candidatus* "Nitrospira inopinata",[9] *Candidatus* "Nitrospira nitrificans", and *Candidatus* "Nitrospira nitrosa".[10] Protein-coding regions from two metagenome-derived AMX genomes that were previously unavailable in *nr* database version 123, *Candidatus* "Brocadia caroliniensis"[22] and *Candidatus* "Scalindua profunda",[23] were also included in the custom database. Filtered reads from each sample were then aligned against this custom database using NCBI's BLASTX. Alignments were curated for minimum identity percentage (80%) and maximum *e* value (1×10^{-10}). Resulting reads in each metagenome were assigned taxonomic classification according to an automated search of NCBI protein databases, with emphasis on the contributions of new and previously indexed CMX and AMX species, as well as AOB and NOB.

Phylogenetic Analysis of Key Nitrification Genes. To confirm the potential for ammonia and nitrite oxidation by CMX rather than AOB, AMX, or NOB, unique representative sequences from each BNR system assigned through BLASTX to CMX *amoA*, *haoB*, and *nxrB* with at least 30× coverage were selected. Single-nucleotide variants (SNVs) were calculated, and a distance matrix was generated using these unique sequences with MAFFT version 7.[24] CMX (*Candidatus* "Nitrospira inopinata", *Candidatus* "Nitrospira nitrificans", and *Candidatus* "Nitrospira nitrosa"), AOB (*Nitrosomonas europaea*, *Nitrosomonas eutropha*, *Nitrosospira multiformis*, and *Nitrosococcus halophilus*), NOB (*Candidatus* "Nitrospira defluvii", *Nitrobacter hamburgensis*, and *Nitrobacter winogradskyii*), and AMX (*Candidatus* "Brocadia caroliniensis", *Candidatus* "Brocadia fulgida", *Candidatus* "Brocadia sinica", and *Candidatus* "Kuenenia stuttgartiensis") reference genomes were also included in the MAFFT alignment to identify likely phylogenetic origins of sequences in the BNR systems studied here (see Table S1 for a complete list of accession numbers used). RAxML version 8.2[25] was used to generate a maximum likelihood tree and calculate branch node support over 100 bootstrap iterations under the GAMMA GTR substitution model.[25] AOB (for *amoA* and *haoB*), NOB (for *nxrB*), and AMX (for *haoB* and *nxrB*) sequences were also included for the sake of completeness and to confirm whether the representative sequences from the 16 BNR systems clustered with, and therefore likely traced to, CMX in each engineered process.

RESULTS AND DISCUSSION

Varying Degrees of Comammox Capability Detected in All Systems. Even prior to the discovery of comammox-capable bacteria, complete oxidation of ammonia to nitrate in a single organism had been previously theorized.[26] Within the framework of engineered BNR and biological wastewater treatment systems, the potential for complex interactions among CMX, AOB, NOB, and AMX could be particularly interesting given the utilization of common substrates (ammonia, nitrite, and even inorganic carbon).[9,10] While targeted 16S rRNA gene analyses can describe only the microbial community structure of a system, shotgun metagenomics can reveal both the community structure and functional potential of engineered bioreactors.

The results reported here include a discussion of the relative abundance [in reads per kilobase mapped per million reads (RPKM)] of key genes involved in nitrification, related to the capability of each BNR process to produce key nitrifying enzymes encoded by these genes. This approach serves as a reference blueprint for similarly broad meta-transcriptomics or meta-proteomics studies as well as more focused targeted studies. Additionally, phylogenetic analyses are used here to complement the metagenomics approach and provide improved resolution for taxonomic assignment of sequences from organisms with limited genomic reference availability.

Previous work has identified CMX in drinking water or wastewater systems through a combination of next-generation sequencing and phylogenetics.[15,16,18,19] However, unique to this study is the characterization of the contribution of CMX to the overall protein-producing capability of full-scale wastewater treatment plant metagenomes in a wide variety of reactor configurations. The systems assessed here have been designed for conventional BNR or partial nitritation by AOB followed by deammonification by anammox (Table 1). Conventional BNR systems included those from Singapore (SG Biofilm, SG AS) and Virginia (VA BNR 1 and 2). DEMON and ClearGreen systems from California (EB DEMON, SF DEMON, and PDR 1 and 2) were designed and operated for partial nitritation, and the partial oxidization of ammonia to nitrite by AOB, followed by anammox. These systems used granules enriched with AMX due to resultant substrate and oxygen gradients formed therein and treated both sidestream (EB DEMON and SF DEMON) and mainstream (PDR 1 and 2) wastewater.[27−29] The nitritation−anammox MBBRs from California and Virginia (EB MBBR, SF MBBR, and VA MBBR 1 and 2) similarly contained biofilms enriched with AMX and consisted of either single-stage simultaneous partial nitritation−anammox treating sidestream wastewater (as in EB or SF) or two-stage decoupled operation treating mainstream wastewater (as in VA), in which an aerated nitritation process was followed by the anaerobic deammonification MBBR.[30] The hydrocyclone-based process in Denmark (samples denoted DK ALT, overflow, and underflow) was utilized to selectively retain granular biomass in a mainstream BNR system.[28]

In total, 59.65 million filtered contigs were available for this study, with an average of 3.73 million sequences and a 393 bp contig length across the 16 samples (Table S1). For each sample, the number of total coding DNA sequences (CDS) was calculated as the number of contigs aligned with the expanded protein database above the described score thresholds. According to this definition, the 16 samples contained on average 3.14 ± 0.48 million total CDS (Table S1). Interestingly, despite high variability in microbial community profiles across the different samples [0.59−14.35% AMX, 2.25−7.98% AOB, and 2.39−3.47% NOB (Table 1 and Table S1)], CMX-assigned coding regions remained consistently between 0.28 and 0.64% of total CDS.

Phylogenetic analyses confirmed that each BNR process contained sequences aligned with key nitrification genes originating from CMX for *amoA* encoding the ammonia monooxygenase α-subunit, *haoB* encoding the hydroxylamine oxidoreductase β-subunit, and *nxrB* encoding the β-subunit of nitrite oxidoreductase. Each system also contained AOB, NOB, and AMX functional gene sequences, but only contigs aligned with CMX through BLASTX are included here for evidence of potential CMX functionality. SF MBBR, SG BNR AS and Biofilm, and VA MBBR 2 contained *amoA* sequences clustered with *Candidatus* "Nitrospira inopinata" [$P = 0.71$ (Figure 2A)]. EB DEMON and MBBR, SF DEMON and MBBR, DK overflow and inoculum, and VA BNR 2 samples contained *amoA* sequences that clustered with *Candidatus* "Nitrospira nitrosa" ($P = 0.99$). Interestingly, many samples (EB DEMON and MBBR, SF DEMON and MBBR, PDR 2, DK overflow,

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ALT, and inoculum, and VA MBBR 1 and 2) contained *amoA* sequences clustered closely with *Candidatus* "Nitrospira nitrificans" (P = 1). EB DEMON, SF DEMON and MBBR, DK ALT and inoculum, and VA BNR 1 contained *haoB* sequences that clustered with *Candidatus* "Nitrospira inopinata" (P = 0.97) (Figure 2B). EB DEMON, SF DEMON, DK overflow and inoculum, SG BNR AS and biofilm, and VA MBBR 2 *haoB* clustered with *Candidatus* "Nitrospira nitrosa", while EB MBBR, SF DEMON, PDR 1, DK underflow and inoculum, and VA MBBR 2 sequences clustered with *Candidatus* "Nitrospira nitrificans" *haoB* 1 and 2 (P = 0.83).

Previously, it was theorized that CMX have high growth yields rather than high specific growth rates associated with AOB and NOB, leading to a competitive advantage at low substrate concentrations and conditions favoring microbial aggregation.[26] Additionally, previous work by van Kessel et al. suggested functional interplay between CMX and AMX due to tight clustering of functional gene sequences,[10] which was also observed here to an extent (Figure 2). The data presented here may support these hypotheses, as the processes promoting biofilm growth, including nitritation−anammox, biofilm, and anammox inoculum samples, contained more unique CMX *amoA* and *haoB* sequences than the conventional BNR and hydrocyclone systems did (Figure 2A,B). Future analysis will be necessary to quantify the abundance of CMX *amoA* and *haoB*, aided partly by CMX *amoA*-targeted polymerase chain reaction primers.[31]

Each BNR process also contained contigs that clustered with CMX *nxrB* sequences rather than NOB or AMX (Figure 2C). EB DEMON and MBBR, SF DEMON and MBBR, DK overflow and inoculum, and VA BNR2 samples had *nxrB* contigs that clustered closely with *Candidatus* "Nitrospira nitrosa" *nxrB* 1 and 2 (P = 0.97). SF MBBR, SG BNR AS and Biofilm, VA BNR 1, and VA MBBR 1 and 2 contained contigs related to *Candidatus* "Nitrospira inopinata" *nxrB* (P = 0.82). EB DEMON and MBBR, SF DEMON and MBBR, PDR 1 and 2, DK overflow, underflow, and inoculum, SG BNR AS and biofilm, and VA MBBR 1 and 2 all contained *nxrB* contigs that clustered with *Candidatus* "Nitrospira nitrificans" *nxrB* 1, 2, and 3 (P = 0.98). Of note, CMX-assigned *nxrB* contigs from EB MBBR, DK inoculum, VA BNR 1, and VA MBBR 1 and 2 were revealed through phylogenetic analysis to be more closely related to the non-CMX, canonical NOB *Candidatus* "Nitrosomonas defluvii" (P = 1). Thus, relying solely on BLAST-based alignment could lead to overestimation of CMX, highlighting the need for integrated metagenomics approaches that include phylogenetic interrogation for closely related organisms or those without published references. NOB out-selection is important in deammonification systems primarily because of the competition between NOB and AMX for nitrite.[4] Similarly, CMX utilization of nitrite could impact deammonification by AMX. Therefore, it is critical to note the potential for each nitritation−anammox process to produce *nxrB* by CMX to develop and apply more accurate out-selection strategies.

Nitrification by CMX Is Possible in Engineered Water Systems. The emergence of newly discovered microorganisms capable of full nitrification is understandably exciting. Detection of comammox in such a wide array of systems does reveal the need for re-evaluation and adjustment of current BNR models given these newly available metabolic pathways. Metagenomic analysis alone, though, cannot determine if comammox is active within engineered nitrogen removal systems, given the operation conditions and competitive microbial communities involved. Lab-scale enrichment of CMX under appropriate conditions could improve our understanding of CMX thermodynamics, biokinetic parameters, and electron flow.[32] Subsequently, calculation of theoretical relative abundances of CMX, AOB, and NOB required for nitrification[33,34] and improved CMX-inclusive BNR modeling would be possible. Such directed studies will be required to compare the likelihood of comammox to that of conventional two-step nitrification or anammox under different conditions, link the presence of CMX and functionality to reactor operational parameters, and ultimately quantify the contribution of CMX to overall nitrogen turnover in BNR systems. These insights will be key in both the diagnosis of existing systems and the design of new nitrogen removal processes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.7b00577.

Details regarding contig assembly, filtering, and database alignment (Table S1), reference NCBI Genome Assembly and GenBank accession numbers used in the phylogenetic analyses (Table S2), raw sequencing reads that have been submitted to the NCBI Sequencing Read Archive (SRA) (BioProject PRJNA377913), and all phylogenetic trees that have been visualized through the Interative Tree of Life (iTOL) viewer (https://itol.embl.de/)[35] (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Department of Earth and Environmental Engineering, Columbia University, 500 W. 120th St., New York, NY 10027. E-mail: kc2288@columbia.edu. Phone: (212) 854-9027. Fax: (212) 854-7081.

ORCID

Vikram Kapoor: 0000-0002-7159-0883

Kartik Chandran: 0000-0002-7526-3724

Present Addresses

#M.K.A.: Infectious Diseases Division, Department of Medicine, Columbia University, New York, NY 10032.

§V.K.: Department of Civil and Environmental Engineering, The University of Texas, San Antonio, San Antonio, TX 78249.

Notes

The authors declare no competing financial interest.

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