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Complete List of Authors:	Metch, Jacob; Virginia Polytechnic Institute and State University, Via Department of Civil and Environmental Engineering Wang, Hong; Tongji University, College of Environmental Science and Engineering Ma, Yanjun; University of Illinois System Miller, Jennifer H.; Virginia Tech Vikesland, Peter; Virginia Polytechnic Institute and State University, Civil and Environmental Engineering Bott, Charles; Hampton Roads Sanitation District, HRSD Higgins, Matthew; Bucknell University, Murthy, Sudhir; DCWater Pruden, A.; Virginia Tech, Civil & Environmental Engineering

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Jacob W. Metch[a], Hong Wang[b], Yanjun Ma[c], Jennifer H. Miller[d], Peter J. Vikesland[d], Charles Bott[e], Matthew Higgins[f], Sudhir Murthy[g], Amy Pruden[d]

Water Impact Statement:

An improved understanding of nitrifying microbial communities in wastewater treatment is imperative for proper design and operation of biological nutrient removal systems. Here we investigated nitrifying microbial community shifts in a starvation activated sludge reactor at various temperatures. *Nitrospira* was the only nitrifying genus detected consistently, and phylogenetic analysis showed close relation of the most abundant *Nitrospira* to comammox and mixotrophic organisms.

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Jacob W. Metch[a], Hong Wang[b], Yanjun Ma[c], Jennifer H. Miller[d], Peter J. Vikesland[d], Charles Bott[e], Matthew Higgins[f], Sudhir Murthy[g], Amy Pruden[d]

[a] American Water, Technology and Innovation, 213 Carriage Lane, Delran, NJ 08075, USA

[b] School of Environmental Science and Engineering, Tongji University, Shanghai 200092, China

[c] Department of Civil and Environmental Engineering, University of Illinois, Urbana, IL 61801, USA

[d] Via Department of Civil and Environmental Engineering, Virginia Tech, 418 Durham Hall, Blacksburg, VA 24061, USA

[e] Hampton Roads Sanitation District, 1436 Air Rail Ave., Virginia Beach, VA 23455, USA

[f] Department of Civil and Environmental Engineering, Bucknell University, Lewisburg, PA, 17837, USA

[g] DC WATER, District of Columbia, USA

Abstract:

Although nitrification is widely applied for nitrogen removal in wastewater treatment plants (WWTPs), more information about the microorganisms involved and their corresponding capabilities and limitations is critical to refine kinetic parameters and process design to optimize advanced nutrient removal. Here we carried out a series of ammonia starvation stress experiments and applied a suite of assays to characterize the microbial community response. Illumina sequencing was applied to both DNA and RNA-derived (i.e., cDNA) 16S rRNA amplicons to differentiate responses of functionally-active bacteria, with gene markers corresponding to known nitrifiers and compared to those targeted via quantitative polymerase chain reaction (qPCR). As expected, total bacterial DNA (i.e., 16S rRNA genes) and nitrifier activity potential decayed over the course of the 18-day starvation period. *Nitrospira* was the only known nitrifying genera consistently detected via 16S rRNA amplicon sequencing in all samples. Despite relatively deep DNA sequencing (rarified to 38,000 sequences per sample), *Nitrosomonas* was the only other known nitrifying genera detected (4 of 84 samples), although *Nitrobacter* was detected via qPCR. Relative abundance of *Nitrospira* DNA and cDNA remained relatively constant throughout the starvation experiments and did not vary with temperature. Two *Nitrospira* OTUs were by far most dominant and were most closely related to known *Nitrospira* capable of mixotrophy and comammox. Thus, observed persistence of *Nitrospira* through starvation may be a function of diverse metabolic capability. Recognition that bacteria of diverse metabolic capability can drive nitrogen removal is critical to advance accurate modeling and design of advanced nutrient removal processes.

Keywords: Nitrification, comammox, *Nitrospira*, nitrifying activated sludge, starvation, stress

Introduction:

Variation from Canonical Model of Nitrification

Nitrification is a critical process for nitrogen removal in wastewater treatment plants (WWTPs). Although widely applied, surprisingly little is known about the actual microorganisms involved in nitrification. The canonical assumption has historically been that nitrification is exclusively driven by a two-step process in which ammonia is oxidized to nitrite by ammonia oxidizing bacteria (AOB) and subsequently nitrite is oxidized to nitrate by nitrite oxidizing bacteria (NOB). However, challenges to this paradigm have recently emerged.[1] For example, anaerobic ammonia oxidizing (anammox) bacteria use nitrite as an electron acceptor and ammonia as an electron donor. Anammox bacteria were first discovered in 1995[2,3] and now are already being implemented for the first time in full-scale WWTPs.[4] Nitrifiers also are not exclusive to the bacterial domain, as illustrated by the discovery of ammonia oxidizing archaea (AOA), which are now recognized to sometimes be the dominant nitrifiers in activated sludge.[5] More recently, the comammox process has been described, in which a single microorganism belonging to the genus *Nitrospira* completely oxidizes ammonia to nitrate.[6-9] A recent metagenomic survey of 16 full scale biological nutrient removal WWTPs found comammox bacteria in all systems.[10]

While our understanding of the bacteria involved in the nitrogen cycle is rapidly evolving, it remains unclear the extent to which various processes are the major drivers of

nitrification in real-world, typical nitrifying activated sludge systems or if they are important only under specialized circumstances. Importantly, widely implemented WWTP design parameters, such as growth and decay rate constants, were determined long before these newer discoveries and do not take into account such deviations from the canonical model of nitrification in activated sludge.[11]

Nitrogen removal processes, in particular, tend to require precise manipulation of conditions to achieve desired performance. This is especially true in the case of short-cut denitrification, in which it is desired to stop nitrification at nitrite, which is subsequently directly denitrified. While short-cut denitrification is highly attractive from the standpoint of minimizing aeration and supplemental carbon costs, it is very challenging to implement in practice. In an effort to limit nitrite oxidation, environmental factors such as DO, pH, ammonia and nitrite concentrations must be carefully controlled in order to select for ideal nitrifying microbial communities that exclude NOB.[12-14] However, any deviation from the canonical model of nitrification presents a fundamental obstacle to implementation. In particular, if the dominant organisms are capable of carrying ammonia oxidation all the way through to nitrate, as is in the case of comammox, then it will be impossible to out-select only the bacteria that oxidize nitrite.

Stress Response and Nitrifying Microbial Communities

Increased knowledge of the dominant nitrifiers and their behavior in wastewater treatment will inherently inform and benefit process design and optimization. To this end, stress tests can provide a useful tool not only for assessing the resilience of a microbial community in the face of reactor upset, but also for informing strategies for selecting a microbial community composition that is capable of performing the desired

process. Nitrification is known to be very sensitive to several types of stress, including variations in salinity,[15] temperature,[16] DO,[17] and substrate concentration.[18] Reactor upsets due to influent variation and environmental conditions are a common and often expensive nuisance for WWTP management.[19] Nitrifying bacteria cope with stress using various mechanisms. For example, prior studies of responses to low substrate concentration in pure and mixed culture lab scale reactors identified increased intracellular ammonia concentrations,[20] dormant or resting states,[21,22] and elevating ribosomal content.[23] In response to light or acetylene exposure to inactivate ammonia monooxygenase (*amo*), the enzyme responsible for ammonia oxidation, it was found that *Nitrosomonas europaea* maintained multiple copies of *amo* with varying ammonia activity allowing recovery in various substrate concentrations.[24] Variations in stress response mechanisms can lead to selective advantages for certain nitrifying microorganisms over others and thus influence key process parameters, such as activity and decay rates. Indeed, varying operational parameters have been shown to impact nitrifying communities, with *Nitrosomonas* and *Nitrobacter* thriving in an alternating aerobic/anaerobic reactor and *Nitrosospira* and *Nitrospira* dominating in strictly aerobic reactors.[12] Also, some *Nitrospira* and *Nitrobacter* species are able to oxidize formate as an alternative to nitrite,[25-28] making them more resilient to nitrite limitation. With improved understanding of the impacts of stresses to nitrifying microbial communities, it may be possible to minimize nitrification reactor upsets and improve recovery after reactor upset or dormancy by selecting for nitrifying microorganisms best suited to handle stresses in a given wastewater.

Recently, we sought to refine estimates of AOB and NOB decay parameters through modeling the decline in nitrate production rate (NPR) and NO_x production rate

113 (NO_xPR) during ammonia starvation of nitrifying activated sludge.[11] While apparent
114 AOB decay rates were similar to those reported in the literature, NOB decay rates were
115 much slower. This is one potential reason why out-selecting NOBs for processes such as
116 short-cut denitrification has been found to be challenging to implement in domestic
117 wastewater treatment.[29] Interestingly, we further observed that the decay rates estimated
118 based on disappearance of gene markers (i.e., DNA) of known nitrifiers was very low
119 compared to the observed rates of activity decay. Such work highlights the importance of
120 understanding exactly who the dominant nitrifying microorganisms are in a given WWTP
121 and their various metabolic capabilities and constraints.
122 The purpose of this study was to gain insight into the structure and function of
123 typical nitrifying activated sludge communities through a series of ammonia starvation
124 experiments. Starvation is typically used to assess decay rates of nitrifying bacteria, and
125 therefore is an important stress response to investigate as it impacts key reactor design
126 parameters. We used the Washington D.C. Blue Plains Advanced WWTP, one of the
127 largest in operation in the world, as a source of stable and high-performing nitrifying
128 activated sludge. The sludge was assayed over a period of several months and multiple
129 seasons, with starvation trials conducted over a range of operating temperatures (14-30
130 °C). We applied a combination of nitrifier activity potential assays with molecular
131 profiling to identify shifts in nitrifying microorganisms with time and temperature within
132 the context of this real-world, complex activated sludge community. Illumina sequencing
133 was applied to both DNA and RNA-derived (i.e., cDNA) 16S rRNA amplicons to
134 differentiate responses of functionally-active bacteria, and gene markers corresponding to
135 known nitrifiers were compared to those targeted via qPCR. Improving our understanding

of the dominant nitrifiers active in activated sludge WWTPs; especially in terms of their metabolic capabilities and constraints, response to stress, resilience, and upset recovery patterns, is critical to future optimization of design and operation of advanced nutrient removal processes.

Materials and Methods:

Experimental Set-Up

Mixed liquor suspended solids (MLSS) was collected from nitrification basin effluent at DC Water Blue Plains Advanced WWTP (Figure 1) and immediately transported to the laboratory at Virginia Tech (Blacksburg, VA ~5 hours) with aeration maintained using an aquarium-pump. The MLSS was transferred to a 25-L Starvation Reactor and maintained in a constant temperature room with aeration controlled by a HACH SC100 sensor controller coupled with an LDO™ dissolved oxygen (DO) sensor (HACH, Loveland, CO) to maintain DO between 2.5 and 4 mg/L. pH was manually adjusted within a range of 7.2-7.8 using $NaHCO_3$ throughout the experiment. Samples were collected from the Starvation Reactor for DNA and RNA extraction and NPR/NO_xPR assays. Logistically, technical replicates of Starvation Reactors were not possible so the experimental design instead emphasized biological repeatability with different seed sludges, along with technical duplicates of the NPR/NoxPR assays.

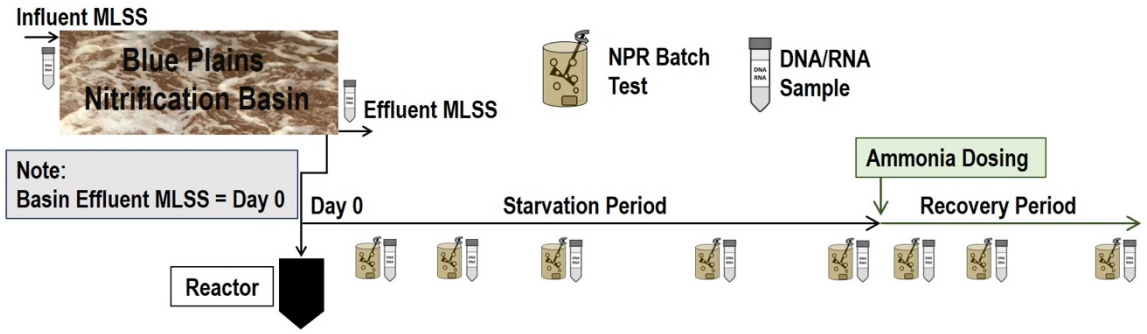


Figure 1 Schematic overview of "Starvation" and "Recovery" Trials. First three Starvation trials (14 ^{0}C Trial 1, 20 ^{0}C Trial 1, and 30 ^{0}C Trial 1) included analysis of WWTP basin effluent MLSS along with samples collected during the starvation period, while Recovery trials (14 ^{0}C Trial 2, 20 ^{0}C Trial 2, and 27 ^{0}C Trial 1) all included WWTP basin influent MLSS, effluent MLSS, and Day 0 sampling, along with samples collected during the Recovery period.

Initial "Starvation" trials were performed at three temperatures typical of seasonal cycles experienced in temperate regions (14 °C Trial 1, 20 °C Trial 1, and 30 °C Trial 1) and were run for 16-18 days. To observe recovery of nitrifiers following starvation experiments, follow-up "Recovery" trials were conducted at similar temperatures (14 °C Trial 2, 20 °C Trial 2, and 27 °C Trial 1) in which ammonium was re-introduced into the Starvation Reactors and maintained between 20-50 mg/L NH_3-N at the conclusion of the Starvation trials. At the start of the starvation trials for these Recovery trials, "Influent" of the DC Water nitrification basin was also sampled (Figure 1). These samples were taken in order to compare the succession of microbial community composition in the laboratory to that experienced during the conditions of the sequential nitrification aeration basins in the field. This comparison of laboratory and field microbial community successions would likely be impacted by the difference in scale of the reactors. The seed sludge was also sampled directly from the basin effluent in the Recovery trials and is denoted as "Day 0" (Figure 1). While these Recovery trials were run at similar temperatures as the

176 former Starvation trials, it should be noted that these are not technical replicates, but are
177 biological replicates as a new seed sludge was used to start the Starvation reactor in every
178 trial. See Table S1 for sludge collection dates and details on sampling frequency for each
179 trial.

180 **Nitrifier Activity Potential Assays**

181 After a ≈36 h acclimation time, 1,300 mL of MLSS was withdrawn from the main
182 Starvation Reactor and transferred to aerated batch reactors to conduct nitrifier activity
183 potential assays. $(NH_4)_2SO_4$ and $NaNO_2$ were dosed simultaneously to the batch reactors,
184 in duplicate, to reach an initial concentration of ≈50 and ≈15 mg-N/L, respectively. DO
185 was maintained at 2 to 4 mg/L during the batch tests by adjusting the air flow to aeration
186 stones using aquarium aerators with controlling valves. pH was also monitored and
187 maintained between 7.2 to 7.9 through addition of $NaHCO_3$, as needed. Aqueous samples
188 for analysis of ammonia, nitrite, and nitrate were collected every 20 min for 2 hours and
189 filtered through 0.45 μm VWR Polyethersulfone membrane filters (Arlington Heights,
190 IL). Nitrate was measured using a Dionex (Sunnyvale, CA) DX-120 ion chromatography
191 system (IC) according to Standard Method 4110.[30] Ammonia and nitrite were measured
192 using the HACH Salicylate Method (TNTplus 832) and HACH Diazotiaztion method
193 (TNTplus 840), respectively. Nitrate concentration was plotted with respect to time and
194 the linear line of best fit was calculated – the slope of which was the NPR. Similarly, the
195 sum of nitrate and nitrite concentrations were plotted with respect to time, and the line of
196 best fit for this plot was the NO_xPR. The assays were repeated over seven time points
197 throughout the Starvation trials. In the Recovery trials, nitrifying microorganism activity
198 was monitored following the Starvation trials, after dosing $(NH_4)_2SO_4$ and $NaNO_2$ into

199 the Starvation Reactor at a concentration of ≈50 and ≈15 mg-N/L, respectively.
200 Ammonia, nitrite and nitrate were measured every 20 min for 2 hours.

201 **DNA and RNA Extraction**

202 MLSS samples for DNA and RNA extraction were preserved with RNAlater®
203 solution (Life Technologies, Grand Island, NY) upon sampling, flash frozen using a dry
204 ice-ethanol bath, and stored at -80 °C prior to further processing. DNA was extracted in
205 duplicate from 175 µL of preserved sludge using a FastDNA® Spin kit for soil (MP
206 Biomedicals, Solon, OH) according to the manufacturer's protocol. RNA was also
207 extracted in duplicate from 175 µL of sample using a MagMAX® Total Nucleic Acid
208 Isolation Kit (Life technologies, Grand Island, NY), DNA was removed using a TURBO
209 DNA-free™ Kit (Life Technologies Corporation, Grand Island, NY) and reverse
210 transcription performed using an iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA)
211 on the same day according to the manufacturer's instructions.

212 **Quantitative Polymerase Chain Reaction**

213 AOB, as represented by the bacterial ammonia monooxygenase gene (*amo*A),
214 along with *Nitrospira*, *Nitrobacter*, and total bacterial 16S rRNA genes were enumerated
215 by qPCR using previously published methods.[31] Reactions were performed in technical
216 triplicate in 10 µL volumes containing 1×SsoFast® Probes or Evagreen® supermix (Bio-
217 Rad, Hercules, CA), 400 nM primers, and 1 µL template. Samples were analyzed at 1:10
218 or 1:50 dilutions to minimize qPCR inhibition based on dilution curves.

16S rRNA Gene Amplicon Sequencing and Analysis

A cross-section of samples was selected from the experimental trials (see Table S1 for sample selection) and subjected to 16S rRNA amplicon sequencing. The V4 region of the 16S rRNA gene from DNA and cDNA samples was subject to PCR amplification using universal bacterial/archaeal barcoded primers 515F/806R[32] following the Earth microbiome protocol (http://www.earthmicrobiome.org). For QA/QC purposes, a DNA extraction blank, cDNA extraction blank, and PCR blank were subjected to sample processing in parallel and included in the sequencing pools. Two sets of pooled samples were sequenced by the Biocomplexity Institute's Genomics Research Laboratory (Blacksburg, Virginia) using a paired-end 250 Illumina MiSeq platform, one pool included initial Starvation trials (14 °C Trial 1, 20 °C Trial 1, and 30 °C Trial 1) and the second included follow-up Recovery trials (14 °C Trial 2, 20 °C Trial 2, and 27 °C Trial 1) as well as blank controls. Paired-end sequence reads were joined using PANDAseq[33] and then processed using Quantitative Insights Into Microbial Ecology (QIIME) version 1.8.0.[34] The *pick_de_novo_otus.py* script was used to perform *de novo* operational taxonomic unit (OTU) picking using the uclust_ref method and taxonomy assignments of OTUs were generated using Greengenes 13_8 reference database, aligned at 97%. The resulting OTU table was filtered to remove chimeric sequences using the Chimera Slayer method,[35] and singletons and organelle sequences using the *filter_taxa_from_otu_table.py* script. This procedure produced an OTU table consisting of 12 million sequences from 84 samples and 3 blank controls. The DNA extraction blank, cDNA extraction blank, and PCR blank had 9,135 sequences, 14,109 sequences, and 24,136 sequences respectively. These sequence counts were much lower than the

samples in the second DNA pool sequences (sample with lowest sequence count of 71,924). Blank control sequences mainly consisted of OTUs identified as *Proteobacteria* (76-94%) which is consistent with normal reagent contamination of extraction kits.[36] The lowest number of sequences in a sample was 38,561 and therefore the OTU table was rarefied to 38,000 sequences per sample for all analyses, unless otherwise stated. Similarity of microbial community compositions was quantified using weighted UniFrac distances computed using the Greengenes reference tree within QIIME.[37] A weighted UniFrac distance matrix was exported to Primer 6 software (Primer-E, Plymouth, UK), where multidimensional scaling (MDS) ordination was conducted. A phylogenetic tree of *Nitrospira* OTUs was created using the maximum likelihood method, bootstrapped 100 times in MEGA7 software[38] and plotted using FigTree software version v1.4.3 (tree.bio.ed.ac.uk/software/). Known *Nitrospira* 16S rRNA sequences were included as reference sequences and the tree was rooted using *Leptospirillum ferrodiazotrophum*, a species within the *Nitrospiraceae* family, but not in the *Nitrospira* genus (see Electronic Supporting Information for sequences used).

Results and Discussion

Shifts in AOB and NOB Activity Potential with Starvation and Recovery

Nitrifier activity potential assays provided a means to measure shifts in microbial community function, in terms of observed ammonia oxidation and nitrite oxidation rates, in response to stress during the Starvation and Recovery trials. Aliquots of MLSS were removed, spiked with ammonium sulfate and sodium nitrite, then NO_3^- and NO_2^- concentrations were measured with time to determine the NO_xPRs and NPRs,

respectively (Figure 2). In this manner, NO_xPR and NPR are gross indicators of AOB and NOB activity potential respectively.

Nitrifier activity potential decayed with time during the Starvation trials, as expected, reaching a stable baseline rate across all temperature conditions after about 10 days. Baseline NO_xPRs ranged from 13-58 mg-N/L-d while NPRs ranged from 64-134 mg-N/L-d by day 16-18. Nitrification rates are known to be temperature sensitive and are generally thought to increase linearly over the range of 14 to 30 °C according to the Arrhenius relationship.[39] However, in the present study, NO_xPRs and NPRs did not trend consistently with temperature under which the Starvation Reactors were incubated (Figure 2). We note that we hypothesized that 30 °C was outside of the linear range expected from the Arrhenius relationship due to the fact that the nitrification rate at 30 °C was less than that at 20 °C. For this reason, we reduced the initial temperature to 27 °C for the Recovery trial.

Response of Nitrifying Microbial Communities to Starvation and Recovery

During the 16-18 day Starvation period, the Starvation Reactor essentially functioned as an aerobic digester. Nitrifiers were intentionally starved by not providing soluble ammonia, although some low level of ammonia resulting from biomass decay theoretically would remain available. It is important to note that soluble organic carbon

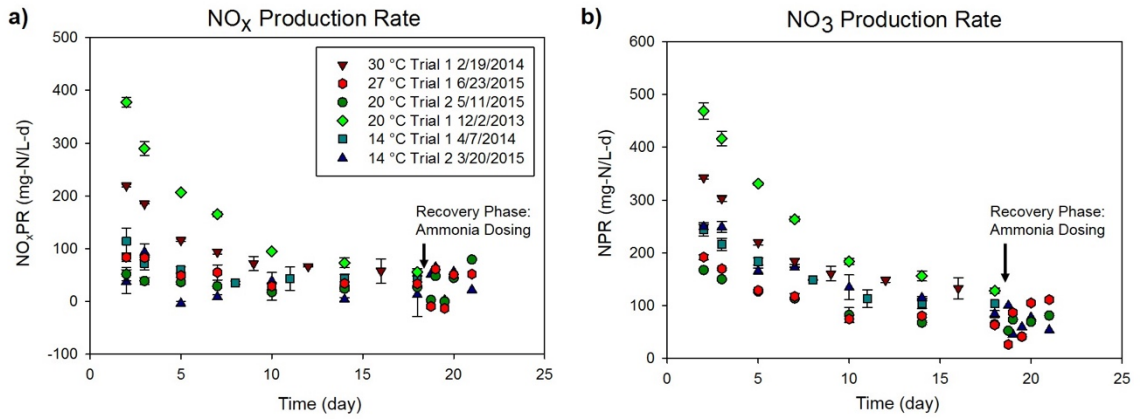


Figure 2 Nitrifying activity potential assays carried out during ammonia Starvation and Recovery trials from 14 ^{0}C – 30 ^{0}C. a) NO_xPR is indicative of AOB activity potential and b) NPR is indicative of NOB activity potential. Error bars indicate standard deviation of duplicate assays, conducted on aliquots of MLSS from Starvation Reactors in batch mode. Replicates assays were not conducted during Recovery trials.

was also deprived and therefore shifts in heterotrophic organisms towards those capable of hydrolysis would be expected. Microorganisms that were not adaptive to these conditions might become inhibited, dormant, or die, thus influencing the observed nitrifier activity potential discussed above. In the case of death, the microorganisms may exhibit variable decay rates, depending on amenability of cell envelope to digestion, and therefore DNA from dead cells may persist at varying rates. Thus, molecular profiling of microbial communities included both DNA- and RNA-derived profiling, with the latter providing a more direct indicator of which microbes were active.

Overall, qPCR of 16S rRNA genes (i.e., DNA) indicated net decay of microbes across Starvation trials, with a total loss of 2.1×10^{8}-5.6×10^{9} gene copies/mL over the 16-18 days (Figure S1). Interestingly, the 14 °C Trial 1 consistently contained the greatest density of 16S rRNA genes, as well as *amo*A and *Nitrospira* gene copies, whereas this trial ranked in the middle in terms of nitrifier activity potential and rate of decline. The 20 °C Trial 1 displayed the sharpest decrease in 16S rRNA gene copies (Figure S1) and

correspondingly yielded the highest initial nitrifier activity potential and also the steepest decline (Figure 2). Figure S2 summarizes the results of a Spearman correlation analysis among NO_xPR and NPR rates and gene copies targeted by qPCR. Ammonia oxidation rates (i.e., NO_xPR) correlated with *amoA* concentrations (p-value 0.00017), but did not correlate with universal 16S rRNA concentrations (p-value 0.056). Nitrite oxidation rates (i.e., NPR) correlated significantly with both *Nitrospira* 16S rRNA gene concentrations (p-value 0.017) and universal 16S rRNA concentrations (p-value 0.0015), but did not correlate with *Nitrobacter* 16S rRNA gene concentrations (p-value 0.27). The tendency for nitrifier activity potential to correlate with gene copy numbers of total bacteria, as well as those corresponding to known AOBs and NOBs suggests that there is a general trend of death of nitrifying organisms through the Starvation trial.

Microbial Community Structure and Activity During Starvation and Recovery

MDS ordination based on the weighted Unifrac distance matrix revealed general trends in how the microbial community compositions shifted in response to Starvation and Recovery (Figure 3). A comprehensive MDS plot (Figure S3) indicated that DNA-derived and RNA-derived microbial community profiles formed two distinct clusters, with two RNA-based profiles appearing as outliers (20 °C Trial 2: 12 hr Recovery and 48 hr Recovery). This observation is expected as DNA-derived and RNA-derived sequencing would be distinct due to differences in starting nucleic acids and in the protocol of sample processing and sequencing. Still, both DNA-derived and RNA-derived microbial community compositions shifted over a similar trajectory during the Starvation trials, with a tandem shift toward the upper right quadrant of the plots (Figure

3, increasing red shading). Importantly, the initial time points of the Starvation experiments were distinct across different trials (Figure S3). Although technical variance could not be assessed based on this study due to logistical constraints on operating technical replicates of Starvation trials, distinct initial microbial community structure across trials is consistent with distinct trends in terms of nitrifier activity potential as a function of temperature (Figure 2). Even slight differences in microbial community composition could influence the rates at which nitrifiers died, went dormant, or switched metabolism during starvation. Differences between DNA- and RNA-derived analyses indicate that a substantial portion of the DNA-based analysis represents either dead or dormant organisms.

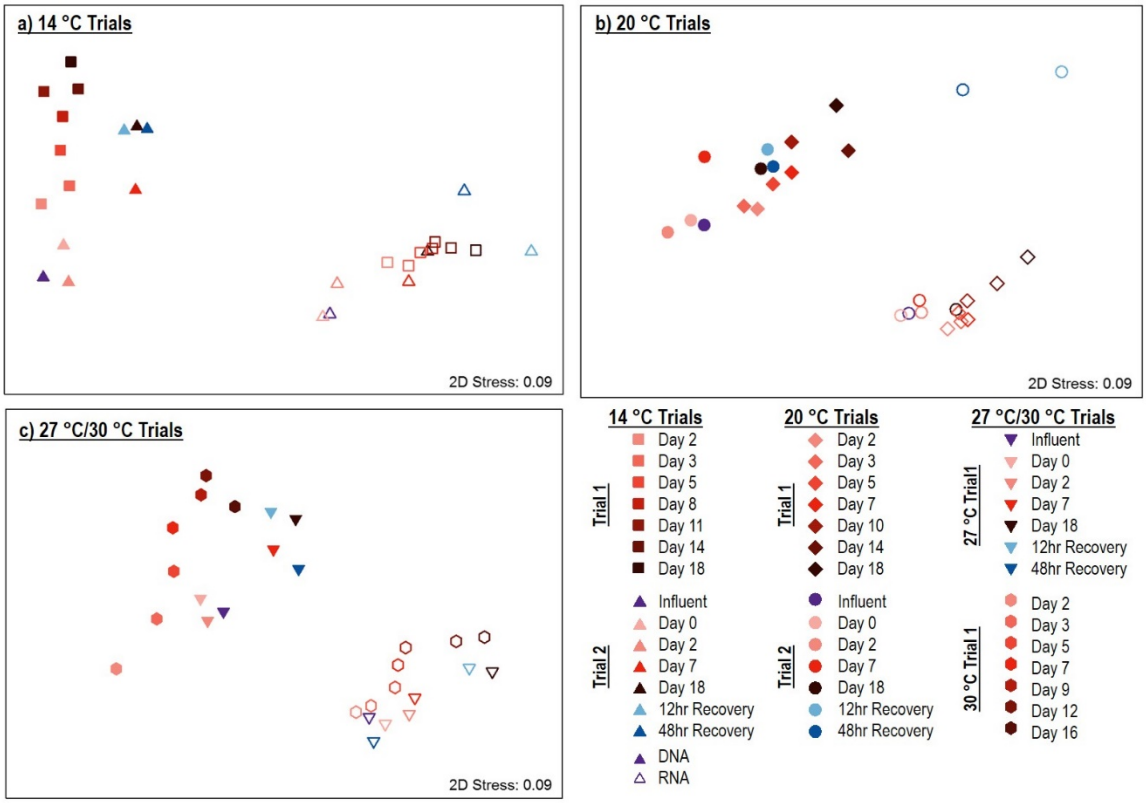


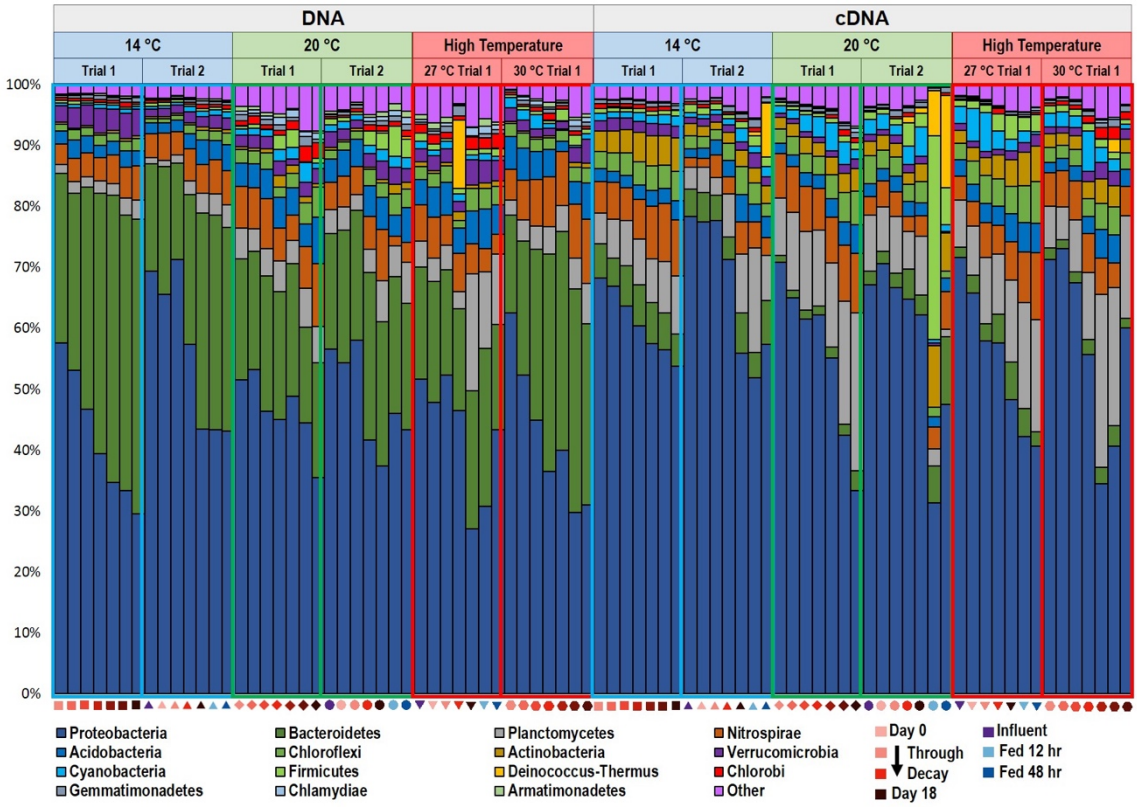
Figure 3 Comparison of microbial community composition (DNA) and activity (RNA) changes with time during Starvation and Recovery trials as a function of temperature based on MDS analysis. MDS ordination of Unifrac distance matrix of OTU table were rarified

to 38,000 sequences per sample. a: 14 °C trials b: 20 °C trials c: 27 °C/ 30 °C trials. Combined plot is available in SI (Figure S3). Intensity of red shading increases with time in Starvation Reactor, purple points indicate MLSS samples collected from the Influent to the WWTP nitrification basin. Blue points represent Recovery samples collected after commencing ammonium feed to the Starvation Reactor.

Comparison of RNA- versus DNA-based community profiling provided a sense of which microbes were stimulated by ammonia addition during the Recovery trials. According to DNA profiling, the microbial community composition remained relatively similar to the Day 18 condition after ammonia dosing, which is not unexpected during a short 48 hour incubation. However, characterization based on RNA indicated much more pronounced, though highly variable, response. This general trend was consistent across temperatures. The contrast between the RNA during recovery and the RNA from the Starvation period of the experiments was most apparent in 14 °C Trial 2 and 20 °C Trial 2 (Figure 2, panels a and b, open blue triangle and open blue circle, respectively). However, in 27 °C Trial 1 experiment (Figure 2, panel c), the RNA shift after commencing ammonia feeding returned to a state that resembled that of the microbial community comprising the "influent" MLSS at the beginning of the WWTP aeration basin (see open upside-down blue triangles).

Examining the relative abundances of the various phyla detected by amplicon sequencing provided insight into potential underlying factors driving the trajectories of microbial community shifts through time and the underlying causes of the bifurcation in DNA and RNA-based analyses shown in Figure 3. The microbial communities characterized by DNA were dominated by *Proteobacteria* and *Bacteroidetes*, as is expected in activated sludge.[40-42] Notably, a trend of distinct relative abundances among various phyla, as estimated by DNA versus RNA-based analysis, was observed across all

366 trials. Specifically, DNA-based measurements consistently indicated increased
367 *Bacteroidetes* and decreased *Proteobacteria* (Figure 4). RNA-based measurements, on
368 the other hand, indicated that *Planctomycetes* and *Cyanobacteria* were greater in relative



369

370 **Figure 4** Relative abundances of the Phyla detected by 16S rRNA gene amplicon
371 sequencing in each trial with time. Symbols along x-axis correspond with those in Figure
372 3, where intensity of red shading indicates increase in time in Starvation trial, purple points
373 indicate samples collected from the "influent" of the DC Water nitrification basin, and blue
374 points represent Recovery samples collected after feeding of ammonium to reactor.
375

376 abundance relative to the DNA dataset, especially at 20 °C and above. Increase in
377 *Bacteroidetes* relative abundance in the DNA dataset during this aerobic experiment was
378 surprising, given that most of these organisms are known to be obligate anaerobes.
379 Importantly; however, relative abundance of *Bacteroidetes* in the RNA dataset was much
380 lower and less variable, suggesting that these organisms were not very active.[43] Thus,

high abundance of *Bacteroidetes* probably relates to their dominance in fecal matter, with perceived "increase" being an artifact of greater resilience to decay relative to other phyla.

In order to identify OTUs driving the observed trends, the variance of the OTU relative abundances within each trial and nucleic acid type (i.e., DNA or RNA) were calculated and ranked (Figure S4). This revealed substantial differences in the ten most variable OTUs based on DNA versus RNA datasets among the trials. For example, in the 14 °C trials, *Bacteroidetes* and *Betaproteobacteria* OTUs were among the most variable OTUs compared to mainly *Deltaproteobacteria* OTUs being the most variable in the RNA dataset. The trends observed in Figure 3 are supported by the 10 most variable OTUs in each trial for DNA and RNA (Figure S4). In general, most of these OTUs belonged to genera with species associated with organic carbon degradation. It has been shown that influent COD composition and carbon source are strong drivers of activated sludge microbial community composition.[44] Therefore, the variation in microbial community composition during the starvation trials may largely be due to the switch from a soluble to a mostly particulate carbon source.

Of particular interest in this study, a *Nitrospira* OTU was among the top ten most variable OTUs in 5 of the 6 trials for the DNA dataset (all but 14 °C Trial 2), and 4 of the 6 trials for the cDNA dataset (all but 20 °C Trial 2 and 27 °C Trial 1). Thus, *Nitrospira*, a well-recognized genus of nitrifiers, were consistently identified as among the most responsive microbes to the ammonia Starvation and Recovery conditions imposed.

Further Identification of Nitrifiers in 16S rRNA Amplicon Sequence Libraries

Following taxonomic assignment of OTUs using the Greengenes reference database, the resulting OTU table was mined to identify known nitrifying microorganisms. Genera known to contain AOBs searched included *Nitrosomonas, Nitrosospira, Nitrosococcus, Nitrosolobus,* and *Nitrosovibrio*.[46] Genera known to contain NOBs searched included *Nitrospira, Nitrobacter, Nitrococcus, Nitrospina, Nitrotoga, Nitrolancea,* and *Nitromaritima*.[1] Since no OTUs were identified as *Thaumarchaeota*, which is thought to be the only phylum containing AOA,[47] it was concluded that archaea did not play a significant role in nitrification in this system. *Nitrosomonas* were detected in only four samples out of the rarefied dataset (84 samples), and were found at low relative abundance (less than 10^{-3}) (Figure 5). *Nitrospira*, however, were detected at high relative abundance in all trials across temperature variations and remained relatively stable throughout starvation experiments, ranging from 1.4% to 11% of the total DNA sequences.

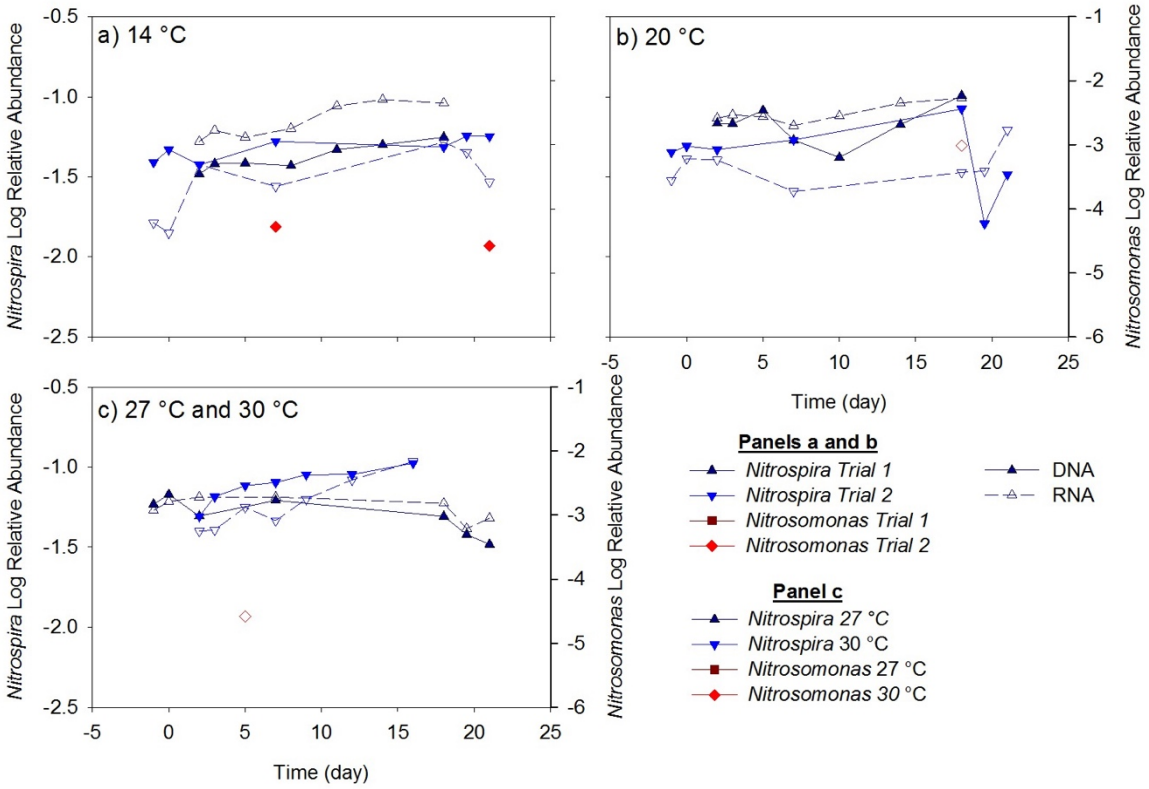


Figure 5 Relative abundance of nitrifying genera during Starvation experiments based on Illumina 16S rRNA gene amplicon sequencing. Relative abundance was calculated based on abundance in rarefied OTU table, normalized to total rarefied sequences (38,000 sequences). Influent WWTP nitrifying aeration basin samples were plotted and labeled as a reference, with ammonium dosing initiated at about day 18.5 of the experiment, as indicated on the plots.

Quantification of AOB and NOB Gene Markers by qPCR

AOB, *Nitrospira*, and *Nitrobacter* were also examined using qPCR to quantify *amo*A as a functional gene marker for known AOB and 16S rRNA gene markers specific to the known NOB, *Nitrospira* and *Nitrobacter* (Figure S1). *Nitrospira* 16S rRNA gene copies were consistently much greater than *amo*A gene copies, averaging 2.0 log higher in absolute abundance (ranging from 0.26-3.9 log). This suggests that canonical AOBs are very low in relative abundance, which would explain absence of all canonical AOB genera, except for four instances of *Nitrosomonas* detection, in the16S rRNA amplicon

sequence libraries. On the other hand, 16S rRNA genes corresponding to *Nitrobacter*, traditionally considered to be an NOB, were consistently detected at sufficiently high relative abundance (ranging from 0-23%) to be detected using 16S rRNA amplicon sequencing, but they were not detected in any of the libraries. This suggests that the "universal" 16S rRNA gene primers used for amplicon sequencing may be biased against *Nitrobacter,* though they matched *Nitrobacter* in NCBI.

***Nitrospira* OTU Analysis**

The *Nitrospira* genus comprised 159 unique OTUs, which is consistent with a prior study that found 120 unique *Nitrospira* OTUs in an activated sludge WWTP.[25] However, two were consistently more abundant (greater than 1 log) than the others (OTU ID# 4460870 (henceforth OTU A) and OTU ID# 1491 (henceforth OTU B)) (data not shown). OTU A was consistently more abundant than OTU B, with a few exceptions (See Figure 6). In the 14 °C trials, OTU A and OTU B trends were similar to each other. However, the RNA-based relative abundance for OTU A decreased after 48 hrs of feeding, while the RNA-based relative abundance for OTU B increased at the same time. Similarly, in the 20 °C trials, both OTUs were stable in relative abundance throughout the starvation period, but after 12 hrs and 48 hrs of feeding, RNA-based relative abundance of OTU A sharply decreased while the RNA-based relative abundance of OTU B increased. In the 27 °C and 30 °C trials *Nitrospira* abundance and activity throughout Starvation seemed to be dominated by OTU A, with the exception of Day 7 of 27 °C Trial 1 in which the relative abundance of OTU A DNA decreased and the relative abundance of OTU B increased to control overall *Nitrospira* trends at that point.

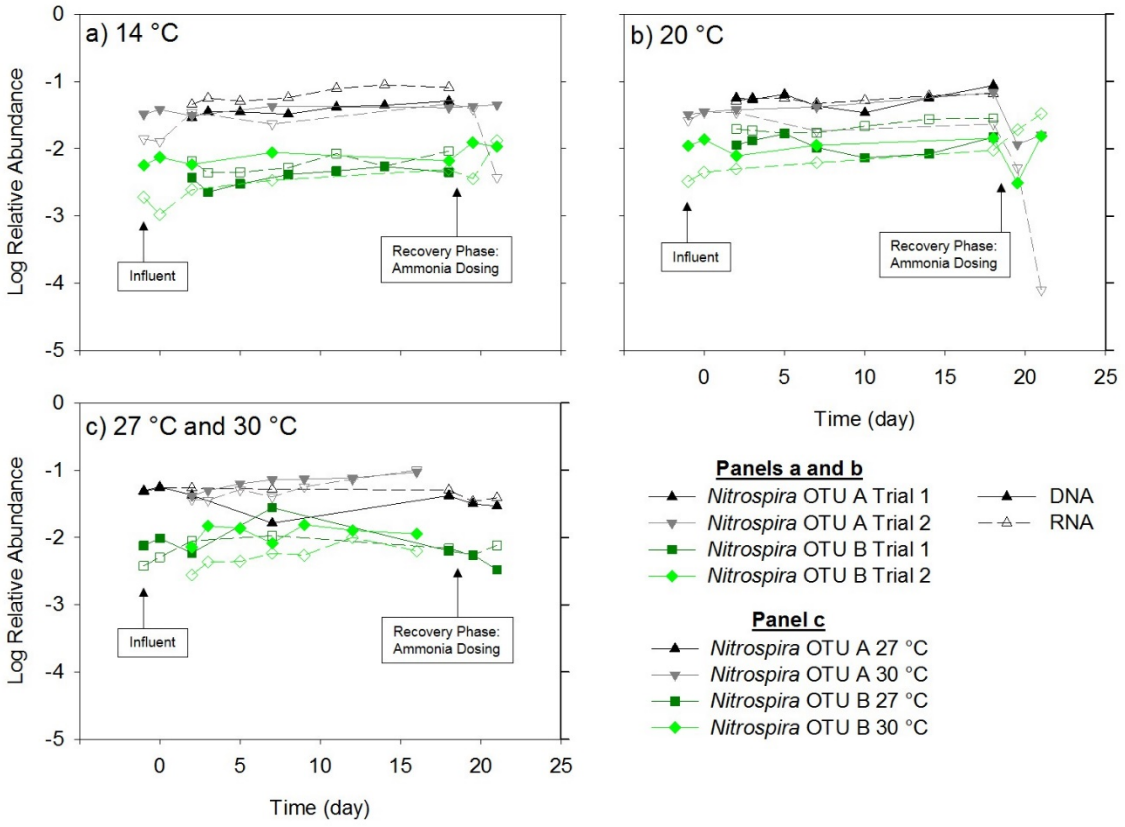


Figure 6 Relative abundance of dominant *Nitrospira* OTUs based on Illumina 16S rRNA amplicon sequencing. Relative abundance was calculated based on abundance in rarefied OTU table, normalized to total rarefied sequences (38,000 sequences). Influent WWTP nitrifying aeration basin samples were plotted and labeled as a reference, with ammonium dosing initiated at about day 18.5 of the experiment, as indicated on the plots.

To better understand the potential roles of OTU A and OTU B, a phylogenetic tree was constructed consisting of the *Nitrospira* species found across the entire dataset relative to reference *Nitrospira* spp. (Figure 7). OTU A and OTU B were found to be closely related to *Nitrospira defluvii* and *Nitrospira nitrosa*, respectively. Indeed, the amplified 16S rRNA sequences from OTU A and OTU B (152 bp) matched the corresponding sequence locations of *N. defluvii* and *N. nitrosa,* respectively (see Electronic Supporting Information).

The discovery of *Nitrospira* as by far the most dominant and persistent known nitrifier in this study provides a logical explanation for the resilience of nitrifying

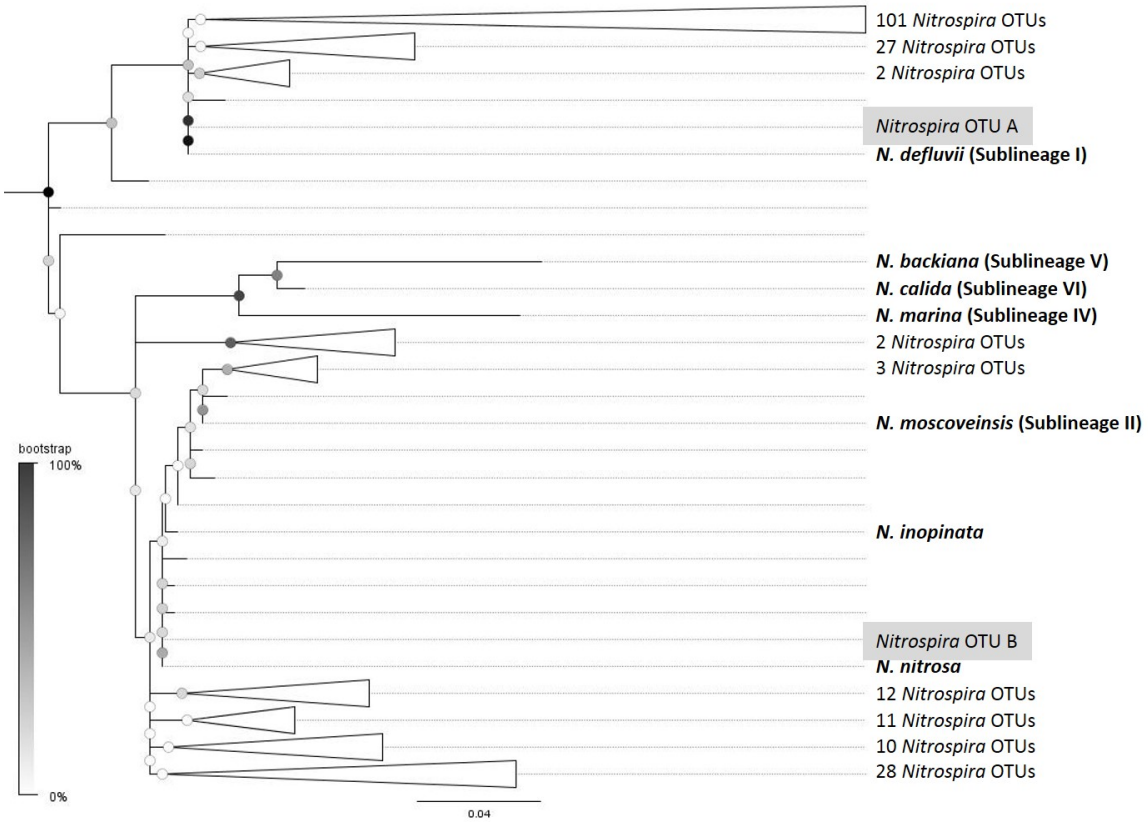


Figure 7 Phylogenetic analysis of *Nitrospira* OTUs based on maximum likelihood analysis of 152bp segment of V4 region of 16S rRNA genes captured by amplicon sequencing. OTU A and OTU B branches are labeled and reference *Nitrospira* spp. are highlighted in bold, unlabeled branches represent *Nitrospira* OTUs other than OTU A and OTU B. 159 total Nitrospira OTUs from this study were included in this analysis, many of which truncated along the same branch of the tree, as indicated. Tree was rooted to *Leptospirillum ferrodiazotrophum*. Reference sequences are available in the Electronic Supporting Information.

activity, which was revivable during recovery as well as the fact that known AOBs were scarcely detectable by amplicon sequencing or qPCR of *amo*A genes. Specifically, the assumed interdependence between AOB and NOB has recently been challenged by the discovery of *Nitrospira* species capable of ammonia oxidation[6,8] and *Nitrospira* spp. capable of ureolytic and heterotrophic activity using oxygen or nitrate as electron

acceptors have also been described.[26] Given such newly discovered metabolic capabilities, it is entirely possible for *Nitrospira* to be the dominant or sole nitrifying genus in an activated sludge system. As it turns out, *N. nitrosa*, with which OTU B most closely matched, is a known comammox bacterium. Also, the *amoA* gene in comammox *Nitrospira* differs phylogenetically from those targeted by qPCR in this study and thus would explain why ammonia oxidation readily occurred in the presence of such low levels of *amo*A gene copies.[8,48] Similarly, Camejo et al. recently found comammox *Nitrospira* derived *amoA* genes were more abundant than AOB derived *amoA* genes in lab scale activated sludge sequencing batch reactors.[49]

N. defluvii, with which OTU A most closely matched, is a member of *Nitrospira* sublineage I and has been found to be capable of heterotrophic metabolism via catabolism and assimilation of acetate, pyruvate, and formate.[27,50] The metabolic versatility of *N. defluvii* provides a good explanation for the stability of OTU A in the absence of addition of traditional substrates (ammonia and nitrite), since it could switch to heterotrophic metabolism.

While *N. nitrosa* is a known comammox organism, possessing genes responsible for both ammonia oxidation and nitrite oxidation,[8,27] unlike *N. defluvii*, it is not known to be a mixotroph. Thus, its survival and maintenance of stable levels through the Starvation phase would likely have to be driven via other means. *N. nitrosa* have been found to possess genes responsible for urea cleavage to ammonia and multiple copies of *amoA* genes demonstrating their diverse metabolic capabilities,[27] possibly lending to their stability in the present study. The relation of OTU B to *N. nitrosa* may also explain the sharp increase of OTU B RNA-derived relative abundance during the Recovery period of

several trials, as the increase in ammonia would likely increase activity and may have given a competitive advantage to the comammox-like organism.

Potential limitations of 16S rRNA amplicon sequencing are important to acknowledge. For example, multiple, diverse AOBs may have been present, each individually at a relative abundance below detection, but collectively sufficient to drive ammonia oxidation. Limitations in the "universal" primers used in this study (515F/806R) to amplify 16S rRNA genes corresponding to AOB are also possible. Such limitations were apparent in the case of *Nitrobacter*, which was detected by qPCR within 1 log of *Nitrospira* (Figure S1), but were not detected in the 16S rRNA amplicon sequencing dataset. Similarly, these primers have been found to have a bias that leads to under-representation of certain phyla, including *Thaumarchaea*,[51] which contains all known AOA.[47] Also, there may be other nitrifying archaea or bacteria present in adequate abundance to be sequenced, but were not identified due to limitations of genomic data in the reference database.[52] Thus, it is important to bear in mind limitations of amplicon sequencing and employ multiple methods for assaying the targets of interest, as was carried out in the present study.

Operational Conditions Shaping the Microbial Community and Role of *Nitrospira*

As microbial community profiling is increasingly being applied to gain insight into the composition of nitrifying activated sludge communities, it is apparent that there is a vast range in composition.[1] As was in the case in this study, a lab-scale nitrifying reactor and a WWTP were similarly found to be dominated by *Nitrosomonas* and *Nitrospira* based on pyrosequencing analysis; in that particular study *Nitrosomonas* represented 15.54% of the DNA sequences in the reactor and 0.05% in the WWTP.[40] It

533 is important to consider how factors in this study, such as WWTP operational conditions
534 and those of the Starvation and Recovery trials, might have shaped the composition of the
535 microbial community. Operational conditions are known to shape nitrifying microbial
536 community composition, for example, with slower growing *Nitrosospira and Nitrospira*
537 found to be more dominant in a strictly aerobic reactor relative to sequential
538 anaerobic/aerobic reactors.[12] *Nitrospira* has been found to be sensitive to changes in
539 temperature as well[17]; however they were observed to be remarkably stable in this study.
540 16S rRNA gene amplicon sequencing of both DNA and RNA as well as qPCR targeting
541 of *Nitrospira* told a consistent story of their persistence at substantial levels (1.4% to 11%
542 of 16S rRNA sequences and 6.3 to 9.3 log genes/mL 16S genes via qPCR) through
543 multiple trials carried out over nearly 2 years of sampling and multiple seasons. They
544 also persisted through Starvation and were capable of regaining activity during the
545 Recovery phase.

546 Various *Nitrospira* OTUs responded distinctly to the introduction of ammonia
547 during the Recovery trials. Both the 14 °C Trial 2 (Figure 6, panel a) and 20 °C Trial 2
548 (Figure 6, panel b), OTU A DNA and OTU B DNA relative abundance responded
549 similarly to introduced ammonia. However, relative abundance of these OTUs derived
550 from RNA analysis had opposing reactions to introduced ammonia (Figure 6). While
551 OTU A was dominant during starvation, after feeding started the relative abundance of
552 OTU A RNA decreased, while OTU B RNA relative abundance increased and became
553 the dominant *Nitrospira* OTU. This effect was not observed at 27°C or 30°C, which
554 suggests different selective pressures between these two OTUs at elevated temperatures
555 and is consistent with the nitrifying potential assays not fitting the trend at these

556 temperatures. Stronger response of OTU B RNA may be explained by the fact that, being
557 more closely related to the comammox bacterium *N. nitrosa*, it may gain an advantage
558 when ammonia is reintroduced to the environment.

559 **Conclusions:**

560 Here we examined shifts in microbial community composition and activity of
561 nitrifying activated sludge representative of a large, stable advanced WWTP during an
562 imposed period of ammonia starvation followed by introduction of ammonia during a
563 recovery phase. Several key observations were made:

564 • Starvation experiments simulated aerobic sludge digestion, correspondingly with
565 net decline in total bacterial populations represented by 16S rRNA genes.
566 • Nitrification activity potential, in terms of NO_x and nitrate production rates,
567 declined as starvation ensued, but temperature was not the sole driver of rates of
568 decline, with sludge collection date also an important factor.
569 • Relative abundances of various phyla shifted distinctly, depending on whether
570 DNA or RNA was profiled by 16S rRNA gene amplicon sequencing, suggesting
571 that some bacteria are killed, with varying rates of decay, while others persist via
572 dormancy or versatile metabolism.
573 • Known AOB were not abundantly detected, either through qPCR of *amo*A genes
574 or 16S rRNA gene amplicon sequencing.
575 • The only known nitrifier consistently detected throughout the Starvation or
576 Recovery trials was *Nitrospira*, traditionally thought of as an NOB, but now
577 known to contain comammox and mixotrophic members. The most dominant
578 OTUs were most similar to known comammox and mixotrophic strains, providing

an explanation for the anomalous activity decay trends with starvation and temperature.

Together these results suggest that major WWTPs employing traditional nitrifying activated sludge nitrification can contain nitrifying bacterial populations that deviate from the canonical model of nitrification. The dominance of likely comammox and mixotrophic nitrifiers is a critical consideration for improving design and operation of WWTPs, particularly in the context of advanced and alternative nutrient removal strategies seeking to out-select nitrite oxidation. Effort is needed to improve the accuracy of kinetic parameters employed for nutrient removal given such deviations from past assumptions and better optimize process parameters, such as aeration, food to microorganism ratio, and wasting rates. Such accuracy will be vital to robust design and implementation of advanced nutrient removal processes. Further, this study can help understand how nitrifying activated sludge communities respond to and recover from upset such as starvation. Ultimately, deeper knowledge of microbial community structure can serve to inform designs that are more efficient as well as more resilient to upset.

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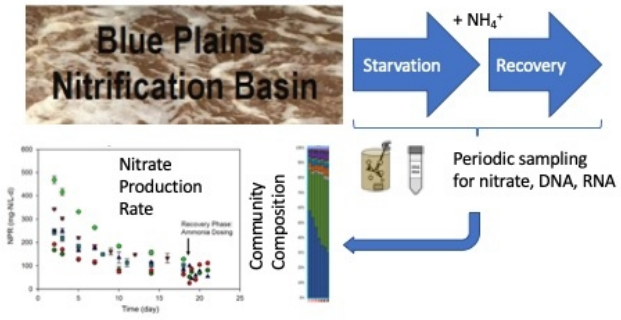

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