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Impact of Varying Electron Donors on the Molecular Microbial Ecology and Biokinetics of Methylotrophic Denitrifying Bacteria

Vladimir Baytshtok, Huijie Lu, Hongkeun Park, Sungpyo Kim, Ran Yu, Kartik Chandran

Earth Microbiology Initiative, Department of Earth and Environmental Engineering, Columbia University, 500 West 120th Street, New York, New York 10027; telephone: 212-854-9027; fax: 212-854-7081; e-mail: kc2288@columbia.edu

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ABSTRACT: The goal of this study was to identify bacterial populations that assimilated methanol in a denitrifying sequencing batch reactor (SBR), using stable isotope probing (SIP) of ^{13}C labeled DNA and quantitatively track changes in these populations upon changing the electron donor from methanol to ethanol in the SBR feed. Based on SIP derived ^{13}C 16S rRNA gene clone libraries, dominant SBR methylotrophic bacteria were related to *Methyloversatilis* spp. and *Hyphomicrobium* spp. These methylotrophic populations were quantified via newly developed real-time PCR assays. Upon switching the electron donor from methanol to ethanol, *Hyphomicrobium* spp. concentrations decreased significantly in accordance with their obligately methylotrophic nutritional mode. In contrast, *Methyloversatilis* spp. concentrations were relatively unchanged, in accordance with their ability to assimilate both methanol and ethanol. Direct assimilation of ethanol by *Methyloversatilis* spp. but not *Hyphomicrobium* spp. was also confirmed via SIP. The reduction in methylotrophic bacterial concentration upon switching to ethanol was paralleled by a significant decrease in the methanol supported denitrification biokinetics of the SBR on nitrate. In sum, the results of this study demonstrate that the metabolic capabilities (methanol assimilation and metabolism) and substrate specificity (obligately or facultatively methylotrophic) of two distinct methylotrophic bacterial populations contributed to their survival or washout in denitrifying bioreactors.

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KEYWORDS: stable isotope probing; methylotrophic denitrification; microbial ecology; biokinetics

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), and eventually to dinitrogen (N_2) gas or under extremely anaerobic conditions to ammonium-nitrogen (NH_4^+-N) (Knowles, 1982). Although recently developed autotrophic processes for engineered biological nitrogen removal (BNR), such as anaerobic ammonia oxidation (ANAMMOX) (Mulder et al., 1995), completely autotrophic nitrogen removal over nitrite (CANON) (Third et al., 2001), and oxygen limited autotrophic nitrification and denitrification (OLAND) (Kuai and Verstraete, 1998) are novel and cost effective alternates to conventional nitrification and denitrification, such processes are most suited for high nitrogen containing streams such as anaerobic digestion reject water. While there is significant interest and ongoing research in understanding the constituent metabolic pathways of some of these more novel processes (with the genome of the first ANAMMOX enrichment sequenced recently (Strous et al., 2006)), autotrophic aerobic nitrification followed by heterotrophic denitrification is still by far the most prevalent strategy followed by wastewater treatment plants to achieve BNR.

Wastewater utilities typically practice addition of external organic electron donors to enhance the rates of denitrification (Grady et al., 1999). Of these external electron donors, methanol is one of the most widely used, mainly owing to its lower cost than alternates such as acetate and ethanol (Louzeiro et al., 2003). Despite extensive research and practical implementation of denitrification, a mechanistic understanding of "active" denitrifying microbial fractions in activated sludge fed with different carbon and electron sources is lacking. Consequently, full-scale implementation of heterotrophic denitrification is still guided by a somewhat empirical understanding of its microbial ecology and

Correspondence to: K. Chandran
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biokinetics, which in turn limits efforts to optimize denitrification reactor design, monitoring and modeling. Although biokinetic parameters for methylotrophic denitrification in activated sludge have been extensively reported (dos Santos et al., 2004; Gauntlett, 1979; Her and Huang, 1995; Janning et al., 1995; Lee and Welander, 1996; Louzeiro et al., 2002; Mulcahy et al., 1981; Purtschert and Gujer, 1999), the actual abundance and diversity of organisms in activated sludge that actually metabolize methanol and alternate carbon and electron sources has only recently been identified (Ginige et al., 2004, 2005; Osaka et al., 2006, 2008). The reason for such sparse data on the identity of bacterial populations denitrifying using specific electron donors is that unlike nitrifying bacteria, which are phylogenetically closely related (Purkhold et al., 2000), denitrifying bacteria are distributed widely across taxonomic groups (Zumft, 1997). Thus, methods that directly inspect the uptake and metabolism of these electron donors during denitrification are needed.

With increasing methanol prices, it is conceivable that wastewater utilities may adopt alternate carbon sources, which though expensive can foster significantly higher denitrification rates (e.g., ethanol). Therefore, from an engineering perspective, it is also essential to determine the impact of changing between external carbon sources for denitrification on the constituent microbial ecology of activated sludge. The ability to link changes in the microbial ecology with resulting reactor performance and biokinetics could lead to better understanding of the "black-box" of heterotrophic denitrification in activated sludge and result in better reactor operation, monitoring, and control.

Given the limited diversity of methylotrophic organisms recognized to date and their specific nutritional requirement for methanol and other single-carbon compounds as energy sources (Anthony, 1982), we hypothesized that switching the carbon source from methanol to ethanol in a denitrifying reactor would result in the washout of methylotrophic organisms and the subsequent enrichment of a more diverse non-methylotrophic ethanol degrading population.

The specific objectives of this study were to:

1. Determine the microbial ecology of a denitrifying sequencing batch reactor (SBR) fed with methanol followed by ethanol, using stable isotope probing (SIP) and 16S ribosomal RNA gene sequence enabled phylogenetic interpretation of ^{13}C labeled DNA.
2. Quantitatively track the performance, biokinetics, and microbial ecology of the SBR upon switching the electron donor in the influent stream from methanol to ethanol.

Materials and Methods

A methylotrophic enrichment consortium was cultivated in a SBR ($V = 9.2$ L, hydraulic retention time, HRT, $= 1$ day, solids retention time, SRT, $= 10$ days) operated at 21°C. Each SBR cycle was 6 h long with 1 h 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 periods. SBR cycles were automatically controlled via a digital controller (Chrontrol Corp, San Diego, CA). The pH of the SBR was automatically controlled at 7.5 ± 0.1 using concentrated hydrochloric acid. The SRT of the SBR was maintained by manually wasting biomass once a day during the end of the react phase, just prior to the settle phase. Wastage volume was calculated using weekly average reactor and effluent total chemical oxygen demand (tCOD). The seed biomass for the SBR was kindly provided by the New York City Department of Environmental Protection and obtained from a pilot-scale BNR reactor treating domestic wastewater fed with methanol. The SBR was operated for 225 days with methanol and 260 days on ethanol using nitrate as the terminal electron acceptor (Table I). The SBR feed medium was made up in tap water and in addition to the organic electron donor and acceptor, contained (per liter), 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.02 g of $CaCl_2 \cdot 2H_2O$, 0.087 g of K_2HPO_4, 1 mL of trace elements solution (10 mg of $Na_2MoO_4 \cdot 2H_2O$, 172 mg of $MnCl_2 \cdot 4H_2O$, 10 mg of $ZnSO_4 \cdot 7H_2O$, 0.4 mg of $CoCl_2 \cdot 6H_2O$ in a total volume made up to 100 mL with distilled water).

Microbial Ecology of Denitrification on Methanol and Ethanol via SIP

The overall schematic of the SIP enabled identification and quantitative tracking of denitrifying microorganisms using different COD sources is summarized in Figure 1. SIP was used to determine the ecological diversity of the SBR microbial community capable of utilizing methanol or ethanol for denitrification, as described previously (Neufeld et al., 2007). For SIP experiments, 2 L biomass was

Table I. Operating conditions of the denitrifying SBR.

	Reactor operation phases	
	Phase 1: Methanol + nitrate	Phase 2: Ethanol + nitrate
Influent COD (mg COD/L)	500	500
Influent N (mg-N/L)	100	100
Period of operation (days)	225	260
Percentage of nitrogen removal	92.5 ± 11.6	98.5 ± 2.5
SRT	10 ± 0.0	10.3 ± 3.7

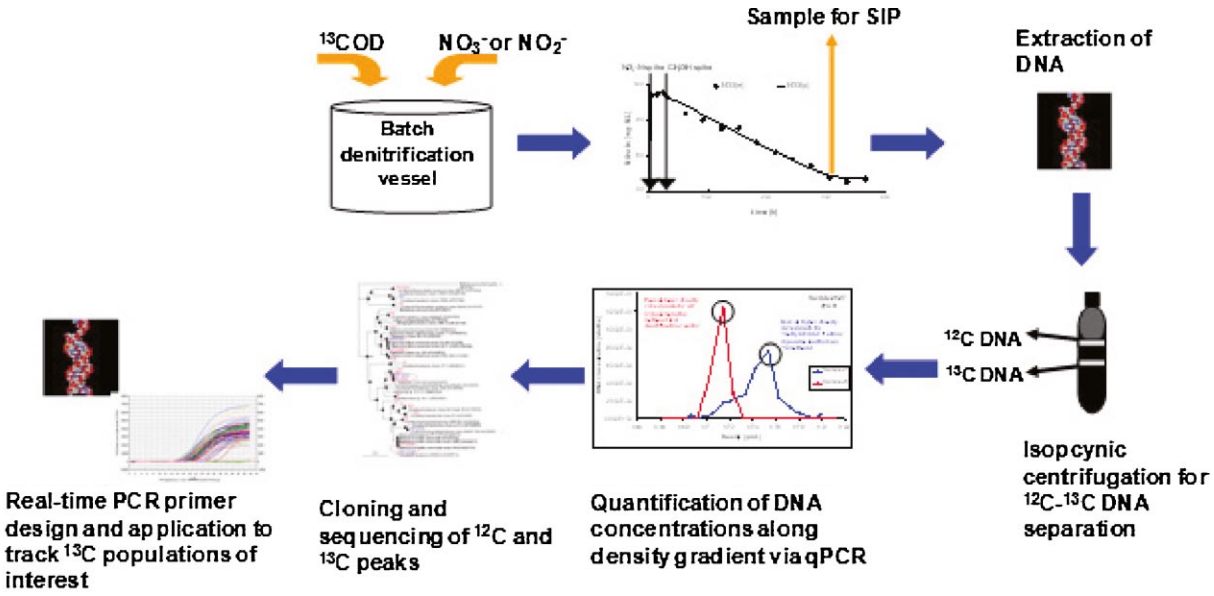

Figure 1. Schematic of SIP enabled identification and quantitative tracking of denitrifying bacteria using different carbon sources.

withdrawn from the SBR just prior to the start of the "settle" phase and washed by centrifugation at 1,000*g* for 20 min at room temperature and resuspending in COD and nitrate free feed medium. SIP experiments were initiated by spiking the biomass with 250 mg COD/L of ^{13}C methanol (day 124 of operation) or ^{13}C ethanol (day 485 of operation) and 100 mg NO_3^--N/L to identify the dominant microbial communities assimilating these organic carbon sources. Samples for phylogenetic characterization of the specific communities metabolizing ^{13}C methanol or ^{13}C ethanol via SIP were obtained just at the point of nitrate depletion during the SIP batch assay. It was independently determined for methanol that collecting samples at the point of nitrate depletion resulted in good discrimination of ^{12}C and ^{13}C DNA with longer incubations resulting in increased secondary ^{13}C uptake (data not shown). An unspiked sample was also obtained just before the ^{13}C spike ($t = 0$) to characterize the "overall" community in the reactor. Genomic DNA was extracted (DNeasy Blood & Tissue Kit, Qiagen, Valencia, CA) and subjected to isopcynic density gradient ultracentrifugation (55,000 rpm, $T = 20^{\circ}$C, 22 h). Sixteen density-gradient fractions per sample were collected and quantified by real-time PCR (qPCR) using eubacterial primers (Suzuki et al., 2000) at conditions described previously (Ahn et al., 2008) (BioRad *iQ*5, Hercules, CA). Out of the 16 DNA fractions (Fig. 2), the fraction containing the highest ^{13}C concentration of DNA (e.g., at $t = 22.4$ h, corresponding to a density of 1.753 g/mL, Fig. 2) and the fraction containing the highest ^{12}C concentration of DNA (e.g., at $t = 0$ h corresponding to a density of 1.714 g/mL, Fig. 2) were amplified against eubacterial 16S rRNA primers 11f (Kane et al., 1993) and 1492r (Weisburg et al., 1991). Amplicons were cloned (TOPO TA Cloning® for Sequencing, Invitrogen, Carlsbad, CA) and plasmid inserts were sequenced (Molecular Cloning Laboratories, San Francisco, CA). Sequences were aligned, edited manually, and screened for chimera (CHIMERA_CHECK, http://rdp8.cme.msu.edu/html/). The closest matching sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov). ClustalX (InforMax, Inc., North Bethesda, MD) software was used to establish and bootstrap phylogenetic trees. The Neighbor Joining (NJ) method (Saitou and Nei, 1987) was used for tree construction and positions with gaps were excluded and multiple substitutions were corrected. The tree was subjected to 1,000 bootstrap trials. The rooted bootstrapped tree was rendered using 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 identification of *Hyphomicrobium* spp. and *Methyloversatilis* spp. related bacteria as the dominant methylotrophic populations during phase 1 of this study (Fig. 3), qPCR assays were designed, experimentally optimized, and applied for determining their abundance over the three phases of reactor operation. qPCR primer sets were designed using PrimerQuest® software package (Integrated DNA Technologies, Coralville, IA) specifically targeting clones related to *Hyphomicrobium* spp. (Hzf-ACAATGGGCAGCAACACAGC and Hzr-ATTCACCGC-

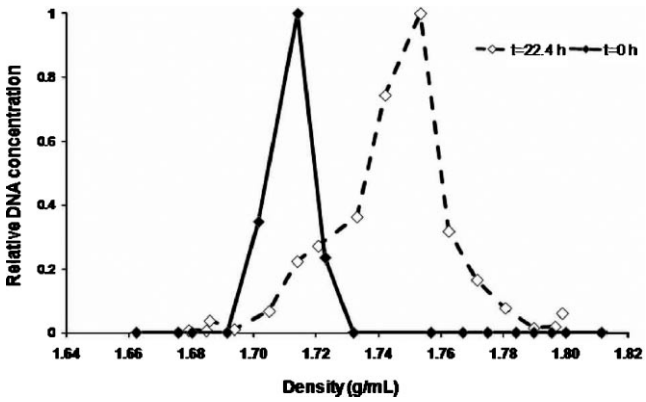

Figure 2. SIP profiles of methylotrophic biomass samples before the ^{13}C methanol spike ($t=0$, continuous line and shaded symbols) and after the ^{13}C methanol spike (dashed line and open symbols, obtained at $t=22.4$ h after the spike). DNA concentrations have been normalized to the maximum concentration for each respective profile.

GCCATGCTGAT) and *Methyloversatilis* spp. (Muf- AAGG-CCTACCAAGGCAACGA and Mur-ACCGTTTCGTTCC-TGCCGAA). Experimental optimization of qPCR assays was performed using genomic DNA from monocultures of *Hyphomicrobium zavarzinii* strain ZV620 (ATCC 27495) and *Methyloversatilis universalis* 500 (ATCC BAA-1314) as

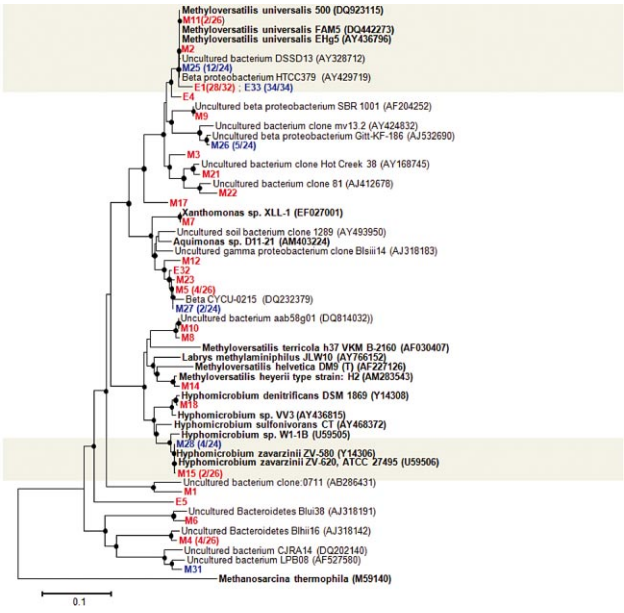

Figure 3. Phylogenetic tree depicting dominant overall populations (RED) and populations assimilating either ^{13}C methanol in phase 1 (denoted by "M", blue) or ^{13}C ethanol in phase 2 (denoted by "E", blue) of SBR operation. Numbers in parentheses represent fraction of clones most closely associated with a given phylogenetic lineage. Shaded rectangles indicate 16S rRNA gene sequences with >97% similarity.

standards. Monocultures of *H. zavarzinii* ZV 620 and *M. universalis* 500 were grown at 37°C in nutrient broth to stationary phase as per ATCC instructions. Genomic DNA was extracted and purified from the cultures (DNeasy, Qiagen, Valencia, CA) and serially diluted to prepare standard curves for qPCR.

Briefly, optimization of qPCR assays was performed by conducting multiple qPCR reactions across an annealing temperature gradient from 50 to 57°C. The selected optimum assay conditions were those that resulted in the highest SYBR Green conferred relative fluorescence units (RFU) just at the onset of the plateau region of the qPCR amplification curve corresponding to the lowest threshold cycle number (C_t) value. Optimized qPCR conditions were as follows: denaturation at 94°C for 5 min, annealing at 94°C for 30 s (step 1), 55°C for 40 s for *Methyloversatilis* spp. and 58°C for 40 s for *Hyphomicrobium* spp. (step 2), 72°C for 30 s (step 3), extension at 72°C for 5 min, and final extension at 50°C for 10 s. The assays were conducted in triplicate using *i*Q™ SYBR® Green Supermix (BioRad®, Valencia, CA), containing:100 mM KCl, 40 mM Tris–HCl (pH 8.4), 0.4 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), *i*Taq DNA polymerase (50 units/mL), 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein, and stabilizers. The template DNA volume per reaction was 1 μL and the total reaction volume was 25 μL. The primer concentration was 200 nM. High primer specificity and the absence of primer-dimers were confirmed via melt curve analysis (data not shown). qPCR controls consisted of template blanks which did not contain target DNA or standard DNA. Standards consisting of seven decimal dilutions were run on each qPCR plate to ensure that plate specific variability was accounted for. Plasmid DNA standard concentrations, containing the amplicon and vector of known lengths, were measured spectrophotometrically at 260 nm and translated to copies/μL.

Performance and Biokinetics of the Denitrifying SBR

Denitrifying SBR 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 and Smets, 2000) using nitrite or nitrate as electron acceptors. For these specific denitrification rate (sDNR) assays, biomass was withdrawn and processed identical to that for the SIP assays, but instead spiked with nitrate or nitrite and regular (^{12}C) methanol. Initial COD and N concentrations were 250 mg COD/L and 100 mg NO_3^--N/L for the nitrate extant batch assays and 75 mg COD/L and 50 mg NO_2^--N/L for the nitrite extant batch assays. These initial COD:N ratios of 2.5:1 and 1.5:1 for nitrate and nitrite extant batch kinetic assays, respectively, thereby rendered

organic carbon as the limiting nutrient, based on stoichiometric COD:N requirements of 5:1 and 3:1 for nitrate and nitrite, respectively (Grady et al., 1999). tCOD concentrations for the extant batch assays were 901.3 ± 318.4, $n = 97$ for phase 1 and 996.0 ± 328.7, $n = 69$ for phase 2.

The sDNR was computed by linear regression of the nitrate or nitrite depletion profiles normalized to the tCOD of the SBR mixed liquor.

Results

Microbial Ecology of Denitrification Using Methanol and Ethanol

Based on 16S rRNA gene clone libraries, the organisms that assimilated ^{13}C methanol most rapidly during phase 1 (methanol feed) were a subset of the overall SBR biomass, and were most closely related to *H. zavarainii* ZV 620 and *M. universalis* strain 500 (Fig. 3). A third abundant cluster M26(5/24), which was most closely related to uncultured bacteria from uranium mining waste piles (AJ 532690) (Selenska-Pobell et al., 2002) was not pursued further owing to its presumed limited relevance to methylotrophic denitrification in activated sludge systems. The remaining SBR populations did not assimilate ^{13}C methanol rapidly enough during the period of the ^{13}C spike or were potentially sustained on endogenous bacterial metabolites during long-term SBR operation.

The diversity of the ethanol-fed biomass was significantly lower than that of the methanol fed-biomass, and most of the ^{12}C and ^{13}C clones in phase 2 were closely clustered with known bacteria related to *Methyloversatilis* spp. (Fig. 3). These results suggest that the methylotrophic bacteria enriched in the SBR on methanol during phase 1 were indeed mainly sustained by assimilating ethanol during phase 2 and not on secondary metabolites or endogenous biomass products. Significantly, the long-term addition of ethanol to the SBR during phase 2 (260 days) did not result in the enrichment of a necessarily more diverse non-methylotrophic community as initially hypothesized.

Based on the newly developed and optimized qPCR assays, *Methyloversatilis* spp. were in general more abundant than *Hyphomicrobium* spp. during phase 1 of SBR operation (Fig. 4). The change in organic electron donor from methanol to ethanol resulted in a significant decline in the concentrations of *Hyphomicrobium* spp. but not in the concentrations of *Methyloversatilis* spp. (Fig. 4). Thus, both qualitative clone library analysis and quantitative PCR were consistent in pointing to the overall preponderance of *Methyloversatilis* spp. over *Hyphomicrobium* spp. during phase 2 (ethanol feed) as a result of changing the electron donor from methanol to ethanol (Figs. 3 and 4).

The applicability of the primer set targeting *Methyloversatilis* spp. that was designed based on the SIP results during phase 1 in targeting the additional *Methyloversatilis* spp. clones obtained in phase 2 (Fig. 3) was evaluated and confirmed based on a BLASTn search (http://www.ncbi.nlm.nih.gov/blast/). Thus, the *Methyloversatilis* spp. concentrations obtained by qPCR indeed included the entire *Methyloversatilis* spp. populations inferred from both clone libraries (phase 1 and 2). It is to be expected that the ^{13}C ethanol clone library (based on 34 clones) did not reveal *Hyphomicrobium* spp., since by the end of phase 2, their concentrations were three orders of magnitude lower than *Methyloversatilis* spp. On the other hand, the detection of both populations by qPCR was in keeping with a broader operational linear range of qPCR assays from 10^4 to 10^{10} copies/mL.

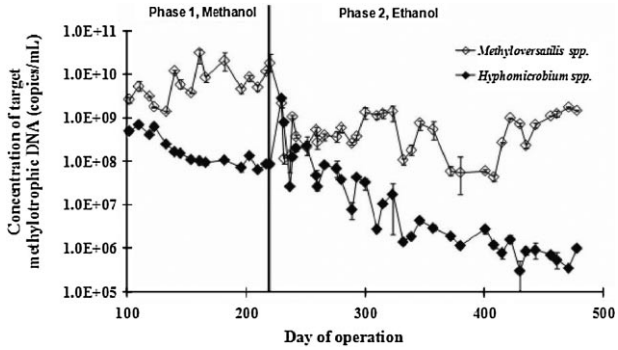

Figure 4. Relative abundance of *Hyphomicrobium* spp. related and *Methyloversatilis* spp. related methylotrophic bacteria in the SBR obtained via triplicate qPCR measurements during phase 1 (methanol feed) and phase 2 (ethanol feed) of denitrifying SBR operation.

Performance and Biokinetics of the SBR

Near complete steady-state nitrate and nitrite removal was obtained during the entire period of SBR operation (Table I). During phase 2, the switch in the electron-donor from methanol to ethanol resulted in a significant decrease in sDNR for methanol-spiked biomass for both nitrate ($P = 9.5E{-}12 < 0.050$) and nitrite ($P = 2.76E{-}10 < 0.050$) as electron acceptor. sDNR values with nitrite as the terminal electron acceptor were in general higher than those with nitrate (Fig. 5) and were in congruence with the lack of nitrite accumulation during the entire period of SBR operation (data not shown). During phase 2, sDNR values from ethanol-spiked biomass were also higher than for methanol-spiked biomass for both electron acceptors (data not shown).

Thus, based on the retention of *Methyloversatilis* spp., which could actively assimilate both methanol and ethanol and sustained SBR denitrification performance during phase 2, our initial hypothesis that the switch from methanol to ethanol would cause the less diverse methylotrophic community to be supplanted by a more diverse and distinct ethanol degrading community, was comprehensively rejected.

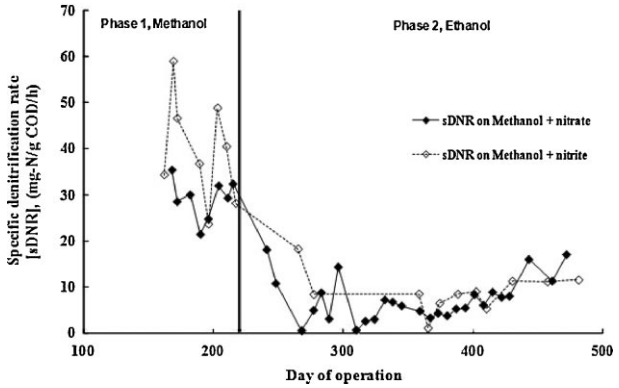

Figure 5. Time profiles of batch nitrate (continuous line and closed symbols) and nitrite (dashed line and open symbols) sDNR during phase 1 (methanol feed) and phase 2 (ethanol feed) of denitrifying SBR operation.

Discussion

The Microbial Ecology of Methylotrophic Denitrification

Notwithstanding the preferred and widespread use of methanol, ethanol, acetate, and now glycerol or sludge fermentate as external carbon sources for enhanced denitrification in BNR facilities, very little is still known about the microbial structure–function link of denitrification relating to these carbon sources. *Hyphomicrobium* spp. have been long speculated to be the dominant microbial group engaged in methylotrophic denitrification based on isolation techniques (Attwood and Harder, 1972; Harder et al. 1978; Knowles, 1982; Sperl and Hoare, 1971; Timmermans and Van Haute, 1983) or direct molecular inspection techniques (Gliesche and Fesefeldt, 1998; Holm et al., 1996; Kloos et al., 1995). However, several recent studies have implicated a considerably higher diversity of methylotrophic denitrification in activated sludge (Ginige et al., 2004; Neef et al., 1996; Osaka et al., 2006). According to two recent SIP based studies, *Hyphomicrobium* spp. were not the dominant active methylotrophic populations in methanol fed denitrifying reactors (Ginige et al., 2004; Osaka et al., 2006). Instead, the reactors were mostly comprised of obligately methylotrophic *Methylobacillus* and *Methylophilus* belonging to the order *Methylophilales* of the *Betaproteobacteria* (Ginige et al., 2004) or a co-culture of *Hyphomicrobium* spp. and *Methylophilaceae* (Osaka et al., 2006). In another study, *Hyphomicrobium* spp. constituted just 2% of the total population of a methanol-fed denitrifying filter (Neef et al., 1996).

Despite the implication of bacteria other than *Hyphomicrobium* spp. in denitrification with methanol (which is in good agreement with the results herein), the impact of long-term changes in the type of carbon source on the ecological diversity and concentrations of these methylotrophic communities, and correlations with their metabolic capabilities has not been determined before. The short-term ability of denitrifying biomass grown on methanol to denitrify using ethanol (via sDNR assays) was indeed recently shown but not explained or attributed to the specific participant microbial communities (Mokhayeri et al., 2008).

From an engineered wastewater treatment standpoint, it is equally important to understand both the identities and metabolic capabilities of dominant methylotrophic denitrifying populations in activated sludge. Such information is critical, for instance to evaluate the feasibility of switching to a higher rate carbon source such as ethanol in lieu of methanol, which could be favored during lower winter temperatures at wastewater treatment plants, or due to the increasing price of methanol.

The observed trend in the dominant methylotrophic populations upon switching from methanol to ethanol in this study can be explained based on their nutritional modes. Most characterized *Hyphomicrobium* spp. are restricted facultative methylotrophs that can utilize mainly C1 compounds for growth (McDonald et al., 2001). Therefore, it is expected that the *Hyphomicrobium* spp. related organisms present in the SBR during phase 1 (methanol feed) of this study could not be sustained during phase 2 (ethanol feed). On the other hand, bacteria related to *M.universalis* can utilize several C1 and multicarbon compounds (Kalyuzhnaya et al., 2006). Therefore, the continued higher concentrations of *Methyloversatilis* spp. related organisms upon transition from methanol to ethanol feed correlated well with their broader metabolic capabilities.

Therefore, from a practical perspective, upon switching from methanol to ethanol for denitrification, it is the presence of facultative methylotrophs that can assimilate both ethanol and methanol that results in sustained denitrification and not necessarily the rapid development of a more diverse ethanol degrading community.

The Microbial Ecology of Ethanol Based Denitrification

Compared to methanol, even less is known about the molecular microbial ecology of ethanol-based denitrification. The dominance of bacteria related to *Azoarcus*, *Dechloromonas*, *Thauera*, and *Acidovorax* spp. in an ethanol-fed bioreactor was recently reported (Hwang et al., 2006), although it was not clear which of these communities actually assimilated ethanol during denitrification (as explicitly done in this study). Another recent study reported that the diversity of an ethanol fed denitrifying community was lower than that of a methanol fed denitrifying community, based on sequence information of the nitrite reductase (*nirS* and *nirK*) genes (Hallin et al., 2006). However, again, the specificity of the overall community for methanol or ethanol degradation was not determined. Therefore, in general, a direct comparison

of the microbial ecology of ethanol based denitrification with other studies was precluded by the paucity of information thereof and the fact that the ethanol degrading community in this study had been enriched on methanol a priori.

Techniques for Characterizing the Structure and Function of Methylotrophic Denitrification in Activated Sludge

Elucidating the link between microbial community structure (composition and diversity) and function (reactions catalyzed) represents a singular challenge for microbial ecologists and engineers alike. However, since denitrification capabilities are phylogenetically and taxonomically diverse (Zumft, 1997), it is nearly impossible to "structurally" probe for all denitrifying bacteria in communities such as activated sludge using 16S rRNA targeted methods. Some studies have favored a more "functional" elucidation of denitrification microbial ecology by targeting key nitrogen reductase enzymes in the denitrification pathway including nitrate reductase (*napA* and *narG*) (Chèneby et al., 2003; Flanagan et al., 1999; Gregory et al., 2000), nitrite reductase (*nirS* and *nirK*) (Braker et al., 1998, 2001; Hallin and Lindgren, 1999; Liu et al., 2003; Nogales et al., 2002; Prieme et al., 2002; Song and Ward, 2003; Yoshie et al., 2004), nitric oxide reductase (*norB*) (Braker and Tiedje, 2003), and nitrous oxide reductase (*nosZ*) (Scala and Kerkhof, 1998, 1999). However, it is not possible to differentiate between the use of different COD sources or confirm denitrification activity by looking at the abundance of the nitrogen reductase genes alone (Fig. 6).

An alternate technique, fluorescence in situ hybridization combined with microautoradiography (FISH-MAR) relies upon the uptake of radio isotopic substrates into bacterial cells to infer phylogenetic composition (Lee et al., 1999; Lund Nielsen et al., 2005). However, FISH-MAR is only applicable to identify organisms for which phylogenetic information is available a priori. If such information is not known, one cannot determine which probes to use. Therefore, FISH-MAR alone cannot be used as a discovery tool for functional elucidating microbial ecology, but just as a probing tool.

In contrast, SIP relies upon the incorporation of ^{13}C substrates into cellular macromolecules such as DNA, RNA, phospholipid fatty acids (PLFAs), and proteins, which confirms metabolism of the ^{13}C substrate (Radajewski et al., 2000). When combined with DNA, RNA, or PLFA based phylogenetic mapping, SIP is an effective discovery technique for linking the identity of active bacteria with their function (degradation of specific substrates) (Ginige et al., 2004, 2005; Manefield et al., 2002; Osaka et al., 2006, 2008; Radajewski et al., 2000, 2002; Singleton et al., 2005, 2007). In addition to 16S rRNA genes (as followed in this study), SIP could also be combined with other biomarkers such as messenger RNA (mRNA) of genes coding for methanol anabolic and catabolic reactions including tetrahydromethanopterin-linked formaldehyde oxidation (*fae* and *fhcD*) (Kalyuzhnaya et al., 2004; Nercessian et al., 2005), methanol dehydrogenase (*mxaF*) (McDonald and Murrell, 1997) or alcohol dehydrogenase genes for both

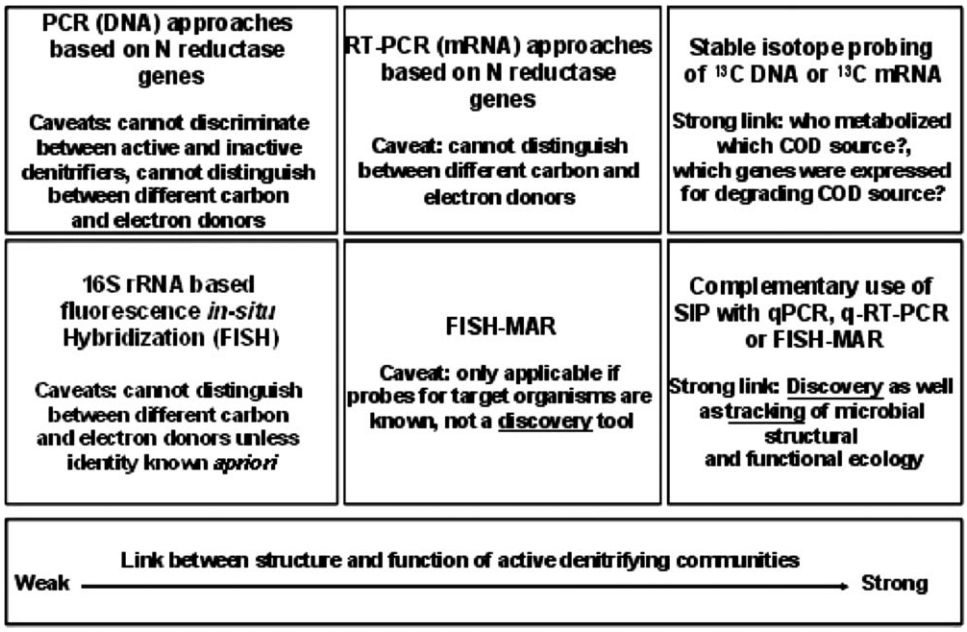

Figure 6. Varying degrees of information obtained on denitrification structure and function from different molecular techniques (after Philippot and Hallin (2005)).

methanol and ethanol (*mdh2*) (Kalyuzhnaya et al., 2008), which could explain the microbial pathways at work (Manefield et al., 2002).

Significance of Monitoring the Concentrations and Specific Activities of Methylotrophic Bacteria in Activated Sludge

The utility of conducting both population and biokinetic monitoring of denitrification was amply illustrated upon switching electron donors in this study. Since, we explicitly measured both population abundance (by qPCR) and activity (by sDNR), we inferred that the lower (methanol + nitrate) sDNR in phase 2 was not just due to a reduction in specific activity of the methylotrophic population but also due to a reduction in the concentrations of *Hyphomicrobium* spp. in the SBR (Figs. 4 and 5). Additionally, the strong parallel decrease in the concentrations of *Hyphomicrobium* spp. and methanol + nitrate sDNR in phase 2 suggests that *Hyphomicrobium* spp. have higher methanol based sDNR (and correspondingly maximum specific growth rates, μ_{max}) values than *Methyloversatilis* spp. This could be one reason why previous cultivation or isolation based studies (which select for rapidly growing organisms) routinely implicated *Hyphomicrobium* spp. as the dominant or even the sole methylotrophic bacteria in their test cultures (Attwood and Harder, 1972; Harder et al., 1978; Knowles, 1982; Sperl and Hoare, 1971; Timmermans and Van Haute, 1983). The lower concentrations of *Hyphomicrobium* spp. during phase 1 could be due to the fact that bacteria with higher μ_{max} values are typically associated with higher half-saturation (K_S) values as well (*r*-strategists) (Pianka, 1970). *R*-strategists cannot scavenge low substrate concentrations (in casu, methanol and nitrate, reflected by near complete nitrate removal in throughout the study) and are out-competed by *K*-strategists, which have both lower μ_{max} and K_S values (in casu, *Methyloversatilis* spp.). Alternately, it may be speculated that *Hyphomicrobium* spp. have a lower biomass yield than *Methyloversatilis* spp.

Conclusions

In sum, this study successfully linked the metabolic capabilities (methanol assimilation and metabolism) and substrate specificity (obligately or facultatively methylotrophic) of two distinct methylotrophic populations to their survival in a denitrifying SBR. Based on these results, it is expected that a change in the electron donor from methanol to ethanol will likely not result in significant disruptions to denitrification performance in BNR reactors, by virtue of the sustained presence and activity of facultatively methylotrophic bacteria therein.

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