Molecular and biokinetic characterization of methylotrophic denitrification using nitrate and nitrite as terminal electron acceptors

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ABSTRACT

Although methanol is a widely employed carbon source for denitrification, relatively little is known on the abundance and diversity of methylotrophic bacteria in activated sludge. The primary aim of this study was to specifically identify bacteria that metabolized methanol in a sequencing batch denitrifying reactor (SBDR), using a novel technique, stable isotope probing (SIP) of 13C labeled DNA. A secondary aim was to quantitatively track dominant methylotrophic bacteria in the SBDR exposed to different terminal electron acceptors. SIP enabled 13C 16S rDNA clone libraries revealed that SBDR methylotrophic populations were related to *Methyloversatilis* spp. and *Hyphomicrobium* spp. Based on newly developed quantitative polymerase chain reaction (qPCR) assays, *Hyphomicrobium* spp. were more abundant than *Methyloversatilis* spp. throughout the period of SBDR operation. The relative population abundance was stable despite a shift in electron acceptor from nitrate to nitrite (keeping the same methanol dose). However, the shift to nitrite resulted in a significant decrease in denitrification biokinetics on both nitrate and nitrite.

Key words | biokinetics, methylotrophic denitrification, microbial ecology, stable isotope probing

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INTRODUCTION

Denitrification is the dissimilatory biochemical reduction of ionic nitrogen oxides such as nitrate-nitrogen (NO_3^--N) and nitrite-nitrogen (NO_2^--N) to gaseous oxides such as nitric oxide (NO) and nitrous oxide (N_2O), which may be reduced further to dinitrogen (N_2) or under extremely anaerobic conditions to ammonium-nitrogen (NO_4^+-N) (Knowles 1982). Methanol is a widely employed supplemental carbon source for denitrification in biological nitrogen removal (BNR) since it is less expensive than sources such as ethanol or acetate. Despite extensive research and practical implementation of denitrification, we still do not have a complete understanding of 'active' denitrifying microbial fractions in activated sludge fed with different carbon and electron sources. Consequently, full-scale implementation of heterotrophic denitrification is still guided by a somewhat empirical understanding of its microbial ecology and biokinetics.

The three primary application oriented questions related to external COD based denitrification that practitioners are interested in answering are:

- How big should the denitrification zone in an activated sludge reactor be?
- How can the carbon dosage for denitrification be minimized while achieving desired N removal ?
- Are there alternative carbon sources available that are more cost-effective and/or sustainable than those used now (methanol) and are equally or more effective?

However, in order to answer these questions, we need to address these fundamental issues pertaining to denitrification

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- Who degrades which carbon sources and what is their concentration in activated sludge?
- What are the specific rates and stoichiometry of degradation for this 'active' fraction?
- Can different carbon sources be degraded by the same activated sludge organisms?
- Do we need to consider modifying denitrification process models to account for 'active' denitrifying fractions associated with the different substrates?

This work is part of a larger study aimed at elucidating the structural and functional microbial ecology of denitrification on several carbon sources and nitrogenous electron acceptors (nitrate and nitrite) in a sequencing batch denitrification reactor (SBDR). The first part of the study that involves long-term methylotrophic denitrification is described here. Ultimately, this overall work is expected to provide significant information about major functional denitrifying groups and their biokinetics in activated sludge using different electron donors and acceptors. Such information will be extremely useful in minimizing external electron donor addition, while achieving mandated total nitrogen removal goals.

OBJECTIVES

The objectives of this study were to:

- Determine the structural and functional microbial ecology of a methylotrophic sequencing batch denitrification reactor (SBDR) using SIP and 16S ribosomal RNA based phylogenetic interpretation of ^{13}C labeled DNA.
- Quantify the biokinetics, yields and performance of methylotrophic denitrification as a function of terminal electron acceptor (nitrate and nitrite) in the influent.

METHODS

A methylotrophic enrichment consortium was cultivated in a SBDR ($V = 10$ L, hydraulic retention time = 1 d, solids retention time = 10 d) operated at room temperature. Each cycle of the SBDR was six hours long with 1 h anoxic feed and react, 3.5 h anoxic react, 0.5 h aerobic mixing (to strip out any dinitrogen gas and improve settling), 0.75 h settle and 0.25 h decant phases. In phase1 (225 days), the reactor feed contained 100 mg nitrate-N/L and 500 mg methanol COD/L. In phase2 (95 days), the reactor feed contained 166–250 mg nitrite-N/L and 500 mg methanol COD/L.

Microbial ecology of the denitrifying SBDR via SIP

SIP was used to determine the ecological diversity of the SBDR as per (Lueders *et al.* 2004) during Phase 1 of this study.

Stable isotope probing (SIP) was used to determine the ecological diversity of the SBDR as described previously (Lueders *et al.* 2004). SIP experiments were initiated in a separate anoxic batch reactor, spiked with 500 mg COD/L of ^{13}C methanol and 100 mg NO_3^--N/L. Samples for phylogenetic characterization via SIP were obtained just at the point of nitrate depletion ($t = 22.4$ h). Additionally, an unspiked sample was also obtained to characterize 'all' the bacteria in the reactor. Genomic DNA extracted from these samples (DNeasy Blood & Tissue Kit, Qiagen, Valencia, CA) was separated via isopcynic density gradient ultracentrifugation (55,000 RPM, $T = 20$C, 22 h). Sixteen density-gradient fractions per sample were collected and DNA concentration was measured using real-time PCR (MX 3005P, Stratagene, La Jolla, CA). Out of the sixteen DNA fractions (Figure 1), the fraction containing the highest ^{13}C concentration of DNA (from $t = 22.4$ h sample, peak in 1.753 g/mL) and one fraction containing the highest ^{12}C concentration of DNA (from $t = 0$ h sample) were amplified against the eubacterial 16S rRNA primers 11f (Kane *et al.* 1993) and 1492r (Weisburg *et al.* 1991) with 25 Polymerase chain reaction (PCR) cycles. The amplicons

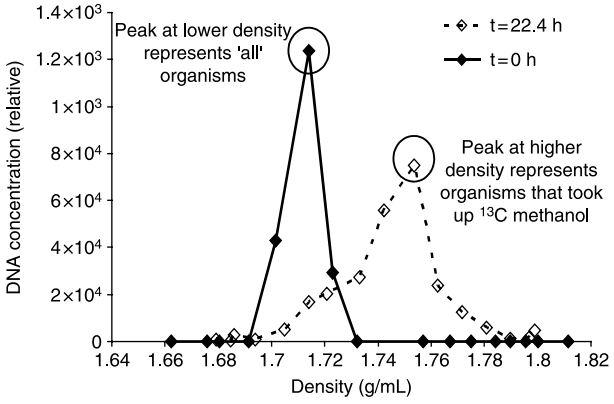

Figure 1 | SIP profiles of methylotrophic biomass samples spiked with ^{12}C methanol (continuous line and shaded symbols) relative to that spiked with ^{13}C methanol (dashed line and open symbols).

were cloned (TOPO TA Cloning® for Sequencing, Invitrogen, Carlsbad, CA) for 16S rRNA gene sequencing (Molecular Cloning Laboratories (South San Francisco, CA) employing the above primers. The sequences were aligned and edited manually to get near complete sequence information. The closest match database sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov). The phylogenetic tree for the clones was developed and bootstrapped with ClustalX (InforMax, Inc., North Bethesda, MD) software using the Neighbour Joining (NJ) method (Saitou & Nei 1987). Positions with gaps were excluded and multiple substitutions were corrected. The tree was then subjected to 1,000 bootstrap trials and the rooted phylogenetic tree was rendered using the TreeView software (http://taxonomy.zoology.gla.ac.uk/rod/rod.html) with *Methanosarcina thermophila* as the outgroup.

Development of quantitative PCR assays for tracking methylotrophic biomass concentrations

Based on the dominance of *Hyphomicrobium* spp. and *Methyloversatilis* spp. related clones (Figure 2), quantitative

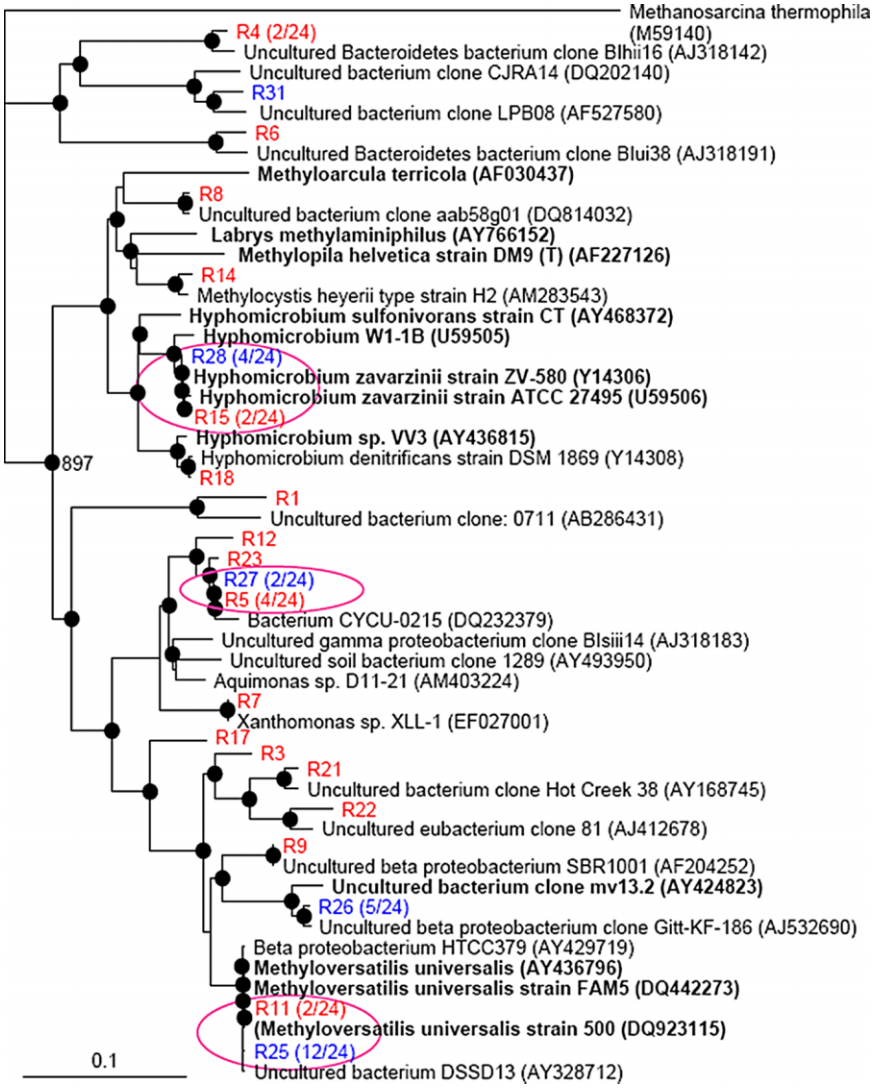

Figure 2 | Phylogenetic tree depicting dominant Overall populations (RED) and Methylotrophic populations (BLUE) of the SBDR. Subscribers to the online version of *Water Science and Technology* can access the colour version of this figure from http://www.iwaponline.com/wst.

polymerase chain reaction (qPCR) primer sets were developed for determining their abundance over different modes of reactor operation. Briefly, primer sets were developed using the PrimerQuest® online software package from Integrated DNA Technologies (Coralville, IA) for *Hyphomicrobium* spp. and *Methyloversatilis* spp.. Additional qPCR related constraints imposed during primer design were, an amplicon size between 80–200 base pairs, primer size of 24 ± 6 nucleotides, optimum primer melting temperature of 60 ± 5°C and optimum primer GC content of 50 ± 15%. Out of the resulting primer sets, the ones with the highest specificity (checked using the BLASTn subroutine, http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) were selected for experimental optimization.

Experimental optimization of qPCR based determination of methylotrophic abundance was performed using monocultures of *Hyphomicrobium zavarzinii* strain ZV580 and *Methyloversatilis universalis 500* (both from ATCC, Manassas, VA). Monocultures of *H. Zavarzinii* ZV 580 and *M. Universalis* 500 were grown at 37°C in nutrient broth to stationary phase. Genomic DNA was extracted from the cultures (DNeasy, Qiagen, Valencia, CA) and serially diluted to prepare the standard curves for qPCR. qPCR optimization was directed at determining the melting temperature at which PCR amplification was most rapid and was conducted on a Bio Rad iQ5 real-time gradient thermal cycler (Bio Rad, Hercules, CA). The newly developed primer sequences and optimal qPCR conditions are given in Table 1.

Performance and biokinetics of the SBDR

SBDR performance was determined via influent and effluent nitrite (diazotization), nitrate (ion-selective electrode, Accumet®) and influent total and effluent soluble COD measurements, all according to standard methods (Eaton *et al.* 2005). Denitrification biokinetics were determined via 'extant' batch assays (similar to (Chandran & Smets 2000)) using nitrite or nitrate as electron acceptors. Initial COD:N ratios were 2.5:1 and 1.5:1 for nitrate and nitrite, respectively, thereby rendering methanol as the limiting nutrient. Additional parallel measurements of ORP and pH were also performed to corroborate the end of the batch assays based on visual inspection of inflexion points in their time series profiles. Specific denitrification rates (sDNR) on nitrate or nitrite were computed via linear regression of the nitrate or nitrite depletion profiles normalized to the biomass concentration (COD).

In select experiments, the maximum specific growth rate (μ_{max}), methanol half saturation constant ($K_{S,MeOH}$) and (if applicable) nitrite self-inhibition constant (K_{I,NO_2^-}) were estimated by non-linear least squares minimization, as described previously (Chandran & Smets 2000). The biomass yield coefficient was experimentally calculated via electron balances from experimental data (Chandran & Smets 2001) and compared with theoretical thermodynamic estimates based on dissipation energy (Heijnen *et al.* 1992).

RESULTS AND DISCUSSION

Microbial ecology of methylotrophic denitrification

In unspiked biomass samples (representative of all bacteria in the reactor), one DNA concentration peak was obtained at a density of 1.714 g/mL. In the biomass sample spiked with ^{13}C methanol and harvested at $t = 22.4$ h, a clearly distinguishable DNA peak at a higher density of 1.753 g/mL was obtained (Figure 1). The phylogenetic tree constructed based on cloned 16S rRNA sequences of the ^{12}C and ^{13}C DNA samples showed that the organisms that took up ^{13}C most rapidly were a small specialized subset of the overall SBDR biomass, consisting of *Hyphomicrobium*

Table 1 | Newly developed qPCR primers and conditions for tracking two dominant methylotrophic populations in the SBDR

Primer pair	Sequence	Target	TM	Primer concentration (nM)
Muf	AAGGCCTACCAAGGCAACGA	*Methyloversatilis* spp.	55°C	200
Mur	ACCGTTTCGTTCCTGCCGAA			
Hzf	ACAATGGGCAGCAACACAGC	*Hyphomicrobium* spp.	57°C	200
Hzr	ATTCACCGCGCCATGCTGAT			

and *Methyloversatilis* spp. (Figure 2). Therefore, a truly novel result of this study is that even in a reactor fed solely with methanol, a fraction of the population did not assimilate ^{13}C methanol rapidly enough during the period of the C spike and was potentially sustained on endogenous bacterial metabolites long-term SBDR operation.

Based on qPCR based quantitative tracking, revealed that *Hyphomicrobium* spp. were in general more abundant than *Methyloversatilis* spp. over the entire course of reactor operation. Further, there were no systematic changes in population abundance of either community when reactor was shifted to nitrite rather than nitrate as the electron acceptor (Figure 3). Another significant finding is that *Hyphomicrobium* spp., which are speculated to be (Layton *et al.* 2000), did in fact constitute the major portion of the organisms that took up ^{13}C methanol in the SBDR enrichment culture.

Performance and biokinetics of the SBDR

Near complete nitrate and nitrite removal was obtained during SBDR operation (data not shown). Changing the electron acceptor from nitrate (Phase 1) to nitrite (Phase 2) in the SBDR reactor resulted in statistically significant lower specific denitrification rates (mg-N/gCOD/hr) in phase 2 for denitrification of both nitrate ($p = 7.22 \times 10^{-7} < 0.05$) and nitrite ($p = 0.002 < 0.05$), respectively (Figure 4). In general, sDNR values of both nitrite and nitrate decreased in phase 2. Possible mechanisms for this reduction (attributed to nitrite toxicity) might be at the gene or enzyme expression levels since we have conclusively determined that the

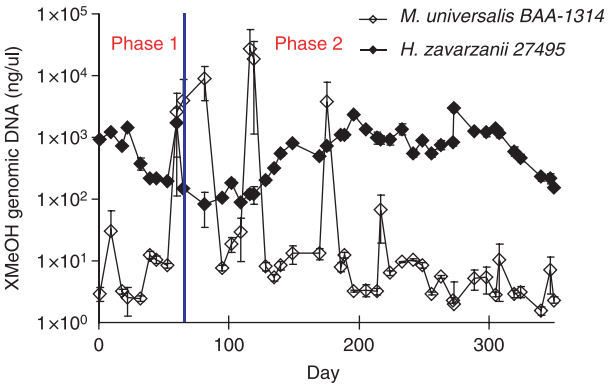

Figure 3 | Relative abundance of *Hyphomicrobium* and *Methyloversatilis* spp. in the SBDR.

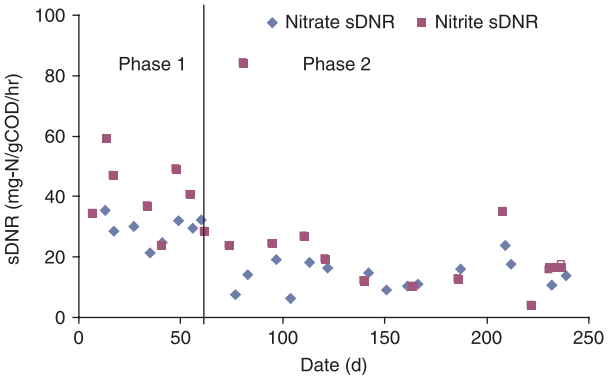

Figure 4 | Time profiles of batch nitrite (squares) and nitrate (diamonds) sDNR using NO_3^--N (Phase 1) or NO_2^--N (Phase 2) as the terminal electron acceptor.

methylotrophic population abundance itself did not change after the shift to nitrite (Figure 3).

There is little information on maximum specific growth rates for methylotrophic denitrification in activated slude; most data are reported in terms of the maximum specific denitrification rates, normalized against the total biomass concentrations. Nevertheless, our estimates for the maximum specific denitrification rate (μ_{max}, Table II) on nitrate compare favorably with that from BioWin 3 ($1.3\,d^{-1}$), a widely used process simulator. The high values of KS estimates in this study are likely due to practical identifiability limtations of Monod type functions and the need for additional data points near the K_S region (Chandran & Smets 2005). The significantly lower estimates of nitrite reduction biokinetics cannot be not due to nitrite self-inhibition, since initial rate tests performed at three initial nitrite concentrations (25, 50 and 100 mg-N/L) yielded similar specific denitrification rates of 50.9 ± 1.6, 58.1 ± 0.6 and 52.1 ± 3.5 mg-N/g COD/h, respectively. Thus, the overall kinetics of methylotrophic denitrification were presumably governed by both dual-limitation by nitrate and nitrite reduction kinetics in the SBDR. Biomass yield coefficients measured from extant denitrification profiles for nitrate compared favorably with theoretical thermodyanmics based calculations (Table 2). However, experimental estimates for nitrite were significantly lower than the corresponding theoretical estimates. It is likely that production of gaseous intermediates including nitrous and nitric oxide may have contributed to this reduced yield, since nitrite preferentially induces nitrite reductase over the

Table 2 | Parameter estimates for NO_3^--N and NO_2^--N reduction obtained from extant denitrification assays. ($\mu \pm \sigma$)

Electron acceptor	μ_{max} (d^{-1})	K_S ($mgCODL^{-1}$)	Y (mg COD/mg COD) from e^- balance	*n*	Y (mg COD/mg COD) from dissipation energy
NO_3^--N	0.94 ± 0.13	31.7 ± 25.9	0.41 ± 0.068	3	0.55
NO_2^--N	0.28 ± 0.31	17 ± 2.74	0.3 ± 0.3	3	0.61

other enzymes involved in the denitrification cascade (Korner & Zumft 1989).

The utility of conducting both population and biokinetic monitoring was amply illustrated upon switching electron acceptors in phase of this study. If one had followed the conventional practice of just monitoring sDNR, it would not be possible to discriminate whether the lower sDNR values during phase 2 were due to the same populations functioning at lower specific rates or because of a combined change in population abundance and specific growth rates. However, since, we explicitly measured both population abundance and sDNR, we could conclude that the reduction in sDNR was mainly due to reduction in cell activity rather than reduction in population abundance (Figures 3 and 4). The merits of dual population abundance and activity monitoring can be extended further to another likely scenario in full-scale BNR facilities. Many utilities might consider intermittent addition of methanol or other carbon sources to meet nitrogen limits. In the case of methanol, a 'lag' in denitrification performance has typically been observed. It has been speculated that the lag maybe associated with the growth of specific methylotrophic bacteria not present abundantly prior to methanol addition. Such a speculation is well-founded since bacteria that degrade organic compounds containing 1 carbon atom (such as methanol) are phylogenetically and metabolically distinct (Nercessian *et al.* 2005; Kalyuzhnaya *et al.* 2006). Our study in fact indicates that the methylotrophic bacteria in the test SBDR were indeed phylogenetically distinct and coherent relative to the overall SBDR population (Figure 2). Thus, a more appropriate strategy to expedite methylotrophic denitrification at full-scale might be to 'seed' methylotrophic biomass from an enrichment reactor rather than wait for the population to build up from base activated sludge levels. Additionally, further studies are needed to determine how the developed methylotrophic populations behave when subjected to prolonged methanol starvation and exposure to a different carbon source. Such information would be useful in the scenario that a less expensive or a higher rate carbon source needs to be employed *in lieu* of methanol. Yet another relevant consideration is the elucidation of methylotrophic behavior under aerobic conditions. The questions to be addressed are: *Do the same organisms use methanol under aerobic and anoxic conditions* and *what is the associated N speciation with such varying aerobic and anoxic activity?* Such information would be relevant in controlling methanol addition strategies. If methylotrophic denitrifying organisms are exclusively anoxic then a strict degree of control in methanol pumping rates to the bioreactor would need to be exercised to prevent bleed through into aerobic zones of the reactor. On the other hand, if methylotrophic denitrifying organisms are facultatively anaerobic then it might be beneficial to allow for some degree of aerobic methanol utilization and once again 'seed' the anoxic zones of the reactor with this developed consortium. Using SIP and downstream techniques such as qPCR in conjunction with biokinetic assays, we are now uniquely poised to answer such questions.

From a modeling perspective, based on our results, it seems appropriate to introduce additional terms describing the activity methylotrophic microorganisms. The kinetics of the two dominant methylotrophic organisms are currently under investigation in our lab to determine whether they could be lumped into one set of equations or whether they should be treated explicitly.

CONCLUSIONS

In conclusion, we successfully applied SIP in conjunction with qPCR to diagnose and quantitatively track the 'active' methylotrophic fraction in a denitrifying sequencing batch reactor. *Methyloversatilis* and *Hyphomicrobium* spp. emerged as the dominant groups metabolizing methanol in the SBDR. The transition from nitrate to nitrite as the electron acceptor did not translate into changes in the

population size but rather in their biokinetics. Based on the sDNR and biokinetic estimates, overall denitrification was subject to dual rate limitation by both nitrate and nitrite reduction.

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