Comparison of Partial and Full Nitrification Processes Applied for Treating High-Strength Nitrogen Wastewaters: Microbial Ecology through Nitrous Oxide Production

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Supporting Information

ABSTRACT: The goal of this study was to compare the microbial ecology, gene expression, biokinetics, and N2O emissions from a lab-scale bioreactor operated sequentially in full-nitrification and partial-nitrification modes. Based on sequencing of 16S rRNA and ammonia monoxygenase subunit A (amoA) genes, ammonia oxidizing bacteria (AOB) populations during full- and partial-nitrification modes were distinct from one another. The concentrations of AOB (X_{AOB}) and their respiration rates during full- and partial-nitrification modes were statistically similar, whereas the concentrations of nitrite oxidizing bacteria (X_{NOB}) and their respiration rates declined significantly after the switch from full- to partial-nitrification. The transition from full-nitrification to partial nitrification resulted in a protracted transient spike of nitrous oxide (N2O) and nitric oxide (NO) emissions, which later stabilized. The trends in N2O and NO emissions correlated well with trends in the expression of nirK and norB genes that code for the production of these gases in AOB. Both the transient and stabilized N2O and NO emissions during partial nitrification were statistically higher than those during steady-state full-nitrification. Based on these results, partial nitrification strategies for biological nitrogen removal, although attractive for their reduced operating costs and energy demand, may need to be optimized against the higher carbon foot-print attributed to their N2O emissions.

INTRODUCTION

The increasing regulatory demands to achieve greater nutrient removal from wastewater treatment plant effluents, while minimizing infrastructure investments and operating costs, has resulted in the development of several innovative biological nitrogen removal (BNR) processes. Partial nitrification based processes such as the single reactor system for high ammonium removal over nitrite (SHARON) and its variants are attractive for treating high-strength nitrogen waste streams such as anaerobic digestion reject water or centrate, owing to their reduced consumption of energy (for aeration) and organic carbon (for denitrification). Indeed, separate treatment of centrate via partial nitrification is one of the options for limiting nitrogen discharges to Jamaica Bay in New York City and is part of PlaNYC, a sustainability plan for New York City targeted for 2030.

The energy and carbon savings of partial nitrification processes for nitrogen removal are by virtue of restricting ammonia oxidation to nitrite rather than to nitrate. On the other hand, nitrite is a known trigger for nitrous oxide (N2O) and nitric oxide (NO) production via nitrification pathways and denitrification pathways. Full-scale measurements also point to nitrite as a factor in N2O production. Low (but not zero) dissolved oxygen concentrations were initially implicated as a significant factor for N2O and NO emissions from nitrification. However, the production of N2O by nitrifying bacteria under aerobic conditions has also been shown. Recent reports suggest that N2O and NO emissions by ammonia oxidizing bacteria are related to imbalances in their metabolism and gene-expression patterns.

Given that the greenhouse impact of N2O is about three hundred times that of carbon dioxide and both N2O and NO contribute to ozone layer depletion, it needs to be determined whether N-removal processes based on transient nitrite accumulation are systematically greater contributors of N2O and NO than full nitrification based processes. The mechanisms of such differential N2O production from partial and full-nitrification systems at the microbial level also need to be understood. Therefore, the overarching goal of this study was to compare the microbial ecology, gene expression, biokinetics, and N2O emissions from a lab-scale bioreactor operated sequentially in full-nitrification and partial-nitrification modes. It was hypothesized that operation in partial nitrification mode would result in higher N2O and NO emissions than operation in full nitrification mode. It was additionally hypothesized that the high emissions of the gases would parallel the sustained elevated expression of the genes coding for their production.

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MATERIALS AND METHODS

Reactor Operation and Monitoring. Nitrifying cultures were cultivated in a lab-scale bioreactor (V = 11 L) at a hydraulic retention time (HRT) of 1.1 d and a target pH of 7.5 ± 0.10. The reactor was fed with a nutrient medium containing 500 mg-N/L ammonium and devoid of any organic carbon as described previously.18 Prior to this study, the reactor had been operated to achieve partial nitrification by maintaining the reactor DO concentration in the range 1.5 ± 0.87 mg O2/L and a target SRT of 3.0 days.18 This present study was initiated by changing the DO to 3.8 ± 0.38 mg O2/L and increasing the target SRT to 8.0 days to promote full-nitrification. After operation for 104 days (=13*SRT) in full-nitrification mode the DO concentration and target SRT were reduced back to 1.1 ± 0.38 mg O2/L and 3.0 days, respectively, to promote partial nitrification.

Reactor performance was monitored twice a week by measuring ammonia (gas-sensing electrode, Corning, Corning, NY), nitrite (diazotization-colorimetry),19 and nitrate (ion selectoelectrode, Fisher, Waltham, MA) concentrations. Reactor biomass concentrations were approximated using total chemical oxygen demand (tCOD, Hach Chemical Co., Loveland, CO), since no organic carbon was added in the feed. The tCOD measures are an approximation due to the likely presence of microbially derived soluble COD in the reactor. DO concentrations were measured in real-time using polarographic Clark type electrodes (YSI 5331A, Yellow Springs Instruments, OH), connected to a dual-channel DO meter (YSI 5300) and interfaced to a personal computer. Online data acquisition was performed using virtual instrument codes implemented on LABVIEW, version 8.0 (National Instruments, Austin, TX). Gaseous N2O (gas filter correlation, Teledyne API 320E, San Diego, CA) and NO (Chemiluminescence, CLD 64, Ecophysics, Ann Arbor, MI) concentrations were measured twice a week, each over a two hour period at a frequency of 1 per minute and time-averaged.

DNA Extraction and Quantification. DNA was extracted from biomass samples collected twice a week using a DNeasy Blood & Tissue kit (Qiagen, Inc., Germantown, MD). The concentrations of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) were quantified by real time polymerase chain reaction (qPCR) based on 16S rRNA targeted primer sets (Supporting Information, Table S–I), conducted on an iCycler iQ5 (Bio-Rad Laboratories, CA), as described previously.18 Standard curves for quantifying AOB, Nitrobacter spp., and Nitrospira spp. concentrations were constructed using genomic DNA from Nitrosomonas europaea ATCC 19718 (ATCC, Manassas, VA), genmic DNA from Nitrobit aber winogradskyi ATCC 25391, and a custom synthesized amplicon, from the partial sequence AB117711 (IDT, Coralville, IA), respectively.18 Primer specificity and the absence of primer-dimers were confirmed via melt curve analysis, performed for each and every qPCR assay conducted (data not shown).

Cloning, Sequencing, and Phylogenetic Analysis. The overall community composition during full and partial nitrification was elucidated via 16S rRNA clone libraries using primers 11f and 1492r, employed as previously described.18 Amplicons were cloned (TOPO TA Cloning Kit for Sequencing,Invitrogen, Carlsbad, CA), and 20 colonies were randomly picked for each sample for sequencing (Macrogen USA, Rockville, MD). For these libraries, partially and fully nitrifying biomass samples were obtained on days 103 and 226, respectively. Phylogenetic trees were constructed using the Neighbor-Joining method with a bootstrap of 1000 replications with Clustal X, using MegAlign software (DNASTAR Inc., Madison, WI) and Jukes-Cantor computational modeling.20 The obtained 16S rRNA gene sequences have been deposited in GenBank under accession numbers (HQ228582-HQ228581).

DGGE, Sequencing, and Phylogenetic Analysis. The impact of reactor operating mode on the diversity of AOB was inferred more frequently using denaturing gradient gel electrophoresis (DGGE) and sequencing, targeting ammonia monooxygenase subunit A (amoA), as described previously21 (Primers described in Table S–I in the Supporting Information). DGGE was conducted at 60 °C in 1 × TAE buffer at 75 V for 13 h on a Decode system (Bio-Rad Laboratories, CA) on a 8% polyacrylamide gel with 30–60% (M/V) gradient of urea-formamide denaturant. Gels were stained with ethidium bromide and visualized under UV transillumination. Specific gel bands were excised with a sterilized scalpel. Upon confirmation of the excisions as single bands via a secondary DGGE run, the bands were reamplified, purified with QIAEX II (Qiagen, CA), and sequenced (ABI3730XL DNA analyzer, Applied Biosystems, CA). Phylogenetic trees were constructed as described for the 16S rRNA clone library. The obtained amoA partial gene sequences have been deposited in GenBank under accession numbers (HQ228576-HQ228581).

RNA Extraction and Quantification. Total RNA was isolated from biomass samples stored at ~80 °C with 1 mL of TRizol solution (Invitrogen, Carlsbad, CA). Transcription of three functional genes coding for ammonia oxidation (amoA), nitrite reduction (nirK), and NO reduction (norB) was quantified by reverse-transcriptase qPCR (q-RT-PCR, Supporting Information, Table S–I). DNA removal and reverse transcription from total RNA was performed using the Quantitect Reverse Transcriptase kit (Qiagen, Valencia, CA). q-RT-PCR was performed in triplicate on an iCycler iQ5 (Bio-Rad Laboratories, Hercules, CA). The q-RT-PCR assays used for quantifying transcript abundance of amoA and nirK were as described previously.18 The assays for quantifying transcript abundance of norB were as follows: 5 min at 94 °C, and 50 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min. Primer specificity and the absence of primer-dimers were confirmed via melt curve analysis, performed for each and every q-RT-PCR assay conducted (data not shown).

Determination of Biokinetic Parameters. Biokinetics of ammonia and nitrite oxidation were estimated via extant respirometry.23 Extant batch respirometric assays were performed under oxygen saturation (35–40 mg-O2/L) to characterize maximum potential ammonia and nitrite specific oxidation rates. Respirometric assays were initiated by a sequential spike of ammonia (5 mg-N/L) followed by nitrite (4 mg–N/L). A spike of sodium acetate (100 mg COD/L) was also performed on a monthly basis, to check for and reject (data not shown) the possibility of substantial heterotrophic activity affecting oxygen utilization in the reactor biomass. Biokinetics were expressed as the maximum specific oxygen uptake rate (sOUR), obtained by linear regression of the obtained respirometers and normalizing to total biomass concentrations (approximated in combination with reactor soluble organic matter as tCOD).

RESULTS AND DISCUSSION

Reactor Performance. Near complete oxidation of ammonia to nitrate (full nitrification) was first achieved on day 54 of this study (Figure 1), whereupon gas-phase and molecular monitoring
of the reactor commenced. During steady state full-nitrification ($t = 54-103$ days), ammonia was predominantly oxidized to nitrate ($98 \pm 6.6\%, n = 15$) and not to nitrite ($0.15 \pm 0.18\%, n = 15$, Figure 1). After switching operating modes on day 104, close to 75% partial nitrification was achieved by day 108. During steady state partial nitrification ($t = 108-376$ days), ammonia conversion ($91 \pm 5.8\%, n = 71$) was primarily to nitrate ($9.4 \pm 5.8\%, n = 71$, Figure 1). Calculated free ammonia (FA, at pH 7.5 and $T = 21^\circ C$) concentrations during partial nitrification were higher ($0.49 \pm 0.35$ mg-N/L, $n = 71$, $t = 108-183$ days) than values inhibitory to NOB ($\leq 0.1$ mg-N/L). Therefore FA inhibition of NOB potentially contributed to nitrite accumulation during partial nitrification. FA concentrations in this range were not expected to inhibit AOB, as documented previously.18

Biokinetics of AOB and NOB during Full and Partial Nitrification. During steady-state full-nitrification ($t = 54-103$ days), the biokinetics of ammonia to nitrite oxidation ($sOUR_{n\rightarrow h}$, $71 \pm 12$ mg O$_2$/tCOD/h, $n = 14$) were significantly higher at the 95% confidence level ($\alpha = 0.05$) than the biokinetics of nitrite to nitrate oxidation ($sOUR_{n\rightarrow 2}$, $18 \pm 2.5$ mg O$_2$/tCOD/h, $n = 14$, Figure 2), pointing to nitrite oxidation limited full-nitrification. After the transition to partial nitrification, $sOUR_{n\rightarrow h}$ remained statistically similar ($74 \pm 28$, mg O$_2$/tCOD/h, $n = 61$). However, $sOUR_{n\rightarrow 2}$ decreased drastically ($1.5 \pm 4.5$, $n = 61$, Figure 2). The low $sOUR_{n\rightarrow 2}$ during partial nitrification was potentially due to the effects of selective FA mediated inhibition and washout of NOB, as similarly observed and discussed elsewhere. 

Population Dynamics of AOB and NOB during Full and Partial Nitrification. Based on 16S rRNA gene sequencing, the AOB found in the reactor during full- and partial-nitrification clustered separately (Supporting Information, Figure S-1). Most AOB during full nitrification were related to *Nitrosomonas europaea*, whereas most AOB during partial nitrification operating were closely related to *Nitroomonas eutropha* (Figure S-1). Similarly, based on *amoA* targeted DGGE and sequencing, the AOB from the full- and partial-nitrification modes were distinct (Figure 3). As the bioreactor transitioned from full- to partial-nitrification, a reduction in AOB diversity was also observed, as indicated by fewer bands in the DGGE profiles (Figure 3). The dominant *amoA* gene sequences obtained during full-nitrification (indicated by cluster of bands 1, 2, 4, and 6) and partial nitrification (indicated by cluster of bands 3 and 5) were both distantly related to *N. eutropha* Nm57 (Supporting Information, Figure 1.

Figure 1. Bioreactor performance during full-nitrification (shaded) and partial-nitrification (unshaded) operating modes.

Figure 2. $sOUR_{nh}$ and $sOUR_{n\rightarrow 2}$ associated with ammonia (shaded) and nitrite oxidation (unshaded) during full-nitrification operating modes. Error bars represent the standard deviation of duplicate measurements.

Figure 3. Population dynamics of AOB and NOB during full and partial nitrification.
tion mode prior to this study were metabolically adapted to the lower and N. europaea Rhodocyclales spp. 29 and appears to be a mechanism to increase activated sludge have also been documented before, for instance with the transition to partial nitri-

communities that allowed some AOB to be sustained well after existed some degree of functional redundancy in the AOB amoA rRNA and DGGE (Figures S-1 and S-2). Notwithstanding the overall changes in AOB ecology captured consistently by both the 16S rRNA and amoA biomarkers, some specific differences between them are to be expected given that these biomarker genes likely evolved at different rates.25

From an ecological perspective, N. eutropha related AOB reportedly have a high half-saturation coefficient for ammonia26 and can thus be enriched in environments with high ammonia concentrations such as the partial nitrification reactor herein (35 ± 25 mg-N/L, n = 71). On the other hand, N. europaea related AOB appear to be metabolically versatile and are distributed over a wider range of ammonia concentrations ranging from activated sludge reactors27 to highly N-loaded reactors.18,21 However, the consistent lack of detection of Nitrosospira spp. (Figures S-1 and S-2), which are also dominant in environments with low ammonia concentrations28 as observed during full-nitrification, (2.5 ± 0.76 mg-N/L, n = 15), was unexpected. Possibly the AOB related to N. europaea and N. eutropha spp. enriched during the partial nitrification mode prior to this study29 metabolically adapted to the lower ammonia concentrations during full-nitrification. Such adaptation might have in turn prevented the establishment of Nitrosopira spp. in the reactor during full-nitrification. Notably, after switching from full to partial nitrification on day 104, significant shifts in the AOB community diversity were only detected after day 219 (Figure 3). Potentially, the AOB exhibited metabolic adaptation to partial nitrification conditions (as discussed above) or there existed some degree of functional redundancy in the AOB communities that allowed some AOB to be sustained well after the transition to partial nitrification. Such functional redundancy between different communities of an overall population in activated sludge have also been documented before, for instance with Rhodocyclales spp.29 and appears to be a mechanism to increase community robustness or respond to changing environments (in casu, from full- to partial-nitrification conditions).

During both partial and full-nitrification, NOB related only to Nitrobacter spp. were detected in the reactor and NOB related to Nitrosira spp. were never detected by either 16S rRNA gene sequencing (Figure S-1) or qPCR (data not shown). The lack of detection of Nitrospira spp. related NOB during partial nitrification is consistent with previous studies18,30,31 and was likely due to the propensity of Nitrosopira spp. to preferentially grow at lower nitrite concentrations.32-34 However, the sustained lack of detection of Nitrospira spp. under low nitrite concentrations during full-nitrification (0.75 ± 0.69 mg-N/L, n = 15) relative to partial nitrification (360 ± 42 mg-N/L, n = 71) was unexpected and again possibly because of competition with a well-established culture of Nitrobacter spp. (from the previous partial nitrification period), capable of growing at low nitrite concentrations.

The concentrations of AOB (X_AOB) during full nitrification (1.0 × 10^9 ± 4.2 × 10^8 copies/mL, n = 13) were significantly higher (at α=0.05) than the concentrations of NOB (X_NOB 0.2 × 10^8 ± 1.7 × 10^8 copies/mL, n = 13), as shown in Figure 4. After transition to partial nitrification, X_AOB did not change statistically (1.3 × 10^9 ± 3.1 × 10^8 copies/mL, n = 22). However, X_NOB decreased significantly to 1.3 × 10^8 ± 7.4 × 10^7 copies/mL (n = 22), in keeping with the unfavorable conditions for autotrophic nitrite oxidation.

During full-nitrification (with a target SRT of 8.0 days), the biomass settling in the downstream clarifier was very good with average effluent COD concentrations in the range 112 ± 145 mgCOD/L (avg. ± sd, n = 38). This necessitated biomass wasting in the range 1.0 ± 0.5 L/d (n = 38) from the reactor. In contrast, during partial nitrification, the biomass settling in the downstream clarifier was very poor, and effluent COD concentrations were in the range 445 ± 70 mgCOD/L (avg. ± sd, n = 38). Thus, the biomass wastage required to attain the target SRT of 3.0 days was much lower in the range 0.32 ± 0.33 L/d. Consequently, the AOB biomass concentration in the reactor during partial nitrification was higher than that expected with adequate settling.

**Emissions of N2O and NO during Full and Partial Nitrification.** Emissions of N2O and NO during steady-state full-nitrification (t = 54–103 days) were 0.13 ± 0.24% (n = 12) and 0.010 ± 0.010% (n = 12), respectively, of the influent ammonia loading (Figure 5). Despite the rapid shift in N-conversion after the transition from full-nitrification to partial-nitrification (Figure 1), high N2O and NO emissions were observed for about 80 days after this transition (Figure 5). During this period (t = 105–183 days), emissions of N2O and NO were 1.9 ± 1.1% and 0.18 ± 0.070% (n = 25), respectively, of the influent ammonia loading. After the initial transient period, the N2O and NO emissions stabilized during t = 187–376 days and constituted 0.57 ± 0.17% (n = 33) and 0.070 ± 0.030% (n = 33), respectively, of the influent ammonia loading. The emissions of N2O and NO during this post-transient period within the partial nitrification mode were statistically lower (α=0.05) than the emissions during the transient period (t = 105–183 days). However, in keeping with our initial hypothesis, N2O and NO emissions during both the transient and post-transient periods of partial nitrification were statistically higher than those during steady-state full-nitrification (t = 54–103 days, α=0.05). The higher emissions during the transition phase from full-nitrification to partial nitrification are consistent with previous findings that dynamic conditions (changes in oxygen or ammonium concentrations) especially lead to production of N2O and NO by AOB.35,36 The results herein also show that periods of transition from one nitrification mode to another could result in higher N2O and NO emissions rather than the given modes themselves. The stabilized N2O emissions during partial nitrification were in the lower end of those reported from full-scale partial nitrification facilities, which have ranged from 0.24–1.7% of the total influent nitrogen load.33
From a broader perspective, the statistically higher emissions of $N_2O$ and NO during partial nitrification in this study (even after stabilization) pose an optimization challenge for treatment of high-strength nitrogen wastes such as anaerobic digestion reject water or centrate. These higher emissions might still be acceptable provided the reduction in the net CO$_2$ footprint of partial nitrification owing to reduced aeration power (for nitrification) and organic carbon (for denitrification) equals or exceeds the increased $N_2O$ footprint. Currently, there are only a few studies characterizing $N_2O$ emissions from partial nitrification processes or downstream autotrophic N-removal processes involving anaerobic ammonia oxidation and its variants. Therefore, additional measurements of $N_2O$ and NO emissions from partial nitrification processes in general are still needed.

**Gene Expression.** In response to the lower operating DO concentrations and increased nitrite concentrations characteristic of partial nitrification, there was a sharp transient increase in the expression of the genes coding for autotrophic nitrite reduction to nitric oxide ($nirK$) and nitric oxide reduction to nitrous oxide ($norB$, Figure 6). Indeed, high nitrite concentrations and low DO concentrations are known triggers for $nirK$ and $norB$ expression in AOB and thus, these observations were consistent with our initial hypothesis. In contrast to our hypotheses, however, after the initial transiently high values, $nirK$ and $norB$ mRNA concentrations reduced to nearly nondetect levels (Figure 6), despite sustained high nitrite and low DO concentrations (Figure 1). It is therefore conceivable that the AOB herein might have adapted to the sustained high nitrite and low DO concentrations resulting in parallel stabilization in both emissions and expression of $nirK$ and $norB$. The parallel trends (both increasing and decreasing) in $N_2O$ and NO emissions and expression of $nirK$ and $norB$ support the recently proposed link.
Figure 6. Relative gene transcript concentrations of amoA, nirK, and norB during full-nitrification (shaded) and partial-nitrification (unshaded) operating modes. Error bars represent the standard deviation of triplicate measurements.

between imbalanced gene expression in AOB and emissions of these gases. 14

The stabilization in nirK and norB mRNA concentrations (Figure 6) was more rapid than that in reactor headspace N2O and NO concentrations (Figure 5). A similar lack of strict temporal correspondence between gene transcript, N2O and NO concentrations after an anoxygenic transition has also been reported recently 14 and is likely due to the influence of post-transcriptional and translational process on N2O and NO emissions, rather than just gene transcription. Nonsystematic increases in nirK transcript concentrations were also observed after the transient phase during partial nitrification (Figure 6) and did not correlate with trends in concurrent NO concentrations (Figure 5). The lack of respiratory responses by heterotrophic bacteria strongly underscored mainly autotrophic production of NO and N2O in this study.

In contrast to nirK and norB, transcript concentrations of amoA during full nitrification and partial nitrification were statistically similar (α=0.05). The trend in amoA expression (Figure 6) paralleled the relatively consistent trend in sOURnh during the two operating modes (Figure 2). Given that sOURnh is the ultimate manifestation of amoA transcription and translation, such a parallel is understandable and has been reported previously for N. europaea. 38 Similar parallels between activity and gene expression are not restricted to AOB and also hold for yeast, anammox, and some heterotrophic bacteria.

Based on these results, our initial expectation of stable higher emissions from partial nitrification was not entirely satisfied, likely due to the mitigating effect of microbial adaptation at the metabolic, ecological, and gene expression scales. Nevertheless, partial nitrification reactors still have a higher propensity for N2O emissions, which municipalities and wastewater utilities need to consider during selection and optimization of low-energy process alternatives for N-removal from centrate or reject water streams.

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■ REFERENCES


■ ASSOCIATED CONTENT

Supporting Information. Table S-I and Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.


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