Factors Promoting Emissions of Nitrous Oxide and Nitric Oxide From Denitrifying Sequencing Batch Reactors Operated With Methanol and Ethanol as Electron Donors

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ABSTRACT: The emissions of nitrous oxide (N₂O) and nitric oxide (NO) from biological nitrogen removal (BNR) operations via nitrification and denitrification is gaining increased prominence. While many factors relevant to the operation of denitrifying reactors can influence N₂O and NO emissions from them, the role of different organic carbon sources on these emissions has not been systematically addressed or interpreted. The overall goal of this study was to evaluate the impact of three factors, organic carbon limitation, nitrite concentrations, and dissolved oxygen concentrations on gaseous N₂O and NO emissions from two sequencing batch reactors (SBRs), operated, respectively, with methanol and ethanol as electron donors. During undisturbed ultimate-state operation, emissions of both N₂O and NO from either reactor were minimal and in the range of <0.2% of influent nitrate-N load. Subsequently, the two reactors were challenged with transient organic carbon limitation and nitrite pulses, both of which had little impact on N₂O or NO emissions for either electron donor. In contrast, transient exposure to oxygen led to increased production of N₂O (up to 7.1% of influent nitrate-N load) from ethanol grown cultures, owing to their higher kinetics and potentially lower susceptibility to oxygen inhibition. A similar increase in N₂O production was not observed from methanol grown cultures. These results suggest that for dissolved oxygen, but not for carbon limitation or nitrite exposure, N₂O emission from heterotrophic denitrification reactors can vary as a function of the electron donor used.

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Introduction

Heterotrophic denitrification is the dissimilatory reduction of ionic nitrogen oxides such as nitrate and nitrite, to nitric oxide (NO), nitrous oxide (N₂O) and ultimately to dinitrogen gas (N₂) using organic electron donors (Knowles, 1982). Sequential actions of several enzymes including nitrate reductase (NaR), nitrite reductase (NiR), nitric oxide reductase (NOR), and nitrous oxide reductase (N₂OR) are involved. The greenhouse gas effect of N₂O is approximately 300 times more potent than carbon dioxide (IPCC, 2000). A more recent involvement of N₂O in direct depletion of the ozone layer has also been shown (Ravishankara et al., 2009). NO also contributes to destruction of the ozone layer and to precursors of photochemical smog (Seinfeld and Pandis, 1998). As one of the two main reactions in engineered biological nitrogen removal (BNR) systems, denitrification is implicated as a potential source of global N₂O emissions (Tallec et al., 2008; Thn and Sensson, 1996). Although autotrophic nitrification can itself contribute to N₂O emissions from wastewater treatment plants (Ahn et al., 2009; Kampschreur et al., 2008), the sole focus of this work was to elucidate potential triggers of N₂O emissions from two distinctly operated heterotrophic denitrifying reactors. The mechanisms behind autotrophic N₂O and NO emissions in response to aerobic–anoxic transitions have recently been elucidated (Yu et al., 2010) and are not included herein.

Several factors have been linked to N₂O and NO generation and emission from denitrifying bioreactors including low pH (Focht, 1974), short solids retention time (Hanaki, 1992), organic carbon limitation (Chung and Chung, 2000; Hanaki, 1992), dissolved oxygen inhibition (Park et al., 2000; Tallec et al., 2008), and nitrite inhibition (Schulthess et al., 1995). However, the impact of the specific carbon source on resulting N₂O and NO generation and
emission has received limited attention. From an engineering perspective, with increasing methanol costs, wastewater utilities may adopt alternate external carbon sources, for example, ethanol, to sustain and enhance denitrification. Although ethanol is currently more expensive than methanol, it fosters specific denitrification rates 2–3 times higher than methanol (Mokhayeri et al., 2008). Therefore, it is conceivable that ethanol might be a viable carbon source to sustain adequate denitrification kinetics and performance during low temperatures (Mokhayeri et al., 2008). However, it is imperative to determine ethanol-associated N2O or NO emissions to ensure minimization of both aqueous and gaseous nitrogenous pollution. Such an evaluation is especially important since it has been recently shown that different organic carbon sources foster distinct microorganisms, even in mixed cultures (Baytshtok et al., 2009; Ginige et al., 2004; Osaka et al., 2008). Thus, it could be hypothesized that the resulting differences in microbial community structure and their tolerance or susceptibility to transient stressors could give rise to different emissions on different carbon sources.

Therefore, the overall goal of this study was to systematically evaluate N2O and NO emissions from denitrification using two organic carbon sources, methanol and ethanol in response to three stressors, transient organic carbon limitation, exposure to high nitrite concentration spikes, and a range of inhibitory oxygen concentrations. These lab-scale experiments are part of an ongoing overall multiscale investigation on mechanisms of N2O and NO generation and emission from wastewater treatment plants and lab-scale nitrifying and denitrifying cultures.

Materials and Methods

Bioreactor Operation

Two denitrifying sequencing batch reactors (SBR) (V=9.2 L) were inoculated with activated sludge from a step-feed BNR reactor at 26th Ward Water Pollution Control Facility in New York City and operated with methanol and ethanol, respectively, using nitrate as the terminal electron acceptor as previously described (Baytshtok et al., 2008, 2009). The solids retention time (SRT) for both SBRs was 10 days and the hydraulic retention time (HRT) was 1 day. This conservatively high SRT was chosen to enrich for methanol and ethanol assimilating biomass in the respective SBRs and ensure complete nitrate removal, as described previously (Baytshtok et al., 2008, 2009). Each SBR had a 6-hr cycle comprising 1 h continuously anoxic feed and react, 3.5 h anoxic react, 0.5 h aerobic mixing (to strip out dinitrogen gas and improve settling), 0.75 h settle, and 0.25 h decant phases. SBR phases were automatically controlled via a digital controller (Chrontrol Corp., San Diego, CA). The influent COD and NO3 -N concentrations for both SBRs were 500 mg chemical oxygen demand (COD)/L (methanol or ethanol) and 100 mg NO3 -N/L, respectively. The SBR feed medium contained (per liter): 0.2 g of MgSO4·7H2O, 0.02 g of CaCl2·2H2O, 0.087 g of K2HPO4, 1 mL of trace elements solution (10 mg of Na2MoO4·2H2O, 172 mg of MnCl2·4H2O, 10 mg of ZnSO4·7H2O, 0.4 mg of CoCl2·6H2O in a total volume made up to 100 mL with distilled water). The pH of the SBRs was automatically controlled in the range of 7.3 ± 0.2 using concentrated hydrochloric acid during undisturbed operation, but not during gas measurements, during which, the pH ranged from about 7.3 to 8.1.

Characterization of Ultimate-State and Transient-State Operations

Aqueous and gaseous nitrogen species were measured during individual SBR cycles, corresponding to ultimate-state or transient operations with carbon limitation, nitrite and oxygen inhibition. Each transient condition was imposed at least three times independently upon each of the two SBRs to obtain a measure of biological reproducibility. The transients were specifically imposed as follows:

(1) Carbon limitation: Methanol or ethanol along with nitrate was provided during the first 0.5 h of anoxic feeding phase, followed by 1 h of carbon limitation (but not nitrate limitation) and finally followed by 0.5 h of carbon feeding (without nitrate). In this manner, temporary carbon limitation followed by recovery to non-limiting conditions was imposed. However, the overall carbon and nitrate mass fed during a given SBR cycle during transient limitation and ultimate-state operation were identical.

(2) Nitrite inhibition: Ten milliliters of stock sodium nitrite solution (46 g NO2 -N/L) was spiked into the SBR during the middle of the feeding phase to achieve a peak NO2 -N concentration of 50 mg N/L. Methanol or ethanol and nitrate were fed to the SBR as during ultimate-state operation.

(3) Dissolved oxygen inhibition: Oxygen inhibition in the SBR was achieved by continuously pumping air (0.5 L/min for DO = 2.5 ± 0.5 mg/L; 1 L/min for DO = 5.1 ± 1.2 mg/L) or pure oxygen (0.5 L/min for DO = 9.0 ± 1.1 mg/L) to maintain the desired DO concentration during the entire SBR cycle. Methanol or ethanol and nitrate were fed to the SBR as during ultimate-state operation.

Headspace N2O and NO Measurements

Headspace gas collection was performed in accordance with a newly developed USEPA reviewed protocol for measuring N2O and NO fluxes from open surface wastewater treatment plants (Chandran, 2009). Gas collection was performed using a custom-made plastic flux chamber (volume = 3.5 L), which was sealed to the SBR body. Sweep air was introduced into the chamber at a flow rate of 4 L/min, except during transient oxygen inhibition, where
the sum of the sweep gas flow rate and air (or oxygen) flow rate equalled 4 L/min. Real-time N2O and NO concentrations (ppmv) in the flux chamber were measured via gas-filter correlation (Teledyne API, San Diego, CA) and chemiluminescence (Ecophysics, Ann Arbor, MI), respectively. Nitrite (diazotization), nitrate (ion-selective electrode, Accumet®), pH, ORP, and DO (Yellow Springs Instruments, Yellow Springs, OH) were measured at 30 min intervals. Reactor and effluent biomass COD concentrations were measured based on standard methods (Eaton et al., 2005).

The fraction of influent nitrate emitted as N2O or NO was determined by numerically integrating the real-time profile of N2O or NO emission mass flux (Eq. 1) and normalizing to mass of nitrate fed during a cycle

\[ \frac{M_N}{Q} \frac{M_{WN}}{V_0} t_0 \]

where \( M_N \) is the mass of emitted nitrogen during a cycle as either NO or N2O (mg-N), \( Q \) is the flow rate of sweep air and gas pumped into the flux chamber (4 L/min), \( C \) is the accumulated concentration of N2O or NO during a cycle (ppmv), \( M_{WN} \) is the molecular weight of nitrogen in N2O and NO (14 and 28 g/mol), \( V_0 \) is the molar volume of an ideal gas, 24.05 L/mol at 1 atm and 22°C, and \( t_0 \) is the duration of one cycle (6 h).

**Extant Biokinetics of Denitrification**

Batch experiments were conducted as described previously to determine denitrification kinetics with methanol and ethanol at ultimate state and exposure to three DO concentrations: 2, 5, and 9 mg O2/L (comparable to DO concentrations transiently imposed upon the SBRs) (Baytshtok et al., 2008). Briefly, 500 mL biomass samples were withdrawn from the SBRs toward the end of the react cycle, washed, and resuspended in nitrate and COD free medium and sparged with N2 gas to render them anoxic (DO <0.2 mg/L). Biokinetic assays were conducted by spiking the biomass samples with non-limiting concentrations of nitrate and COD (methanol or ethanol) and tracking the resulting nitrate and nitrite profiles over time. In selected assays, air or pure oxygen was introduced into the batch denitrification vessels, to achieve different DO concentrations. Specific denitrification rates (sDNR) were computed via linear regression of the nitrate depletion profiles versus time and normalizing to total biomass COD concentrations.

**Results and Discussion**

**Ultimate-State Performance and Emissions of N2O and NO**

During ultimate-state operation, near complete nitrate removal was observed in both SBRs (methanol: 92.5 ± 11.6%; ethanol: 98.5 ± 2.5%) with minimal nitrite accumulation (<1 mg N/L). Little N2O (methanol: 0.11 ± 0.02%; ethanol: 0.10 ± 0.01%) or NO (methanol: 0.02 ± 0.01%; ethanol: 0.01 ± 0.00%) was emitted (Fig. 1a and b). In keeping with the sequential production of the two species during denitrification, NO concentrations peaked before N2O concentrations during any given SBR cycle (Fig. 2a(1) and b(1)).

Under ultimate-state operation, factors leading to incomplete denitrification have generally been attributed to N2O production. For instance, in a recent study, complete denitrification resulted in 0.1% of the removed nitrate emitted as N2O. In contrast, the extent of emissions was significantly higher (1.3%) as nitrate removal dropped to 66% (Tallec et al., 2006). These results are consistent with the low ultimate-state N2O and NO emissions from the either of both SBRs, wherein nitrate removals higher than 90% were observed without concomitant nitrite accumulation. The fraction of influent nitrate removed that was emitted as N2O for methanol (0.12%) was comparable with previous results in the range of 0.2–1.3% with methanol (Park et al., 2000). Emissions with ethanol enriched denitrifying bacteria have not been reported previously and thus cannot be directly compared.

**Impact of Transient Carbon Limitation**

Transient carbon limitation resulted in transient nitrate accumulation for both methanol- and ethanol-fed SBRs. Relatively lower nitrate accumulation was observed during ethanol limitation than during methanol limitation (data not shown), which can be explained by higher denitrification biokinetics for ethanol than methanol (Baytshtok et al., 2009). Nitrite accumulation was similar for both COD sources and much lower than nitrate accumulation (data not shown). However, owing to the long react phase and the operating SRT of 10 days, complete nitrate removal was eventually observed by the end of the overall cycle for both reactors. N2O and NO emissions during a cycle were statistically lower than ultimate-state control for the methanol-fed SBR but were largely similar in the ethanol-fed SBR (Fig. 1a and b).

The lack of significant N2O emissions during carbon limitation is in contrast to some previous reports (Chung and Chung, 2000; Itokawa et al., 2001). It has been postulated that the higher electron affinities of two upstream denitrification enzymes, NaR and NiR, relative to downstream NOR and N2OR enzymes could be the reason for N2O accumulation during carbon limitation (Betlach and Tiedje, 1981; Knowles, 1982). While specific enzyme affinities were not directly measured in this study, it is possible that the distinct populations fostered by methanol and ethanol (as described previously by Baytshtok et al., 2009) might possess more uniform and high affinities across the sequential reductive nitrogen cascade, leading to the lack of N2O and NO emissions during carbon limitation.
The possession of high affinities could be due to the high operating SRT of the SBRs for over 2 years, which could have resulted in long-term enzymatic adaptation to low substrate (carbon and nitrate) concentrations. Indeed, minimal N\textsubscript{2}O emissions were observed from acetate-limited denitrifying reactors operated at high SRT values (10 days; Hanaki, 1992). Additionally, adaptation of \textit{Alcaligenes faecalis} cultures to cycling between feast and famine resulting in lower N\textsubscript{2}O production has also been shown (Schalk-Otte et al., 2000). Therefore, these results show that the link between carbon limitation and N\textsubscript{2}O emission may not be universal for all carbon sources and operating conditions and needs to be evaluated more specifically.

**Impact of Nitrite Inhibition**

Exposure to nitrite led to statistically higher nitrate accumulation at the end of the SBR cycle for both carbon sources, indicating feedback inhibition of nitrate reduction by nitrite (data not shown). However, near complete nitrite reduction was still achieved in the ethanol-fed SBR but not in the methanol-fed SBR (76.5 ± 3.2%). The nitrite transient also resulted in slightly elevated secondary peak of NO (Fig. 2a(3) and b(3)) compared to ultimate-state (Fig. 2a(1) and b(1)) for both SBRs. Nevertheless, N\textsubscript{2}O emissions were not impacted and the resulting fractions of nitrate converted to N\textsubscript{2}O and NO were statistically similar (\(a = 0.05\)) to those at ultimate state (Fig. 1a and b).

It has been previously suggested that N\textsubscript{2}O\textsubscript{R} is more sensitive to nitrite inhibition compared to other enzymes in denitrification, thus leading to N\textsubscript{2}O production under nitrite exposure (Knowles, 1982; Miller et al., 1986). Besides the direct impact of nitrite, N\textsubscript{2}O\textsubscript{R} inhibition can also be due to NO, which is formed from nitrite reduction (Goretski et al., 1990). Indeed, accumulation of N\textsubscript{2}O and NO during denitrification in the presence of nitrite was observed with acetate and yeast extract fed denitrifying cultures, with an inhibitory threshold nitrite concentration of approximately 10 mg N/L (Hanaki, 1992; Schulthess et al., 1995). However, at the same nitrite concentration, little N\textsubscript{2}O production was observed from activated sludge with sucrose as the sole carbon source.
carbon source (Thn and Sensson, 1996). Another study using pure cultures of Alcaligenes sp. and Pseudomonas fluorescens grown on nutrient broth as carbon source also reported no impact of nitrite pulses on N₂O accumulation (Betlach and Tiedje, 1981). The differences in N₂O production as a function of nitrite exposure in these different studies could be possibly due to the different carbon sources used or the mode of cultivation used. Therefore, the previous results and this study essentially underscore the lack of generality in the link between nitrite exposure and N₂O production, from denitrification using different carbon sources.

Impact of Oxygen Inhibition

In both methanol- and ethanol-fed SBRs, a rapid initial accumulation of nitrate was observed upon the introduction of air or oxygen (Fig. 3). Higher inhibition of oxygen on nitrate reduction occurred in methanol-fed SBR. In contrast, significant (but delayed) nitrate removal occurred in the ethanol-fed SBR at all DO concentrations. As expected, there was a positive correlation between DO concentration and the extent of nitrate accumulation for both carbon sources. High nitrite accumulation was also observed in both SBRs but was more pronounced in the
ethanol-fed SBR due to ongoing nitrate reduction therein. N₂O emission was significant in the ethanol-fed SBR (Fig. 4b–d) and the highest emissions were at DO = 9.0 mg/L, where as much as 7.1% of influent nitrate load was emitted as N₂O (Fig. 1b). NO emissions were much lower but displayed a similar positive correlation with increasing DO concentrations. In contrast, methylotrophic denitrification did not result in significant N₂O or NO emissions at any DO concentration tested (Fig. 3a).

The relative production of N₂O by the two SBRs could not be entirely described by a reduction in their specific nitrate depletion sDNR values (Fig. 5). Though the sDNR values for the ethanol SBR were consistently higher than those for the methanol SBR, the extent of reduction due to oxygen inhibition was statistically similar (P = 0.79) and not in correspondence with much higher N₂O production from the former (Fig. 4). The inability of nitrate sDNR values to describe the extent of N₂O emissions is expected and can be attributed to inhibition of not just NaR but also the other nitrogen reductases by oxygen.

It is reported that N₂OR is more sensitive to oxygen inhibition than the remaining upstream nitrogen reductase...
enzymes, thus leading to selective N₂O production (Knowles, 1982; Korner and Zumft, 1989). Based on the results of this study, differential N₂O production could also be related to differential NaR inhibition by oxygen. In the methanol-fed SBR, complete cessation of NaR-mediated nitrate reduction occurred at the highest oxygen concentration tested (Fig. 3a(3)). Therefore, the lower level of nitrite, N₂O, or NO production in the methanol-fed SBR was in fact mainly due to less upstream nitrate reduced than in the ethanol-fed SBR (Fig. 2a(4)). It should be pointed out that downstream nitrogen reductases (NOR and N₂OR enzymes) could also have been inhibited in the methanol-fed SBR but could not be discerned due to the lack of accumulation of their substrates. On the other hand, the NaR system in the ethanol-fed SBR was seemingly more robust, as reflected in near-complete albeit delayed nitrate reduction (Fig. 3b). However, such ongoing nitrate reduction under oxygen inhibiting conditions resulted in N₂O production.

It is acknowledged that DO concentrations close to saturation are not common in most activated sludge systems. However, in several modular BNR processes, such as non-optimally operated step-feed BNR or five-stage Bardenpho processes, the effluent end of the primary aerobic zone is typified by high DO concentrations as high as 4–5 mg O₂/L, especially during complete nitrification (Ahn et al., 2009). In such cases, in the absence of a swing-zone to scavenge oxygen concentrations, there could be a carryover of high DO concentrations into the downstream zone of carbon addition.

Given increased evidence for autotrophic N₂O and NO production via nitrification (Yu et al., 2010), it could also be argued that the observed emissions were in fact from nitrifying populations in the two SBRs. However, the absence of ammonia oxidizing bacteria in the biomass of both SBRs was confirmed via endpoint polymerase chain reaction (PCR), conducted as per a variation of Hermansson and Lindgren (2001) (data not shown). Additionally, extant resporimetric assays conducted as per Chandran and Smets (2000) revealed negligible ammonia oxidation related
specific oxygen uptake rates (sOUR). The exogenous ammonia-oxidation-related sOUR values for methanol- and ethanol-fed biomass were $(1.1 \pm 0.2) \times 10^{-4}$ and $(4.8 \pm 0.4) \times 10^{-5}$ mg O$_2$/g COD/h, which were statistically similar to the respective endogenous oxygen uptake rates $(8.7 \pm 1.3) \times 10^{-5}$ (P = 0.18) and $(2.9 \pm 1.8) \times 10^{-5}$ mg O$_2$/g COD/h (P = 0.45). Given that autotrophic N$_2$O generation is linked to the specific nitrification rates (Yu et al., 2010), the negligible presence and activity of nitrifying bacteria in both SBRs precludes the contribution of nitrifier denitrification to observed N$_2$O and NO emissions.

Finally, it should be noted that these results may be unique to the specific microbial communities fostered in the two SBRs fed only with methanol and ethanol, respectively. Further, the emissions from anoxic zones of full-scale BNR processes may not be as pronounced (as indeed shown recently, Ahn et al., 2009) given the presence of a broader non-methanol and ethanol degrading consortium therein. Nevertheless, these qualifying factors only underscore the lack of generality in denitrification-related N$_2$O emissions from activated sludge and commonly investigated triggers thereof, especially when applied to different carbon sources.

Conclusions

In summary, this study emphasizes that N$_2$O and NO emissions from denitrification cannot be generalized for all carbon sources and need to be addressed on a case-specific basis. Based on the differences observed, specific mechanisms and pathways of N$_2$O and NO production on different carbon sources also need to be elucidated. Additionally, dosing of ethanol to anoxic zones in BNR processes might need to be strictly controlled not only to minimize ethanol wastage but also to minimize the generation and emission of N$_2$O in downstream aerobic zones.

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References


