Design and Assessment of Species-Level qPCR Primers Targeting Comammox

Natalie K. Beach and Daniel R. Noguera*

Department of Civil and Environmental Engineering, University of Wisconsin–Madison, Madison, WI, United States

Published PCR primers targeting the ammonia monooxygenase gene (amoA) were applied to samples from activated sludge systems operated with low dissolved oxygen (DO) to quantify total and clade-level Nitrospira that perform complete ammonium oxidation (comammox); however, we found these existing primers resulted in significant artifact-associated non-target amplification. This not only overestimated comammox amoA copies but also resulted in numerous false positive detections in the environmental samples tested, as confirmed by gel electrophoresis. Therefore, instead of attempting to quantify comammox diversity, we focused on accurately quantifying the candidate comammox species. We designed specific and sensitive primers targeting 3 candidate species: *Candidatus* (Ca.) *Nitrospira nitrosa*, Ca. N. inopinata, and Ca. N. nitrificans. The primers were tested with amoA templates of these candidate species and used to quantify comammox at the species level in low DO activated sludge systems. We found that comammox related to Ca. N. nitrosa were present and abundant in the majority of samples from low DO bioreactors and were not detected in samples from a high DO system. In addition, the greatest abundance of Ca. N. nitrosa was found in bioreactors operated with a long solids retention time. Ca. N. inopinata and Ca. N. nitrificans were only detected sporadically in these samples, indicating a minor role of these comammox in nitrification under low DO conditions.

Keywords: comammox, *Nitrospira*, nitrification, low dissolved oxygen, biological nutrient removal, qPCR, real-time PCR, PCR primers

INTRODUCTION

The oxidation of ammonium via nitrite to nitrate (i.e., nitrification) was historically considered a two-step process completed by phylogenetically distinct ammonia oxidizers and nitrite oxidizers. Although the complete oxidation of ammonium to nitrate by a single organism was theoretically possible, no microorganism with the ability to carry out both steps had been identified until late 2015 (Daims et al., 2015; van Kessel et al., 2015). These complete ammonium oxidizing (comammox) bacteria belong to the genus *Nitrospira*, which was known to contain only nitrite-oxidizing bacteria (NOB); therefore, an entire group of microorganisms with the ability to oxidize ammonia had been disguised for years (Daims et al., 2015; van Kessel et al., 2015). The discovery of comammox bacteria has redefined a key component of the global nitrogen cycle, initiating a renewed focus of recent nitrogen cycling research in environmental biotechnology (Chao et al., 2016; Palomo et al., 2016; Pinto et al., 2016; Stadler and Love, 2016; Bartelme et al., 2017; Fowler et al., 2017; Hu and He, 2017).
Within the wastewater treatment industry, the application of nitrification under very low dissolved oxygen (DO) concentrations (below 0.2 mg O₂/L) is an exciting energy-saving approach to biological nutrient removal (BNR) (Park and Noguera, 2004; Paredes et al., 2007; Fitzgerald et al., 2015). Previous attempts to identify the key microorganisms responsible for nitrification in low DO bioreactors have been inconclusive, with the presence of known ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA) unable to explain observed nitrification rates (Fitzgerald et al., 2015). Thus, the recent discovery of comammox in a variety of environments (Daims et al., 2015; van Kessel et al., 2015) prompted us to investigate their presence in low DO BNR systems.

The gene encoding the alpha-subunit of ammonia monoxygenase (amoA) has been one of the most widely used markers for detection and quantification of AOB and AOA, as it facilitates functional analysis and reconstruction of phylogenetic relationships (Rotthauwe et al., 1997; Purkhold et al., 2000; Koops et al., 2006; Pester et al., 2012). The amoA of comammox is distinguishable from amoA sequences of AOB and AOA, and thus, it can be used to detect the presence of comammox in environmental samples (Daims et al., 2015; van Kessel et al., 2015; Pinto et al., 2016; Camejo et al., 2017). Recent descriptions of PCR primers targeting the amoA gene of comammox include a primer pair designed specifically for Candidatus (Ca.) Nitrospira inopinata (Daims et al., 2015), a primer pair designed specifically for a comammox-like clone within a freshwater aquaculture system (Bartelme et al., 2017), a primer collection that differentiates two broad clades of comammox within the Nitrospira genus (Pjevac et al., 2017), and a highly degenerate primer pair attempting to encompass all comammox within Nitrospira (Fowler et al., 2017). To date, three candidate comammox species have been described in the literature, namely Ca. N. nitroa, Ca. N. inopinata, and Ca. N. nitrificans (Daims et al., 2015; van Kessel et al., 2015). However, quantifying the contribution of these candidate species to the comammox community in environmental samples has not been possible because of the lack of specific qPCR primer sets. To overcome this limitation, we designed a set of highly specific non-degenerate primers for the independent detection of Ca. N. nitroa, Ca. N. inopinata, and Ca. N. nitrificans. We used these primers to evaluate the contribution of these species to the comammox population in samples from BNR plants operated at low DO conditions.

**MATERIALS AND METHODS**

**Sample Collection, Processing, and DNA Extraction**

Environmental samples used in this study originated from four low DO nitrifying bioreactors: a laboratory-scale sequencing batch reactor (L_SBR), a pilot-scale sequencing batch reactor (P_SBR), a pilot scale continuous flow (P_CF) reactor simulating a University of Cape Town configuration without nitrate recycle, and a full-scale wastewater treatment plant (WWTP) from the Trinity River Authority (TRA) Central Region (Arlington, TX, United States) (Table 1). For comparison, samples were also collected from the full-scale Nine Springs WWTP (NS) at the Madison Metropolitan Sewerage District (Madison, WI, United States), which operates with typical high DO conditions (Table 1). Grab samples with varying volumes were collected from every reactor and were either centrifuged to form a biomass pellet, with supernatant discarded, and the pellet frozen at −80°C (P_SBR, P_CF, TRA, and NS) or were saved in a glycerol mixture prior to placement in a −80°C freezer (L_SBR). Biomass samples were stored at −80°C until DNA extraction. All DNA was extracted using DNeasy® PowerSoil® DNA Isolation Kit (Qiagen, Hilden, Germany) following the manufacturer’s directions. DNA was quantified with a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, United States) and the purity ratio, or ratio of absorbance at 260 and 280 nm, was determined with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). DNA samples were stored at −20°C until further processing.

**Design of Primers to Detect Comammox Ammonia Monoxygenase Gene amoA**

A collection of full-length amoA and full-length particulate methane monoxygenase (pmoA) gene sequences, obtained from the National Center for Biotechnology Information (NCBI) GenBank database (NCBI Resource Coordinators, 2017), were used for primer design. The full-length sequences included in the design are indicated by bold text in the phylogenetic tree in Figure 3, with the corresponding accession number in parenthesis. All amoA and pmoA sequences were aligned using the ‘AlignSeqs’ command in the DECIPHER “R” package (Wright, 2015, 2016). This aligned database was then submitted to DECIPHER’s Design Primers web tool (Wright et al., 2014). Sequences corresponding to Ca. N. nitroa, Ca. N. inopinata, and Ca. N. nitrificans were individually selected as target groups for primer design, which used the following parameters: primer length ranging from 17 – 26 nucleotides with up to 1 permutation, PCR product amplicon length of 100 – 450 bp, 100% target group coverage, and without the Taq 3′-end Model option. The primer design tool also used the same reaction conditions in all cases: [Na+] 70 mM, [Mg²⁺] 3 mM, [dNTPs] 0.8 mM, annealing temperature (Tₐ) of 64°C, [primers] 400 nM. Amplification products were verified by agarose (2%) gel electrophoresis with GelRed™ Nucleic Acid Gel Stain (Biotium, Fremont, CA, United States).

**Quantitative Real-Time Polymerase Chain Reaction (qPCR)**

Quantification of total 16S rRNA genes, total comammox amoA genes, clades A and B comammox amoA, as well as Ca. N. nitroa, Ca. N. inopinata, and Ca. N. nitrificans amoA in each DNA sample was carried out by qPCR. All qPCR assays were performed on a Roche LightCycler® 480 high-throughput real-time PCR system using white LightCycler® 480 multwell plates and the associated LightCycler® 480 sealing foils (Roche Molecular Systems, Inc., Pleasanton, CA, United States). All environmental DNA samples were diluted to 10 ng/µL. Although not specifically
studied here, the applied sample dilutions helped reduce any potential problems with PCR inhibition. All qPCR assays were prepared in Bio-Rad 2x iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, United States), containing 50 U/mL iTaq DNA polymerase, 1.6 mM dNTPs, 100 mM KCl, 40 mM Tris-HCl, 6 mM MgCl₂, 20 nM fluorescein, and stabilizers (10 μL per reaction). Triplicate reactions were prepared for each sample.

Amplification of total comammox amoA was performed according to Fowler et al. (2017), with each reaction containing Bio-Rad 2x iQ™ SYBR® Green Supermix (10 μL), nuclease free water (4.4 μL), environmental DNA or standard (4 μL), and PCR primers (0.8 μL of 12.5 μM each primer) added for a final volume of 20 μL of reaction. The thermal cycling protocol for the total comammox primers was as follows: initial denaturation step at 94°C for 5 min, followed by 40 cycles of initial denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 1 min.

For the novel amoA primers designed in this study (Table 2), nuclease free water (4.4 μL), environmental DNA or standard (4 μL), and PCR primers (0.8 μL of 10 μM each primer) were added to each reaction for a final volume of 20 μL per reaction. The thermal cycling protocol for qPCR using the novel amoA primers designed in this study (Table 2) was as follows: initial denaturation step at 95°C for 3 min, followed by 45 cycles of initial denaturation at 95°C for 30 s, annealing at 52°C for 45 s, and extension at 72°C for 1 min.

For the novel amoA primers designed in this study (Table 2), nuclease free water (4.4 μL), environmental DNA or standard (4 μL), and PCR primers (0.8 μL of 10 μM each primer) were added to each reaction for a final volume of 20 μL per reaction. The thermal cycling protocol for qPCR using the novel amoA primers designed in this study (Table 2) was as follows: initial denaturation step at 95°C for 3 min, followed by 45 cycles of initial denaturation at 95°C for 30 s, annealing at 52°C for 45 s, and extension at 72°C for 1 min.

For the novel amoA primers designed in this study (Table 2), nuclease free water (4.4 μL), environmental DNA or standard (4 μL), and PCR primers (0.8 μL of 10 μM each primer) were added to each reaction for a final volume of 20 μL per reaction. The thermal cycling protocol for qPCR using the novel amoA primers designed in this study (Table 2) was as follows: initial denaturation step at 95°C for 3 min, followed by 45 cycles of initial denaturation at 95°C for 30 s, annealing at 52°C for 45 s, and extension at 72°C for 1 min.

Standard Preparation for qPCR

Full-length amoA from Candidatus Nitrospira nitrosa, Ca. N. inopinata, and Ca. N. nitificans were used as standards for the new species-specific comammox assay as well as the total comammox and clade A comammox assays. Full-length amoA from Nitrospira sp. CG24_E (Palomo et al., 2018) was used as the standard for the clade B comammox assay. These individual amoA standards were generated from synthetic gene

---

**Table 2** | Bioreactor sample characteristics.

| Bioreactor (Sample ID) | Location | Sample Dates | Configuration at time of sampling | Size (gpd) | SRT (days) | Reference |
|------------------------|----------|--------------|-----------------------------------|------------|------------|-----------|
| Lab-scale sequencing batch reactor (L_SBR) | Madison, WI, United States | 3/7/2013 (1), 3/18/2013 (2), 4/17/2013 (3), 10/3/2014 (4) | Sequencing batch reactor (SBR) operated with dissolved oxygen (DO) < 0.2 mg O₂/L | 0.5 | 80 | Camejo et al., 2016 |
| Pilot-scale sequencing batch reactor (P_SBR) | Madison, WI, United States | 9/10/2015 (1), 11/10/2015 (2), 12/15/2015 (3), 4/25/2016 (4) | Sequencing batch reactor (SBR) operated with DO < 0.7 mg O₂/L | 130 | 80 | Camejo et al., 2016 |
| Continuous flow pilot plant (P_CF) | Madison, WI, United States | 9/9/2015 (1), 11/18/2015 (2), 12/30/2015 (3), 4/26/2016 (4) | Modified University of Capetown (UCT) operated with DO < 0.5 mg O₂/L | 1200 | 10 | Keene et al., 2017 |
| Trinity River Authority Central Region Wastewater System Treatment Plant (TRA) | Arlington, TX, United States | 8/9/2016 (1), 7/28/2017 (3) | Anaerobic Aerobic (AO) operated with three zones, Zone 1: DO < 0.6 mg O₂/L, Zone 2: DO < 0.9 mg O₂/L, Zone 3: DO < 1.4 mg O₂/L | 123 M | 10 | This study |
| Madison Metropolitan Sewage District Nine Springs Plant (NS) | Madison, WI, United States | 9/9/2015 (1), 11/18/2015 (2), 12/30/2015 (3), 4/26/2016 (4) | Modified University of Capetown (UCT) operated with DO > 2.0 mg O₂/L | 40 M | 10 | Keene et al., 2017 |

*Number in parentheses after each date is incorporated after the sample ID.

TRA sample 2 and 3 were collected from different aeration basins.

---

*α* | Microbiota (2017), with each reaction containing Bio-Rad 2x iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, United States), containing 50 U/mL iTaq DNA polymerase, 1.6 mM dNTPs, 100 mM KCl, 40 mM Tris-HCl, 6 mM MgCl₂, 20 nM fluorescein, and stabilizers (10 μL per reaction). Triplicate reactions were prepared for each sample.

Amplification of total comammox amoA was performed according to Fowler et al. (2017), with each reaction containing Bio-Rad 2x iQ™ SYBR® Green Supermix (10 μL), nuclease free water (4.4 μL), environmental DNA or standard (4 μL), and PCR primers (0.8 μL of 12.5 μM each primer) added for a final volume of 20 μL per reaction. The thermal cycling protocol for the total comammox primers was as follows: initial denaturation step at 94°C for 5 min, followed by 40 cycles of initial denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 1 min.

Amplification of clade A and clade B comammox amoA was performed using the equimolar primer mixtures according to Pjevac et al. (2017) (Table 2). Four equimolar primer mixtures were created for clade A and clade B forward and reverse primers, by combining 62.5 μL of each of the six individual primer stocks (100 mM) together with 125 μL nuclease free water to a final working volume of 500 μL and final concentration of 12.5 μM each primer. Finally, reactions were prepared in Bio-Rad 2x iQ™ SYBR® Green Supermix (10 μL), nuclease free water (4.4 μL), environmental DNA or standard (4 μL), and equimolar PCR primer mixture (0.8 μL of 12.5 μM each primer) were added to each reaction for a final volume of 20 μL per reaction. The thermal cycling protocol for the clade-level primers was as follows: initial denaturation step at 95°C for 3 min, followed by 45 cycles of initial denaturation at 95°C for 30 s, annealing at 52°C for 45 s, and extension at 72°C for 1 min.

For the novel amoA primers designed in this study (Table 2), nuclease free water (4.4 μL), environmental DNA or standard (4 μL), and PCR primers (0.8 μL of 10 μM each primer) were added to each reaction for a final volume of 20 μL per reaction. The thermal cycling protocol for qPCR using the novel amoA primers designed in this study (Table 2) was as follows: initial denaturation step at 95°C for 10 min, followed by 45 cycles of initial denaturation at 95°C for 10 s, annealing at 64°C for 30 s, and extension at 72°C for 30 s. Fluorescence was measured at 72°C for amplicon quantification. After amplification, an amplicon melting curve was recorded in 0.25°C steps between 65 and 97°C. Melting peaks were obtained by plotting the negative first derivative of fluorescence against temperature. Although 30 cycles is typically sufficient for quantification of targets in qPCR, the thermal cycling protocol was extended to evaluate potential non-specific amplification with the newly designed primers (Wright et al., 2014). Finally, amplification of total 16S rRNA was performed using the 16S rRNA-targeted primer pair 341F/785r according to Thijs et al. (2017) (Table 2).

**Standard Preparation for qPCR**

Full-length amoA from Candidatus Nitrospira nitrosa, Ca. N. inopinata, and Ca. N. nitificans were used as standards for the new species-specific comammox assay as well as the total comammox and clade A comammox assays. Full-length amoA from Nitrospira sp. CG24_E (Palomo et al., 2018) was used as the standard for the clade B comammox assay. These individual amoA standards were generated from synthetic gene....
TABLE 2 | Primers used for qPCR.

| Target gene | Primer name | Forward primer (5′ - 3′) | Reverse primer (5′ - 3′) | Tₘ(°C) | Reference |
|-------------|-------------|--------------------------|--------------------------|--------|-----------|
| Ca. Nitrospira nitrosa amoA | Nitrosa amoA-469F/812R | GCGATTCTGTGTTTATCCCAAGCAAC | CCCTGTGCAAGCGTGCCGG | 64 | This study |
| Ca. Nitrospira inopinata amoA | Inopinata amoA-410F/815R | TCACTTGTGCTAAGAACTGAG | TCCGGCTGACCAATGATG | 52 | Pjevac et al., 2017 |
| Ca. Nitrospira nitrificans amoA | Nitrificans amoA-463F/826R | ATGTCGCGGACCTGTG | CCGAAAGTTCAGCTTTGTCCTT |
| Comammox Nitrospira clade A amoA | comaA-244f_a/659r_a | TACAATCTGGAATTGACACTA | ATGACATGTTGCTAT GTG | 64 Fowler et al., 2017 |
| | comaA-244f_b/659r_b | TATACTGGAATTGACACTA | ATGACATGTTGCTAT GTG |
| | comaA-244f_c/659r_c | TATACTGGAATTGACACTA | ATGACATGTTGCTAT GTG |
| | comaA-244f_d/659r_d | TATACTGGAATTGACACTA | ATGACATGTTGCTAT GTG |
| | comaA-244f_e/659r_e | TATACTGGAATTGACACTA | ATGACATGTTGCTAT GTG |
| | comaA-244f_f/659r_f | TATACTGGAATTGACACTA | ATGACATGTTGCTAT GTG |
| Comammox Nitrospira clade B amoA | comaB-244f_a/659r_a | TAYTTCTGACAGCTTCTA | AFATCCAGACGTTGTTG | 52 | Pjevac et al., 2017 |
| | comaB-244f_b/659r_b | TAYTTCTGACAGCTTCTA | AFATCCAGACGTTGTTG |
| | comaB-244f_c/659r_c | TACTCTGACAGCTTCTA | AFATCCAGACGTTGTTG |
| | comaB-244f_d/659r_d | TAYTTCTGACAGCTTCTA | AFATCCAGACGTTGTTG |
| | comaB-244f_e/659r_e | TAYTTCTGACAGCTTCTA | AFATCCAGACGTTGTTG |
| | comaB-244f_f/659r_f | TAYTTCTGACAGCTTCTA | AFATCCAGACGTTGTTG |
| Total comammox Nitrospira amoA | Ntsp-amoA 162F/359R | GAGATTCTGGTNTGATGGA | WAGTTNGACCCACCTACCA | 48 | Fowler et al., 2017 |
| Total 16S rRNA | 16S-341F/785R | CCTACCGGGGCGGCGAG | GACTAHCYGGCTATAATCC | 53 | Kindworth et al., 2013; Thys et al., 2017 |

Gradient PCR

Gradient PCR was performed with the total comammox (Fowler et al., 2017) and clade-level comammox (Pjevac et al., 2017) amoA primers with environmental DNA samples diluted to three concentrations to determine if an optimal annealing temperature and DNA concentration exists to eliminate or at least minimize the observed non-specific amplification. Gradient PCR was performed on a Mastercycler® nexus gradient PCR cycler (Eppendorf North America, Hauppauge, NY, United States) with Eppendorf twin.tec® 96-well LoBind PCR plates and Eppendorf PCR sealing foils. Reaction mixtures and thermal cycling protocols were identical to the respective qPCR assay, with the exception that annealing temperatures were set to vary across each PCR plate. For the total comammox primers, annealing temperatures were set in small increments between 47.8 and 56°C. For the clade-level comammox primers, annealing temperatures were set in small increments between 51.8 and 60°C. Two samples were selected for each assay and were selected based on the presence of both the target and non-specific amplifications after qPCR. The total comammox gradient assay included DNA from P_SBR-4 and NS-4. The clade A and clade B comammox gradient assays included DNA from P_CF-4 and NS-4. All environmental DNA samples were diluted to 40 ng DNA/reaction, 20 ng DNA/reaction, and 1 ng DNA/reaction. Amplification products were then visualized with agarose (2%) gel electrophoresis with GelRed™ Nucleic Acid Gel Stain (Biotium, Freemont, CA, United States).
Phylogenetic Tree Construction

The database of aligned amoA and pmoA sequences was expanded by including additional comammox amoA sequences obtained from metagenomes available on the JGI Integrated Microbial Genomes (IMG) database (Markowitz et al., 2012) and available comammox Nitrospira bins extracted with mmgenome (Karst et al., 2016). The expanded database was utilized for in silico primer analyses and to construct a consensus phylogenetic tree in Geneious v. 9.1.2 (Kearse et al., 2012) using the Neighbor-Joining method on a Tamura-Nei genetic distance model with bootstrap resampling and 20,000 trials.

Average Nucleotide Identity Calculation

To determine if comammox Nitrospira draft genomes belonged to the same species, we performed pairwise analyses of genome average nucleotide identity (ANI). Initially, open reading frames (ORFs) within each draft genome was predicted with Prodigal (Hyatt et al., 2010). Subsequently, the JSpecies Web Server (Richter et al., 2016) was used to calculate ANI based on BLAST+ (Camacho et al., 2009) from percent of aligned genome ORFs.

RESULTS

Total Comammox and Clade-Level Comammox Detection

For broad comammox amoA quantification, three published primer sets were compared using standards and nineteen environmental samples originating from five bioreactors (Table 1). The Ntsp-amO ∆62F/359R primer set (Fowler et al., 2017), designed to target total comammox Nitrospira amoA (Table 2), amplifies a 198 bp fragment at primer binding regions between positions 162–182 and 339–359 bp (Figure 1). The comaA-244f/659r primer set (Pjevac et al., 2017), designed to target between clades A and B of comammox (Table 2), amplify a 415 bp fragment at amoA primer binding regions between positions 244–261 and 643–659 bp (Figure 1). The standard curves for these assays were linear over a minimum of five orders of magnitude and had high coefficients of determination ($R^2 > 0.99$); although linear range, amplification efficiency, LOD and LOQ varied depending on the comammox amoA standard used (Table 3 and Supplementary Table S1). The results for the total comammox (Fowler et al., 2017) and clade-level comammox (Pjevac et al., 2017) assays are also summarized in decision matrices that were used to consistently evaluate qPCR results between assays (Supplementary Tables S3–S5).

In the total comammox assay, melting curves for the standards were narrow and unimodal above ~10$^1 – 10^2$ copies (Supplementary Figures S1A–C). However, melting curves from the environmental samples (Supplementary Figures S1D–H) and the agarose gel of qPCR products (Supplementary Figure S2) showed non-specific amplifications resulting in three false positive detections (amplification did not correspond

---

1http://madsalbertsen.github.io/mmgencode/
In the clade A comammox assay, all standards with approximately $10^2 \text{ amoA}$ copies or less presented two melting peaks, the expected target peak at approximately 88.9, 86.0, and 88.2°C for Ca. N. nitrosa, Ca. N. inopinata, and Ca. N. nitrificans amoA, respectively, plus a sizeable off-target peak at approximately 70°C (Supplementary Figures S3A–C). Higher copy numbers produced narrow, unimodal melting peaks at the expected $T_m$. The agarose gel performed with the qPCR products showed a single band for the standards; however, eight of the nineteen environmental samples contained multiple non-specific amplified products including a potential target amplicon (Supplementary Figures S3D–H, S4) leading to overestimated gene abundance (Figure 2B and Supplementary Table S4). Amplification of non-specific products in seven additional samples produced artifact-associated fluorescence that was well over the LOQ and translated into false positive predictions of the same order of magnitude as true positive detections (Figure 2B).

Clade B standards with approximately $10^2 \text{ amoA}$ copies or less also contained two melting peaks, an off-target peak at approximately 72°C and the expected target peak at approximately 89°C (Supplementary Figure S5A). In the clade B assay, fifteen samples had a target melting peak at the expected $T_m$ (Supplementary Figures S5B–F), however, the agarose gel revealed that these samples had abundant non-specific products that were approximately 300 bp long—smaller than the expected 415 bp target size (Supplementary Figure S6). Only one sample (P_CF-4) appeared to contain an amplicon of the expected size (Supplementary Figure S6), but also contained several other non-specific amplicons. Thus, none of the samples tested were confirmed to be a true positive for presence of clade B comammox (Supplementary Table S5).

The biggest challenge with the non-specific amplification observed with the total comammox and clade-level comammox primers was the significant artifact-associated fluorescence resulting in $C_q$ values within the same range as samples with abundant comammox (see Supplementary Tables S3–S5), which did not allow us to eliminate samples based on the LOD or LOQ. Two factors that could have contributed to the non-specific amplifications observed with the broad-detection primers are the low design annealing temperatures (48°C for the total comammox primers and 52°C for the clade A and clade B comammox primers) and the total DNA concentration we used in each assay. Therefore, we performed gradient PCR experiments to evaluate whether adjustments to the primer annealing temperature or sample DNA concentration would improve the specificity of the total comammox primers (Fowler et al., 2017) and the clade A and clade B comammox primer sets (Pjevac et al., 2017). In our specific application, the gradient PCR experiments show that small adjustments to annealing temperature or sample dilutions did not make a meaningful difference in the non-specific amplifications. Non-specific products remained in the samples tested at higher annealing temperatures and at all three DNA concentrations tested (40 ng/reaction, 20 ng/reaction, and 1 ng/reaction) with all three published primer sets. In the gradient PCR with the total comammox primers, non-specific products are observed at approximately 300 and 600 bp in the P_SBR-4 sample and at approximately 150, 350, 600, and 700 bp in the NS-4 sample, and do not appear to be dependent on the total DNA concentrations tested in the reaction (Supplementary Figure S7). In the gradient PCR for the clade A comammox primers, non-specific products are observed at approximately 150 and 300 bp in the P_CF-4 sample and at approximately 250 and 300 bp in the NS-4 sample, and the amplification of these non-specific products do not appear to be dependent on the total DNA concentrations tested in the reaction (Supplementary Figure S8). In the gradient PCR for the clade B comammox primers (Supplementary Figure S9), multiple non-specific products were observed, with the most prominent non-specific product consistent with previous observations at approximately 300 bp.

Another challenge we faced when applying the broad-detection comammox primers to our samples was the inability to rely on melting curve analysis alone to differentiate between true positive detections, false positive detections, and overestimated detections. To successfully complete melting curve analysis post qPCR, the melting characteristics of an amplified standard (characterized by the both the shape of the melting peak and the temperature where the peak occurs or $T_m$) is compared to the melting peaks that appear in the environmental samples since the $T_m$ considered a unique feature of a particular DNA fragment (Ririe et al., 1997; Wittwer and Kusakawa, 2004). However, this type of analysis can be difficult with primer sets that target a broad
Beach and Noguera Species-Level qPCR Primers Targeting Comammox

**FIGURE 2** Abundance of amoA genes (gene copies/μg DNA) on a log-scale from comammox Nitrospira using the (A) total comammox primers, (B) clade A primers, (C) clade B primers, (D) the newly designed Ca. N. nitrosa specific primers, (E) Ca. N. inopinata specific primers and (F) Ca. N. nitrificans specific primers from various time series samples originating from five bioreactors. LS, lab-scale sequencing batch reactor; P_SBR, pilot-scale sequencing batch reactor; P_CF, continuous flow pilot plant; TRA, Trinity River WWTP; NS, Nine Springs WWTP. Error bars show the standard deviation of the triplicate samples. The color of each column indicates whether there was a true positive detection, an overestimated detection, or a false positive detection. The median limit of detection and quantification for each assay are represented by horizontal red and blue dotted lines, respectively. The shaded regions surrounding these limits represent the upper and lower bounds of the 95% confidence interval.
number of sequences, especially when the primers are designed to amplify a region with highly variable GC content. In this study, we found that the total comammox primers (Fowler et al., 2017) amplify a region of Ca. N. nitrosa, Ca. N. inopinata, and Ca. N. nitroficans amoA with GC content at 54, 49, and 57%, respectively. This produced melting peaks for Ca. N. nitrosa, Ca. N. inopinata, and Ca. N. nitroficans amoA at a $T_m$ of 86.1°C, 84.4°C, and 87.3°C, respectively. We also found that the clade A comammox primers (Pjevac et al., 2017) amplify a region of Ca. N. nitrosa, Ca. N. inopinata, and Ca. N. nitroficans amoA with GC content at 58, 50, and 56%, respectively. This, in turn, produced melting peaks for Ca. N. nitrosa, Ca. N. inopinata, and Ca. N. nitroficans amoA at a $T_m$ range of 86.9, 86.0, and 88.2°C, respectively. While it is possible to determine a $T_m$ range based on *in silico* analysis of the known comammox diversity, this does not allow us to eliminate non-specific products from the analysis since some may contain similar melting characteristics as the target. For example, the melting peaks for P_CF-4 with the total comammox primers (Supplementary Figure S1F) fall within the $T_m$ range set by the standards used in this study, but only contained a non-specific product between 400 and 500 bp long when verified with agarose gel electrophoresis (Supplementary Figure S2). Since the $T_m$ is also affected by the reaction conditions, one would need to include a much greater number of standards *in situ* to represent a greater variety of known comammox species in order to truly rely on melting curve analysis alone with these broad-detection assays. Since more species are continually discovered, relying on melting curve analysis alone to confirm positive detections will likely be difficult without an array of comammox amoA standards and will require confirmation with an agarose gel electrophoresis of amplified qPCR products.

Overall, the presence, frequency, and influence of unspecific amplification in the total comammox (Fowler et al., 2017) assay and in the clade A and clade B (Pjevac et al., 2017) comammox assays were significant and did not allow for accurate detection and quantification of comammox Nitrospira in the specific environmental samples tested in this study (Figures 2A–C), prompting us to design other options for specific comammox detection.

**Design of Species-Specific Primer Sets**

Since the broad range comammox primers did not provide satisfactory results with our environmental samples, we opted to focus our evaluations on the presence of specific comammox species. With this objective in mind, to detect and quantify comammox belonging to the *Candidatus Nitrospira* species currently described in the literature (Ca. N. nitrosa, Ca. N. inopinata, and Ca. N. nitroficans) we designed qPCR primer sets specifically targeting each of these species. For this design, we use a dataset of 27 full-length amoA gene sequences from AOB, AOA, and comammox, and 15 full-length pmoA gene sequences from methanotrophs. We limited the database to full-length amoA and pmoA sequences in order to allow for discovery of primer-binding regions outside of the fragments amplified by conventional amoA primer sets (Rothhauwe et al., 1997; Meinhardt et al., 2015). We used the Design Primers option in DECIPHER (Wright et al., 2014) with each one of the candidate species as the target group and other amoA/pmoA sequences entered as closely related groups that should not be amplified.

In addition, for all designs, the annealing temperature and PCR conditions were fixed (see Materials and Methods) so that all three species-level primer sets could be simultaneously used in a single thermocycler run. For these species-specific primers, we take advantage of a higher annealing temperature ($T_a = 64°C$) than what was used for the total comammox ($T_a = 48°C$) and clade-level comammox ($T_a = 52°C$) primers, which is a strategy that also helps improve primer specificity.

For Ca. N. nitrosa, the two amoA copies identified in the Ca. N. nitrosa genome (van Kessel et al., 2015) were used as the target group. The design algorithm identified primer binding regions at positions 469–493 and 794–812, predicting amplification of a 344 bp fragment (Figure 1). The target regions for each primer had 5 or greater mismatches to all other sequences in the design database, predicting a 100% specificity to the target group.

A single sequence (Daims et al., 2015) served as the target group for the design of Ca. N. inopinata primers, resulting in primer binding sites at 410–436 and 798–815, for a 406 bp fragment amplification. The target region for each primer had 5 or greater mismatches to other amoA and pmoA sequences used as non-targets in the design database, also predicting 100% specificity.

For Ca. N. nitroficans, two sequences were used as targets; the one in the Ca. N. nitroficans genome (van Kessel et al., 2015) and the one from *Nitrospira* sp. Ga0074138 (Pinto et al., 2016), which had 91.4% sequence identity to the Ca. N. nitroficans sequence. The primers amplified a 374 bp fragment, between positions 463 and 836. The predicted specificity was again 100%, with 5 or greater mismatches per primer to other sequences used in the design database.

Since the sequence dataset used for design was small, we searched for additional amoA sequences that would contain the target regions of the new primer sets. However, this analysis was limited because most of the published amoA sequences contain the target side of the forward primers, but not the site targeted by the reverse primer (Figure 1). In this search, we found 33 nearly full-length sequences that not only clustered with the comammox sequences but were also long enough to contain the target regions of the species-specific primers (Figure 3). This set of nearly full-length amoA sequences included amoA sequences from comammox metagenomes that have been recently described in the literature (Daims et al., 2015; van Kessel et al., 2015; Palomo et al., 2016; Pinto et al., 2016; Camejo et al., 2017; Pjevac et al., 2017).

An *in silico* primer-target analysis (Figure 4) shows that *N. sp. UW-LDO-01* (Camejo et al., 2017), originally described as a strain of Ca. N. nitrosa, has perfect matches to the newly designed primers targeting this species. Two amoA sequences (Full scale WWTP. Univ. of Veterinary Medicine, Vienna, AUT, GCA_001458615.1; Pilot-Scale MBR, Aalborg West, DNK Nitrospira bin 1, CXWJO1000000) clustered together and near the Ca. N. nitrosa cluster (Figure 3). The ANI of their genomes compared to Ca. N. nitrosa is 84.5 and 85%, respectively (Figure 5), lower than the typical cutoff for species definition (ANI > 94%) (Richter and Rossello-Mora, 2009). In agreement,
we predict that their amoA will not be amplified by the nitrosa-
specific primer set due to multiple mismatches with forward and
reverse primers (Figure 4). The amoA sequence of Nitrospira sp.
CG24_B (Palomo et al., 2016) clustered with Ca. N. nitificans
(Figure 3). However, the ANI of these two genomes (86.3%; Figure 5)
is below the typical cutoff for species definition and the in silico analysis predicts that Nitrospira sp. CG24_B amoA will not be amplified with the nitrosificans-specific primers (Figure 4). Three additional amoA sequences clustering with Ca. N. nitificans include those from the draft genomes of Pasty iron sludge GWN N. bin 5, N. bin 9, and N. bin 10 (PREFE10139)’
(Daims et al., 2015) (Figure 3), which contain fewer mismatches (Figure 4) and are predicted to partially amplify (below 56% efficiency) with the CG24_B amoA primer set. The species phylogeny close to Ca. N. nitificans remains unresolved, and therefore, the designed primer set for this species will likely require future refinement.

Sequences from clade B comammox were not included in dataset used for primer design, and therefore, an in silico analysis

**FIGURE 3** | Neighbor-joining consensus tree generated from an alignment of full-length and near full-length amoA and pmcO gene sequences (>600 bp), rooting with archaea amoA. Bootstrap values, shown at the nodes where the value was greater than 50, are based on 10,000 trials. Bold sequence names were included in the new primer design. Blue, red, and green text are amplified by the Ca. N. nitrificans, Ca. N. nitrosa, and Ca. N. inopinata primer sets, respectively. Asterisks indicate that the gene is amplified but with a reduced efficiency due to base pair mismatches. The scale bar indicates the number of nucleotide substitutions per amino acid.
was also performed with these sequences (Figure 4 shows N. sp. CG24_A amoA as an example of clade B comammox). In all cases, the sequences had greater than 4 mismatches per primer and are predicted to not amplify with any of the new species-specific primer sets.

The standard curves for all three primer sets were linear (correlation coefficient $R^2 \geq 0.996$) over 7 orders of magnitude (Supplementary Table S1). The amplification efficiency for Ca. N. nitrosa, Ca. N. inopinata, and Ca. N. nitrificans amoA was 96 ± 3.0%, 90 ± 3.0%, and 92 ± 2.4%, respectively (Table 3). Melting curve analysis of all standards showed amplification of the target product without primer dimer artifacts, represented by a strong fluorescence signal producing a single melting peak at approximately 88.8°C for Ca. N. nitrosa (Supplementary Figures S10A, S11A), 85.6°C for Ca. N. inopinata (Supplementary Figures S12A, S13A), and 86.7°C for Ca. N. nitrificans (Supplementary Figures S14A, S15A).

Environmental Detection of Candidate Comammox Species

Using the new species-specific primer sets developed in this study (Nitrosa amoA-469f/812r, Inopinata amoA-410f/815r, and Nitricans amoA463f/836r), we evaluated the samples originating from the low DO BNR bioreactors (Table 1). With the new primers, positive detections obtained with melting curve analysis (Supplementary Figures S10–S15) correlated well with a positive target amplicon in the agarose gel (Supplementary Figures S16–S18). Additionally, cross-hybridization and primer dimers were not observed following PCR with agarose gel electrophoresis with the standard templates (Supplementary Figure S19). The results from the new species-specific primers are also summarized in decision matrices that were used to consistently evaluate qPCR results between assays (Supplementary Tables S6–S8).

Comammox amoA belonging to Ca. N. nitrosa were detected in all environmental samples. Agarose gel electrophoresis of the qPCR amplified products validated these results, since a single amplicon with the expected length (344-bp) was obtained from all 19 samples (Supplementary Figure S17). Ca. N. nitrosa abundance was greater than $10^3$ copies amoA/ng DNA in nine samples originating from low DO bioreactors L_SBR, P_SBR, P_CF , and TRA (Figure 2D). The maximum number of amoA copies was obtained from L_SBR-1, with approximately $2.5 \times 10^4$ copies amoA/ng DNA (Figure 2D). After 45 cycles, six samples (L_SBR-4, P_CF-4, NS-1, NS-2, NS-3, and NS-4) were estimated

![FIGURE 4](image-url)
to have fewer copies of amoA per ng DNA than the calculated LOD \((M = 11, \ 95\% \ CI \ [9.3, 13] \ \text{copies/ng DNA})\) (Figure 2D and Table 3). However, these positive qPCR detections were confirmed via agarose gel electrophoresis (Supplementary Figures S16A, S17), suggesting that the Ca. N. nitrosa primers are highly sensitive (Supplementary Table S6). The melting curve analysis at 45 cycles (Supplementary Figure S10) and 30 cycles (Supplementary Figure S11) also confirmed the positive qPCR detections.

Compared to the quantification of total comammox (Fowler et al., 2017), Ca. N. nitrosa comprised an average of 340 ± 70%, 96 ± 29%, 67 ± 13%, and 93% of the total comammox population in the true positive detections obtained from L_SBR, P_SBR, P_CF, and TRA, respectively (Figures 2A,D). Although in some cases the lower quantification with the new species-specific primers could indicate the presence of other non-targeted comammox species, we also see that the total comammox primers contain 4 total mismatches to the primer-target region of Ca. N. nitrosa, since mismatches were allowed in the original total comammox primer design (Supplementary Table S2). In addition, we observed reduced total comammox (Fowler et al., 2017) efficiency when Ca. N. nitrosa amoA was present at less than 10^3 copies (Supplementary Table S1). Thus, total comammox (Fowler et al., 2017) primer mismatches to Ca. N. nitrosa amoA may contribute to an overall reduced amplification efficiency and underestimation of this species in environmental samples that contain an abundant comammox population, like L_SBR and P_SBR. All in all, the new species-specific primers show that Ca. N. nitrosa is an important member of the comammox population in wastewater treatment plants and may
that comammox are not relevant to conventional wastewater treatment (Chao et al., 2016; Gonzalez-Martinez et al., 2016). However, since comammox were discovered in low oxygen environments (Daims et al., 2015; van Kessel et al., 2015), they may be able to efficiently utilize oxygen and may be important to low DO BNR, as recently described by Camejo et al. (2017).

Long-term low DO total nitrogen removal has been successfully demonstrated in laboratory (Camejo et al., 2016) and pilot-scale (Camejo et al., 2016; Keene et al., 2017) bioreactors seeded with Nine Springs WWTP activated sludge and operated with DO concentrations less than 0.60 mg O$_2$/L. Samples analyzed from the Nine Springs WWTP showed presence of Ca. N. nitrosa, but below the LOQ. The sludge from this WWTP was the seed for the L_SBR, P_SBR, and P_CF reactors, all of which showed presence of Ca. N. nitrosa, suggesting that the low DO conditions favored accumulation of Ca. N. nitrosa as a potential participant in ammonia oxidation in low DO BNR. Interestingly, Ca. N. nitrosa was also abundant in the full-scale TRA system, which is operated with low DO conditions (Table 1), providing independent support for the hypothesis that Ca. N. nitrosa is an important contributor to ammonia oxidation in WWTP operated with low DO. Ca. N. nitrificans was detected above the LOQ in the L_SBR reactor only, whereas Ca. N. inopinata was not detected above the LOD in any of the low DO reactors; therefore, out of the three candidate species, Ca. N. nitrosa appears to be the only one that becomes enriched under low DO conditions.

When compared to quantification of the total bacterial population by 16S rRNA-targeted qPCR, the greatest relative abundance of Ca. N. nitrosa was 10% and 6% of total bacteria in samples originating from the laboratory-scale and pilot-scale sequencing batch reactors, respectively (Supplementary Figure S20). Although these two reactors were seeded with the same sludge, they were fed from completely different sources (synthetic media for L_SBR and full-scale primary effluent for P_SBR), but operated with low oxygen and a long solids retention time (SRT, both 80 days) (Camejo et al., 2016). The continuous flow reactors (P_CF and TRA) had lower relative abundances of Ca. N. nitrosa, and although operated with low DO, they had a much shorter SRT (both 10 days; Table 1). Thus, in addition to oxygen, SRT may be a factor that contributes to comammox abundance in low DO reactors.

Detection and quantification of microorganisms via real-time PCR relies on designing primers with good specificity to the targeted organisms, good coverage of the targeted group, and good quality of the experimental results. With any newly discovered target group, the quality of primer sets to achieve accurate quantification depends on the quality of the databases used for design and the design considerations. As more sequences of the targeted organisms become available, designs can only improve. The first primer design for comammox quantification described sets for targeting clade A and B amoA with the Nitrospira genus (Pjevac et al., 2017), and subsequently another set of primers was published, aiming for greater coverage to target all comammox amoA (Fowler et al., 2017). Our evaluation of
these primer sets in environmental samples from low DO BNR plants showed challenges with primer dimer formation when target sequences were not abundant (clade A and clade B primers, Pjevac et al., 2017), unspecific amplifications in environmental samples (all sets), and underestimation with some strains due to primer-target mismatches (total comammox, Fowler et al., 2017). These challenges are more important when designing for broad target groups. In general, melting curve analyses of qPCR results are helpful for detection of nonspecific PCR products since different fragments will typically appear as distinct melting peaks (Ririe et al., 1997; Wittwer and Kusakawa, 2004). However, the broad detection and quantification of comammox amoA has high GC content variability among the comammox organisms described thus far (Table 3). Therefore, a wide range of amplicon melting temperatures are expected when using the total comammox (Fowler et al., 2017) or clade-level (Pjevac et al., 2017) assays, making melting curve analyses difficult to interpret. We found that verifying amplified products with the total comammox (Fowler et al., 2017) or clade-level (Pjevac et al., 2017) primers cannot be completed with melting curve analysis alone and will require a subsequent agarose gel electrophoresis of our environmental samples, which is not ideal for routine qPCR applications.

Setting the challenge of broad comammox quantification aside, we aimed at designing primer sets specific to the three candidate species described thus far. Evidently, there is a very small number of sequences representative of these candidate species; therefore, the designed primers inherently have 100% coverage. Specificity depends on finding sufficient differences between target and non-targets sequences (Wright et al., 2014), and the results showed this was possible for the three candidate species targeted. As more sequences become available from metagenome assemblies, the specificity of these primers can be re-evaluated. The beginning of such activity was performed by design, it eliminated the problems of unspecific amplifications that compromised the use of broad comammox primers and resulted in strong experimental evidence in support of Ca. N. nitrosa being a comammox organism in energy-efficient BNR systems operated under low DO conditions.

DATA AVAILABILITY STATEMENT

All datasets analyzed in this study are included in the manuscript and the Supplementary Files.

AUTHOR CONTRIBUTIONS

NB and DN developed the research plan and project goals. NB designed the primers and performed the laboratory work. Both NB and DN drafted the manuscript, tables, and figures. DN contributed sources of project funding. All authors have critically read, corrected, and approved the final version of the manuscript and agreed with the opinions expressed here.

FUNDING

This work was partially supported by funding from the National Science Foundation (CBET-1435661) and the Madison Metropolitan Sewerage District (Madison, WI, United States).

ACKNOWLEDGMENTS

This manuscript was previously submitted to the bioRxiv preprint server for biology (Keene-Beach and Noguera, 2018). We thank Colin Fitzgerald (Jacobs Engineering Group) and Leon Downing (Black & Veatch) for providing collaboration and coordination with the Trinity River Authority (Arlington, TX, United States). We thank Pamela Camejo for providing training for primer design and cloning. We also thank Rahim Ansari, Jonnathan Garcia-Huerta, and Tara Hawes for support with cloning, DNA extraction, and PCR. Finally, we would like to recognize Jackie Bastyr-Cooper for her assistance in the lab with training, protocols and equipment.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00036/full#supplementary-material

REFERENCES

Armbruster, D. A., and Pry, T. (2008). Limit of blank, limit of detection and limit of quantitation. Clin. Biochem. Rev. 29(Suppl. 1), S49–S52.

Bartelme, R. P., McLellan, S. L., and Newton, R. J. (2017). Freshwater recirculating aquaculture system operations drive biofilter bacterial community shifts around a stable nitrifying consortium of ammonia-oxidizing archaea and comammox nitrospira. Front. Microbiol. 8:101. doi: 10.3389/fmicb.2017.01010
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Beach and Noguera. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.