Diagnosis and Quantification of Glycerol Assimilating Denitrifying Bacteria in an Integrated Fixed-Film Activated Sludge Reactor via ^{13}C DNA Stable-Isotope Probing

HUIJIE LU AND KARTIK CHANDRAN*

Department of Earth and Environmental Engineering, Columbia University, 500 West 120th Street, New York, New York 10027, United States

Received June 23, 2010. Revised manuscript received October 14, 2010. Accepted October 22, 2010.

Glycerol, a byproduct of biodiesel and oleo-chemical manufacturing operations, represents an attractive alternate to methanol as a carbon and electron donor for enhanced denitrification. However, unlike methanol, little is known about the diversity and activity of glycerol assimilating bacteria in activated sludge. In this study, the microbial ecology of glycerol assimilating denitrifying bacteria in a sequencing batch integrated fixed film activated sludge (SB-IFAS) reactor was investigated using ^{13}C-DNA stable isotope probing (SIP). During steady state SB-IFAS reactor operation, near complete nitrate removal (92.7 $\pm$ 5.8%) was achieved. Based on ^{13}C DNA clone libraries obtained after 360 days of SB-IFAS reactor operation, bacteria related to *Comamonas* spp. and *Diaphorobacter* spp. dominated in the suspended phase communities. ^{13}C assimilating members in the biofilm community were phylogenetically more diverse and were related to *Comamonas* spp., *Bradyrhizobium* spp., and *Tessaracoccus* spp. Possibly owing to greater substrate availability in the suspended phase, the glycerol-assimilating denitrifying populations (quantified by real-time PCR) were more abundant therein than in the biofilm phase. The biomass in the suspended phase also had a higher specific denitrification rate than the biofilm phase ($p =$ 4.33e-4), and contributed to 69.7 $\pm$ 4.5% of the overall N-removal on a mass basis. The kinetics of glycerol based denitrification by suspended phase biomass were approximately 3 times higher than with methanol. Previously identified methanol assimilating denitrifying bacteria were not associated with glycerol assimilation, thereby suggesting limited cross-utilization of these two substrates for denitrification in the system tested.

Introduction

Methanol is one of the most widely used external organic carbon sources for enhancing denitrification at wastewater treatment plants (*1*−*3*). Of late, glycerol has emerged as an alternative to methanol due to three factors. First, the price of methanol, which is tied to the natural gas price, has been increasing (*4*). Second, the dramatic increase in biodiesel production as a means of moving away from petroleum as an energy source has given rise to significant quantities of glycerol as a waste product (*5*). Third, glycerol has been previously shown to foster higher denitrification kinetics than those of methanol (*6, 7*). Consequently, wastewater treatment plants today are intently considering glycerol as a supplement or replacement for methanol.

From the perspective of wastewater treatment process design, it is essential to determine the fraction of activated sludge bacteria assimilating any given carbon source. It has been shown previously that the type of electron donor applied can strongly impact nitrate removal rates and the molecular microbial ecology of denitrification (*8*−*14*) as well as production of nitrous and nitric oxides in response to disturbances such as inhibition by nitrite or oxygen (*15*). If it is determined that certain carbon sources can only be assimilated by a subpopulation of activated sludge bacteria as in the case of methanol (*8*), and then process models that adequately capture this specific assimilation capability need to be developed and employed. Recent studies have indeed focused on elucidating the ecology of bacteria assimilating other carbon sources such as methanol, acetate, or ethanol (*8*−*10, 13, 16*). It has also been shown that only a fraction of activated sludge bacteria could assimilate methanol, and, out of these, only some could assimilate ethanol as well (*8*). Nevertheless, to date the microbial ecology of glycerol assimilation in mixed microbial communities such as activated sludge has not received adequate attention, although several organisms in *pure culture* have been shown to grow anaerobically using glycerol as the sole carbon and energy source (*5*). Therefore, diagnosing glycerol assimilating bacteria and comparing the microbial ecology of bacteria assimilating methanol and glycerol would be beneficial in evaluating the feasibility of maintaining denitrification performance and kinetics while switching from widely used carbon sources such as methanol to new ones such as glycerol.

Accordingly, the overall focus of this study was to characterize the microbial ecology of glycerol assimilating bacteria in both the biofilm and suspended phases of a denitrifying integrated fixed-film activated sludge (IFAS) reactor. IFAS reactors are being adopted widely for post-denitrification using externally added carbon sources such as methanol, ethanol, and glycerol (*6, 17, 18*). IFAS reactors offer several advantages compared to conventional activated sludge since they can achieve comparable process performance, while operating at a lower footprint (*19*). While the microbial ecology and kinetics of biofilms in IFAS reactors have been described previously (*20*−*22*), there exist few comparisons of community structures and activities in the two distinct phases (suspended and biofilm) based on *direct substrate assimilation*.

In an earlier study, *Methyloversatilis* spp. and *Hyphomicrobium* spp. related species were identified as the dominant populations in a sequencing batch denitrifying reactor with methanol as the carbon source via stable isotope probing (*8*). Given the metabolic specificity of methylotrophic denitrification (*8*), it was hypothesized that the microbial ecology of glycerol enriched biomass would differ from that enriched by methanol. In addition, due to dissimilar nutrient concentration gradients expected in the suspended and biofilm phases, it was hypothesized that distinct glycerol-assimilating bacterial populations would dominate in these two phases. The specific objectives were to (1) identify glycerol assimilating denitrifying bacteria in the suspended and biofilm phases of a sequencing batch IFAS (SB-IFAS) reactor using ^{13}C-DNA stable isotope probing, (2) quantitatively track the abundance

* Corresponding author phone: (212)854-9027, fax: (212)854-7081, e-mail: kc2288@columbia.edu.

10.1021/es102124f
Published on Web 11/05/2010

TABLE 1. Real-Time PCR Primers Employed To Quantify Glycerol Assimilating Bacteria

target group	primer name	sequence (5′ to 3′)	T_m (°C)	amplicon size (bp)
Comamonas spp.	CoF	AGTTGCTACGAAAGGGCACTCTGA	57	120
	CoR	TACCGGCCATTGTATGACGTGTGT		
Tessaracoccus spp.	TeF	GCATGCTACAATGGCCGGTACAAA	59	80
	TeR	AATCCGAACTGAGACCGGCTTTCT		
Diaphorobacter spp.	DiF	GCCGCAAGGTTGAAACTCAAAGGA	59	110
	DiR	TCCTGCCATGTCAAAGGTGGGTAA		
Bradyrhizobium spp.	BrF	ATGGATTCCTTCAGTTCGGCTGGA	56	88
	BrR	TGCGGGACTTAACCCAACATCTCA		

of dominant glycerol assimilating bacteria and two methylotrophic bacteria (*Methyloversatilis* spp. and *Hyphomicrobium* spp.) in the suspended and biofilm phases during start up and steady-state operation of the SB-IFAS reactor, and (3) evaluate the extent of N-removal and kinetics of glycerol induced denitrification in the suspended and biofilm phases.

Materials and Methods

Sequencing Batch Integrated Fixed-Film Activated Sludge Reactor Operation. Biomass was obtained from the methanol-fed anoxic zone of a full-scale wastewater treatment process and used to seed an 8 L denitrifying SB-IFAS reactor containing 30% by volume circular Kaldnes K1 polyethylene carriers (external diameter = 9 mm, specific surface area = 800 m^2/m^3, AnoxKaldnes USA, Providence, RI). The choice of this methylotrophic inoculum also allowed for evaluating possible metabolic and ecological similarities between methanol and glycerol based denitrification. The SB-IFAS reactor was operated for 380 days at 21 °C at a hydraulic retention time of 1 day and a targeted suspended phase solids retention time of 10 days. Each SB-IFAS reactor cycle (6 h) consisted of 1 h anoxic feed and react, 3.5 h anoxic react, 0.5 h aerobic mixing (to strip out dinitrogen gas and improve biomass settling), 0.75 h settle, and 0.25 h decant periods and was automatically controlled via a digital controller (Chrontrol Corp, San Diego, CA). The pH was automatically controlled in the range of 7.3 ± 0.2 using concentrated hydrochloric acid. The feed medium was as described earlier (*8*) and contained (per liter) the following: 0.2 g of $MgSO_4 \bullet 7H_2O$, 0.02 g of $CaCl_2 \bullet 2H_2O$, 0.087 g of K_2HPO_4, 1 mL of trace elements solution (10 mg of $Na_2MoO_4 \bullet 2H_2O$, 172 mg of $MnCl_2 \bullet 4H_2O$, 10 mg of $ZnSO_4 \bullet 7H_2O$, 0.4 mg of $CoCl_2 \bullet 6H_2O$ in a total volume made up to 100 mL with distilled water). The influent concentrations of glycerol and nitrate were 410 mg glycerol/L and 442 mg nitrate/L, respectively. Additional details of SB-IFAS reactor operation are provided in the Supporting Information (SI).

DNA Stable Isotope Probing. DNA stable isotope probing (SIP) was conducted as described previously (*8*). Briefly, on day 360, 500 mL of suspended biomass and 60 biofilm carrier particles were withdrawn from the SB-IFAS reactor just prior to the "settle" phase and washed individually by centrifugation (2050xg, 10 min) and resuspension in 500 mL of nitrate- and glycerol-free medium. The washed biomass samples were spiked with 205 mg of [^{13}C3]-$C_3H_8O_3$/L and 442 mg of nitrate/L and incubated under anoxic conditions at room temperature. 50 mL of suspended biomass samples was obtained from the batch incubations before the spike ($t = 0$, to characterize the overall SB-IFAS populations, S12) and close to the point of nitrate depletion ($t = 20$ h, to characterize the ^{13}C glycerol assimilating populations, S13). Similarly, 6 biofilm carriers were harvested at $t = 0$ (A12) and $t = 20$ h (A13). By sampling close to the point of nitrate depletion (as shown in the SI, Figure S2), cross-feeding on ^{13}C labeled endogenous products was expected to be minimal. Measured oxidation−reduction potentials during the SIP assays in the range −100 − 50 mV (data not shown) also suggested the dominance of denitrification over alternate dissimilatory reduction of sulfate or reduced iron species (*23, 24*).

Approximately 10 μg of genomic DNA was extracted from the 4 biomass samples (DNeasy Blood & Tissue Kit, Qiagen, Valencia, CA), stained with 0.5 μL of 10,000X SYBR Green I (Invitrogen, Carlsbad, CA) and subjected to cesium chloride density gradient ultracentrifugation (45,000 rpm, 20 °C, 70 h) (*25*). The DNA fractions were visualized using UV transillumination at 365 nm and withdrawn from the centrifuge tube using a syringe. A single DNA fraction was withdrawn from the samples taken at time $t = 0$ (S12 and A12), and both light (unlabeled with ^{13}C) and heavy (labeled with ^{13}C) fractions were withdrawn from samples S13 and A13. In order to test the quality of the separation between the labeled and unlabeled fractions, one more sample was withdrawn at a position longitudinally between the two fractions (data shown in the Supporting Information (as shown in the SI, Figure S3)).

DNA recovered from the samples (S12, A12, labeled fraction of S13 and A13) was amplified against eubacterial 16S rRNA primers 11f (5′-GTTTGATCCTGGCTCAG-3′) and 1392r (5′-ACGGGCGGTGTGTRC-3′) as per refs *26 and 27*. Amplicons were cloned (TOPO TA Cloning Kit for Sequencing, Invitrogen, Carlsbad, CA), and 21 colonies were randomly picked for sequencing (Macrogen USA, Rockville, MD). Primers used for sequencing were M13F (5′-GTAAAACGACGGCCAGT-3′) and M13R-pUC (5′-CAGGAAACAGCTATGAC-3′). The partial 16S rRNA sequences obtained were approximately 1400 bp in length. All 16S rRNA gene sequences were checked for chimera using Mallard (*28*). Phylogenetic trees were generated using MEGA 4.0 the neighbor-joining method and the substitution model of Jukes-Cantor. Sequences with the highest number of BLAST hits were included in the tree, and *Methanosarcina thermophila* served as the outgroup. The sequence data generated in this study has been deposited in GenBank under accession numbers HQ232435-HQ232455.

Quantitative PCR Assays for Tracking Glycerol Assimilating Biomass Concentrations. Based on the identification of *Comamonas* spp., *Bradyrhizobium* spp. *Diaphorobacter* spp., and *Tessaracoccus* spp. as dominant glycerol assimilating populations, qPCR primers were designed to specifically target their abundance in both suspended and biofilm phases, using PrimerQuest software (Integrated DNA Technologies, Coralville, IA) (Table 1). Primer specificity was checked using Primer BLAST for both forward and reverse primers (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). qPCR assays were employed in triplicate in 25 μL volumes, containing 12.5 μL of iQ SYBR Green Supermix (BioRad, Valencia, CA), 1 μL each of forward and reverse primers (final concentration of 0.25 μM), 1 μL of DNA template (10−20 ng), and 9.5 μL of dH_2O. For negative and no template controls, 1 μL of *Nitrosomonas europaea* genomic DNA and dH_2O were used, respectively. The optimized PCR conditions were as follows: 5 min at 95 °C, followed by 35 cycles of 95 °C for 1 min, 30 s at a specific annealing temperature (T_m, Table 1), and 72 °C for 1 min and final extension at 72 °C for 5 min.

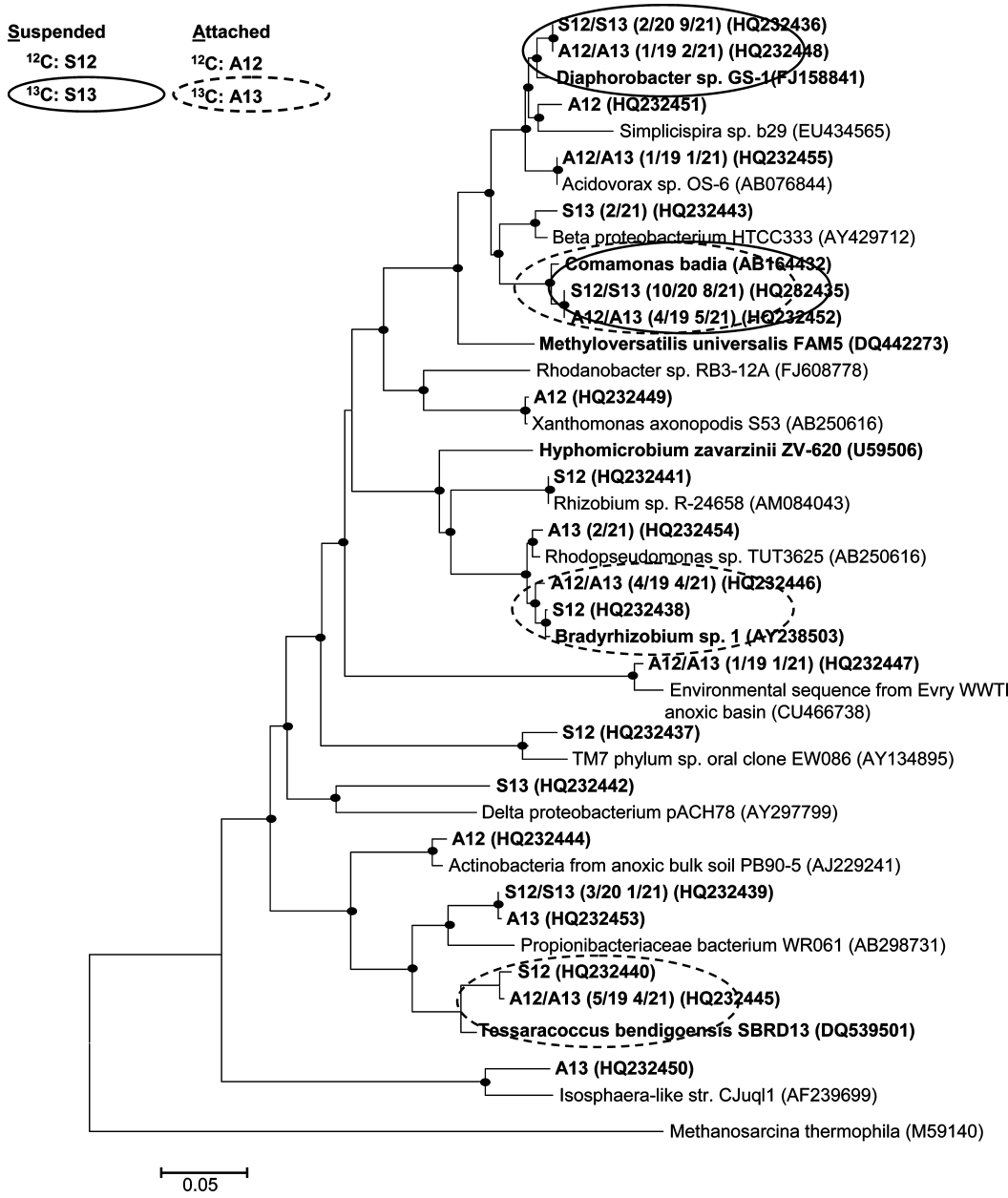

FIGURE 1. 16S rRNA gene based phylogenetic tree depicting (1) overall populations in suspended and attached biomass (denoted by "S12" and "A12", respectively) and (2) populations assimilating ^{13}C glycerol in suspended and attached biomass ("S13" and "A13", respectively). Numbers in parentheses represent the fraction of clones most closely associated with a given phylogenetic lineage (those with two fractions stand for ^{12}C and ^{13}C, respectively). Circles indicate dominant species found in suspended and attached biomass.

Standard curves for qPCR were constructed with serial decimal dilutions of plasmids containing the target amplicon inserts. PCR product specificity for each reaction was confirmed by the presence of a single peak during melt curve analysis and the presence of a single-band of expected molecular size by agarose gel electrophoresis. In selected SB-IFAS reactor biomass samples, concentrations of two methylotrophic populations, *Methyloversatilis* spp. and *Hyphomicrobium* spp., previously found in methanol fed SBRs were also measured as described previously (*8*). The fractions of the different bacterial populations were determined by normalizing the respective 16S rRNA copy number to the copy number of the total eubacterial 16S rRNA gene, which in turn was determined using the primer set UNIf (5′-TCCTACGGGAGGCAGCAGT-3′) and UNIr (5′-GGACTACCAGGGTATCTAATCCTGTT-3′) as per ref *29*.

Performance and Extant Kinetics Measurements. The performance of the SB-IFAS reactor was determined by measuring influent nitrate and effluent nitrite, nitrate, total and effluent soluble chemical oxygen demand (COD, expressed as g O_2 eq/L), all according to Standard Methods (*30*). Denitrification kinetics were determined via extant batch assays (*8*). For suspended phase kinetics, 500 mL mixed liquor samples were withdrawn from the SB-IFAS reactor, centrifuged, and resuspended in nitrogen and carbon free medium (as described in the SI). Biofilm kinetic tests were conducted with 60 Kaldnes K1 carriers. The initial concentration of nitrate and glycerol in the batch kinetic assays were 442 mg nitrate/L and 205 mg glycerol/L, respectively, thereby rendering glycerol as the limiting nutrient based on a stoichiometric carbon:nitrate requirement of 500 mg O_2 eq: 442 mg nitrate (*31*). In selected experiments, methanol was

added *in lieu* of glycerol for comparing kinetics of denitrification with glycerol. The specific denitrification rate (sDNR) was computed by linear regression of the nitrate depletion profiles vs time and normalized to total suspended or biofilm biomass concentrations (expressed as g O_2 eq/L). The relative contributions of biomass in the two phases to the total nitrogen removal were approximated according to eqs 1a and 1b

$$\text{Contribution}_{\text{suspended}} = \frac{\text{sDNR}_{\text{sus}} \times M_{\text{sus}}}{\text{sDNR}_{\text{sus}} \times M_{\text{sus}} + \text{sDNR}_{\text{biofilm}} \times M_{\text{biofilm}}} \quad (1a)$$

$$\text{Contribution}_{\text{biofilm}} = 1 - \text{Contribution}_{\text{suspended}} \quad (1b)$$

where M_{sus} and M_{biofilm} are the mass inventories of total biomass in suspended and biofilm phases, respectively, during steady state operation. $M_{\text{sus}} = 7.6 \pm 0.4$ g O_2 eq ($n = 82$) and $M_{\text{biofilm}} = 3.9 \pm 0.2$ g O_2 eq ($n = 54$).

Results and Discussion

Microbial Ecology of Glycerol Assimilating Denitrifying Bacteria in Suspended and Biofilm Phases. Based on retrieved clone library sequences, the bacteria assimilating glycerol in the suspended phase were most closely related to *Comamonas badia* (8/21) and *Diaphorobacter* GS1 (9/21) (Figure 1). Both *C. badia* and *Diaphorobacter* spp. belong to the family of *Comamonadaceae* in *β-Proteobacteria*. *C. badia* has been implicated in floc-formation in activated sludge (*32*). *Diaphorobacter* spp. has been reported to denitrify using polyhydroxybutyrate as an electron donor (*33*). *Diaphorobacter* spp. has also been implicated in simultaneous nitrification and denitrification under aerobic conditions (*34*). However, the capacity of *C. badia* and *Diaphorobacter* spp. to denitrify while assimilating glycerol has not been demonstrated. The lack of publicly available genomes for these bacteria also precluded *in silico* derived inferences on their dissimilatory nitrogen reduction or carbon assimilation capabilities.

The ^{13}C DNA sequences of the biofilm samples were more diverse and dominated by *Comamonas badia* (5/21), *Bradyrhizobium* sp. 1 (4/21), and *Tessaracoccus bendigoensis* (4/21) related bacteria. *Bradyrhizobia* and *Tessaracocci* belong to the family of *Rhizobiales* in *α-proteobacteria* (*35*) and *Propionibacteriaceae* in *Actinobacteria* (*36*), respectively. Very little is known about the denitrification capability of these bacteria and or their ability to use glycerol as an electron donor. It is notable that the glycerol assimilating bacteria diagnosed and quantified in this study have not been implicated in glycerol metabolism before (as reviewed by ref *5*). A possible explanation for this discrepancy is that the previous studies selected their strains *a priori* for examining glycerol metabolism. In contrast, this study was not biased toward any particular strain. Rather the focus was to elucidate the glycerol assimilating communities in the overall population of a denitrifying IFAS reactor.

By visually and quantitatively analyzing the ^{12}C clone libraries of the communities in two phases, relatively higher overall microbial diversity in the biofilm phase than the suspended phase was inferred in keeping with past results (*37*−*39*). It is generally speculated that the chemical heterogeneity (concentration gradients of substrates, metabolic intermediates and products within the biofilm, owing to biochemical reaction-transport interactions) provides the opportunity for metabolic competition and cooperation between different bacterial groups, thereby allowing more diverse communities to coexist (*40, 41*). In contrast, the relatively more uniform substrate concentrations in the bulk-suspended phase in the IFAS reactor may have contributed to a lower microbial diversity therein, as suggested previously (*41*). The low number of clones used for sequencing (21 clones each for ^{12}C, ^{13}C, biofilm or suspended phases) precluded a more quantitative analyses via calculation of the Chao-1 diversity estimator or Shannon−Wiener indices as measures of diversity.

The concentrations of the four identified glycerol assimilating bacteria in the overall population increased from 19.8 ± 0.8%, based on the 16S rRNA gene fraction, on day 1 to 71.3 ± 1.2% (suspended phase) and 54.4 ± 0.7% (biofilm) on day 363 (Figure 2). The suspended phase was more enriched in glycerol-assimilating bacteria than the biofilm phase possibly due to the higher availability of glycerol in the suspended phase to which it was fed. (Detailed time series profiles of all four glycerol assimilating bacterial concentrations are provided in the SI (Figure S5).)

It was also observed that a significant fraction of the overall population was not related to the four glycerol-assimilating bacterial groups as reflected by their presence only in the ^{12}C 16S rRNA gene sequences (Figure 1). The dominant bacteria that were featured only in the ^{12}C fraction were mostly closely related to *Simplicispira spp.*, *Xanthomonas spp.*, and *Rhizobium spp.* Based on previous studies, these bacteria can all denitrify, but their denitrifying capacities with glycerol as carbon source have not been documented (*42*−*44*). Additionally, since their genomes have not been sequenced, their capacity to assimilate glycerol could not be confirmed or refuted via *in silico* analysis. This non-glycerol-assimilating fraction was possibly sustained in the reactor on products of biomass decay.

Differences in the Microbial Ecology of Glycerol and Methanol Assimilation. Notably, glycerol assimilating bacteria enriched in both suspended and biofilm phases were not related to *Methyloversatilis* spp. or *Hyphomicrobium* spp, which were the two principal methylotrophic populations identified in a methanol fed-SBR (*8*). The lack of glycerol based denitrification by methanol enriched biomass has been shown earlier (*45*), but the direct inspection of glycerol assimilation during denitrification as performed herein is novel. The activated sludge sample used to inoculate the SB-IFAS reactor contained 1.99 ± 0.21% *Methyloversatilis* spp. and 4.32 ± 0.31% *Hyphomicrobium* spp. (as copies of 16S rRNA per copy of the total eubacterial 16S rRNA gene). During the course of exposure to glycerol, the two methylotrophic population concentrations decreased by factors of about 200−1000 (data shown in the SI, Figure S6). The washout of *Hyphomicrobium* spp. related populations is in accordance with their obligately methylotrophic nutritional mode (*8, 46*). Although *Methyloversatilis* spp. have been shown to assimilate both methanol and ethanol during denitrification (*8*), the results of this study show that they might be incapable of assimilating glycerol. Notwithstanding the absence of these two previously detected methylotrophs in the SB-IFAS reactor, the possibility remains that the reactor could have fostered other methylotrophs not characterized to date. However, owing to the focus of this study on glycerol assimilation, no attempts were made to explore ^{13}C methanol assimilation by uncharacterized bacterial communities.

Furthermore, although denitrification can be conducted by archaea as well (*47, 48*), their abundance and contribution to wastewater denitrification processes are not well-documented. As a result, we also did not systematically evaluate the contribution of glycerol assimilating archaea in this study and focused on bacteria alone.

Performance and Kinetics of the Denitrifying SB-IFAS Reactor. Near-complete nitrate removal (92.7 ± 5.8%) with the absence of significant nitrite accumulation (<0.6 mg nitrite/L) was achieved 50 days after initial inoculation of the SB-IFAS reactor (shown in the SI, Figure S7). For suspended biomass, glycerol-sDNR values continuously increased from zero during the study and exceeded those

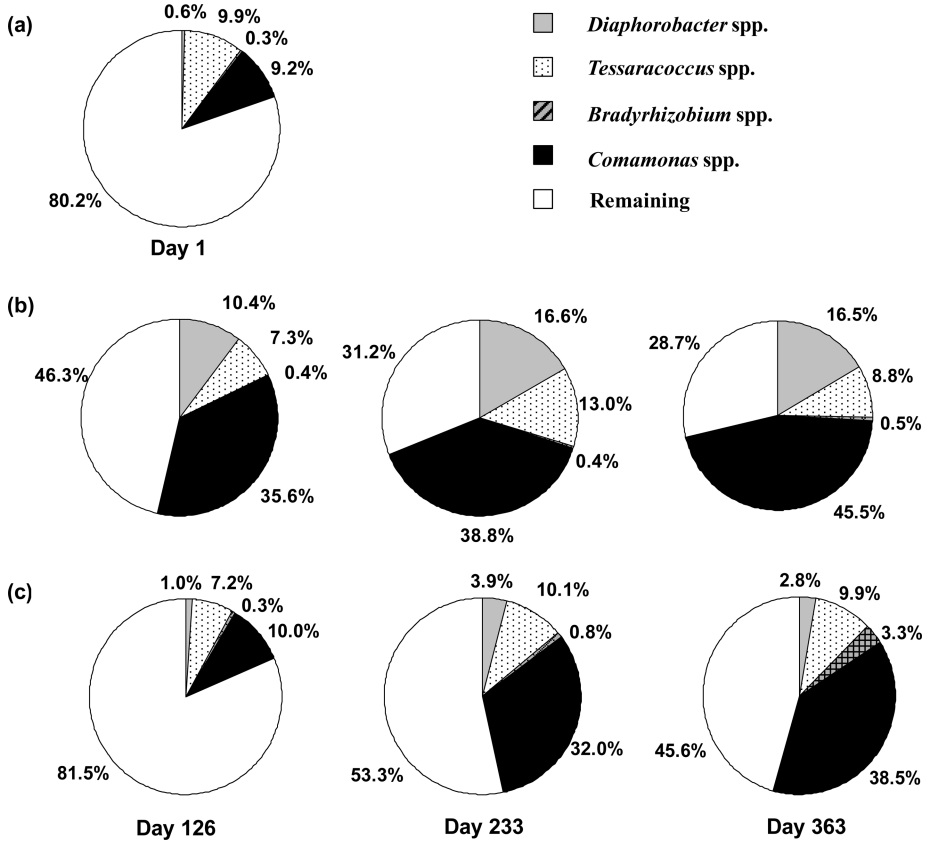

FIGURE 2. Variation in microbial community composition on (a) Day 1 and Day 126, 233, and 363 in (b) suspended and (c) biofilm phases. Pie slices with fractional percentages represent relative abundance of each dominant species as well as the "remaining" untargeted populations.

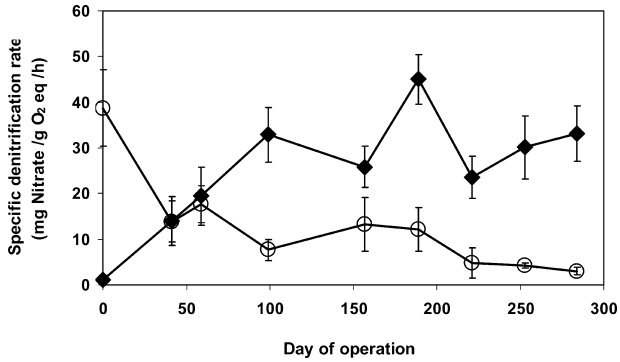

FIGURE 3. Time-series profiles of suspended biomass sDNR values with glycerol (◆) and methanol (⊖) as carbon sources. Error bars indicate one standard deviation of linear regression slopes.

of methanol (1.1 ± 0.4 mg nitrate/g O_2 eq/h, $p = 8.19e\text{-}7$ and therefore statistically significant at $\alpha = 0.05$) on and after day 99 (Figure 3). In general, the suspended-phase glycerol sDNR values measured in this study were higher than those previously reported in the range of 4.42 mg nitrate/g O_2 eq/h (*49*) and might be due to differences in the initial inoculum, reactor operation, or just biological variability in the different systems. A significant decrease of sDNR values on methanol was also observed during this period (Figure 3). The reduction in methanol sDNR values, coupled with the low abundance of methylotrophic bacteria in the suspended and biofilm phases of the glycerol fed SB-IFAS reactor, reflected the progressively declining capacity of the reactor biomass for denitrification using methanol. Therefore, via a combination of SIP and kinetics measurements, the lack of glycerol assimilation by methanol assimilating bacteria was confirmed.

In a side-by-side comparison, sDNR values obtained with suspended biomass on glycerol (20.4 ± 7.1 mg nitrate/g O_2 eq/h) were generally higher than those with biofilm biomass (17.3 ± 5.3 mg nitrate/g O_2 eq/h, $p = 4.33e\text{-}4$ and therefore statistically significant at $\alpha = 0.05$) during steady state operation. The suspended phase biomass contributed to 69.7 ± 4.5% of the total nitrogen removal, calculated using eq 1a. The higher overall N removal in the suspended phase was likely because of higher glycerol availability and degradation therein (*50*). Alternately, the utilization of exogenous and endogenous organic carbon compounds for the production of extracellular polymeric substances (EPS) by biofilm bacteria at the expense of lower energy for growth (*51*) might also have resulted in the lower N-removal therein.

Acknowledgments

This study was supported by the National Fish and Wildlife Foundation and the Water Environment Research Foundation Nutrient Challenge Program.

Supporting Information Available

Details pertaining to IFAS reactor operation; the extent of density gradient separation between ^{12}C and ^{13}C DNA fractions using SIP; specifics of the extant denitrification batch assays; relative concentrations of four dominant glycerol assimilating populations and two methylotrophic populations during this study; SB-IFAS reactor performance and a representative nitrate depletion curve for SIP experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

Literature Cited

(1) Claus, G.; Kutzner, H. J. Denitrification of nitrate and nitric acid with methanol as carbon source. *Appl. Environ. Microbiol.* **1985**, *22* (5), 378–381.

(2) Knowles, R. Denitrification. *Microbiol. Rev.* **1982**, *46* (1), 43–70.

(3) Timmermans, P.; Van Haute, A. Denitrification with methanol: Fundamental study of the growth and denitrification capacity of *Hyphomicrobium* sp. *Water Res.* **1983**, *17* (10), 1249–1255.

(4) Masih, A. M. M.; Albinali, K.; DeMello, L. Price dynamics of natural gas and the regional methanol markets. *Energy Policy* **2010**, *38* (3), 1372–1378.

(5) da Silva, G. P.; Mack, M.; Contiero, J. Glycerol: A promising and abundant carbon source for industrial microbiology. *Biotechnol. Adv.* **2009**, *27* (1), 30–39.

(6) Bill, K. A.; Bott, C. B.; Murthy, S. N. Evaluation of alternative electron donors for denitrifying moving bed biofilm reactors (MBBRs). *Water Sci. Technol.* **2008**, *60* (10), 2647–2657.

(7) Bodík, I.; Blstáková, A.; Sedlácek, S.; Hutnan, M. Biodiesel waste as source of organic carbon for municipal WWTP denitrification. *Bioresour. Technol.* **2009**, *100* (8), 2452–2456.

(8) Baytshtok, V.; Lu, H.; Park, H.; Kim, S.; Yu, R.; Chandran, K. Impact of varying electron donors on the molecular microbial ecology and biokinetics of methylotrophic denitrifying bacteria. *Biotechnol. Bioeng.* **2009**, *102* (6), 1527–1536.

(9) Ginige, M. P.; Hugenholtz, P.; Daims, H.; Wagner, M.; Keller, J.; Blackall, L. L. Use of stable-isotope probing, full-cycle rRNA analysis, and fluorescence *in situ* hybridization-microautoradiography to study a methanol-fed denitrifying microbial community. *Appl. Environ. Microbiol.* **2004**, *70* (1), 588–596.

(10) Ginige, M. P.; Keller, J.; Blackall, L. L. Investigation of an acetate-fed denitrifying microbial community by stable isotope probing, full-cycle rRNA analysis, and fluorescent *in situ* hybridization-microautoradiography. *Appl. Environ. Microbiol.* **2005**, *71* (12), 8683–8691.

(11) Hallin, S.; Pell, M. Metabolic properties of denitrifying bacteria adapting to methanol and ethanol in activated sludge. *Water Res.* **1998**, *32* (1), 13–18.

(12) Osaka, T.; Ebie, Y.; Tsuneda, S.; Inamori, Y. Identification of the bacterial community involved in methane-dependent denitrification in activated sludge using DNA stable-isotope probing. *FEMS Microbiol. Ecol.* **2008**, *64* (3), 494–506.

(13) Osaka, T.; Yoshie, S.; Tsuneda, S.; Hirata, A.; Iwami, N.; Inamori, Y. Identification of acetate- or methanol-assimilating bacteria under nitrate-reducing conditions by stable-isotope probing. *Microb. Ecol.* **2006**, *52* (2), 253–266.

(14) Sage, M.; Daufin, G.; Gésan-Guiziou, G. Denitrification potential and rates of complex carbon source from dairy effluents in activated sludge system. *Water Res.* **2006**, *40* (14), 2747–2755.

(15) Lu, H.; Chandran, K. Factors promoting emissions of nitrous oxide and nitric oxide from denitrifying sequencing batch reactors operated with methanol and ethanol as electron donors. *Biotechnol. Bioeng.* **2010**, *106* (3), 390–398.

(16) Baytshtok, V.; Kim, S.; Yu, R.; Park, H.; Chandran, K. Molecular and biokinetic characterization of methylotrophic denitrification using nitrate and nitrite as terminal electron acceptors. *Water Sci. Technol.* **2008**, *58* (2), 359–365.

(17) Aspegren, H.; Nyberg, U.; Andersson, B.; Gotthardsson, S.; Jansen, J. l. C. Post denitrification in a moving bed biofilm reactor process. *Water Sci. Technol.* **1998**, *38* (1), 31–38.

(18) Labelle, M.-A.; Juteau, P.; Jolicoeur, M.; Villemur, R.; Parent, S.; Comeau, Y. Seawater denitrification in a closed mesocosm by a submerged moving bed biofilm reactor. *Water Res.* **2005**, *39* (14), 3409–3417.

(19) Ødegaard, H.; Rusten, B.; Westrum, T. A new moving bed biofilm reactor-applications and results. *Water Sci. Technol.* **1994**, *29* (10−11), 157–165.

(20) Labbe, N.; Laurin, V.; Juteau, P. Microbiological community structure of the biofilm of a methanol-fed, marine denitrification system, and identification of the methanol-utilizing microorganisms. *Microb. Ecol.* **2007**, *53* (4), 621–30.

(21) Lemmer, H.; Zaglauer, A.; Neef, A.; Meier, H.; Amann, R. Denitrification in a methanol-fed fixed-bed reactor. Part 2: Composition and ecology of the bacterial community in the biofilms. *Water Res.* **1997**, *31* (8), 1903–1908.

(22) Satoh, H.; Yamakawa, T.; Kindaichi, T.; Ito, T.; Okabe, S. Community structures and activities of nitrifying and denitrifying bacteria in industrial wastewater-treating biofilms. *Biotechnol. Bioeng.* **2006**, *94* (4), 762–772.

(23) Madigan, M. T.; Martinko, J. M. *Brock Biology of Microorganisms*, 11th ed.; Prentice Hall: Upper Saddle River, NJ, 2006.

(24) Stumm, W.; Morgan, J. J. *Aquatic Chemistry Chemical Equilibria and Rates in Natural Waters*, 3rd ed ed.; John Wiley and Sons: New York, 1996.

(25) Schwartz, E., Analyzing microorganisms in environmental samples using stable isotope probing with $H_2{}^{18}O$. Cold Spring Harb. Protoc. 2009, doi:10.1101/pdb.prot5341.

(26) Kane, M. D.; Poulsen, L. K.; Stahl, D. A. Monitoring the enrichment and isolation of sulfate-reducing bacteria by using oligonucleotide hybridization probes designed from environmentally derived 16S rRNA sequences. *Appl. Environ. Microbiol.* **1993**, *59* (3), 682–686.

(27) Lane, D. J. *16S/23S rRNA Sequencing in Nucleic Acid Techniques in Bacterial Systematics*; Stackebrandt, E., Goodfellow, M., Eds.; Wiley: New York, 1991; pp 115−175.

(28) Ashelford, K. E.; Chuzhanova, N. A.; Fry, J. C.; Jones, A. J.; Weightman, A. J. New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. *Appl. Environ. Microbiol.* **2006**, *72* (9), 5734–5741.

(29) Nadkarni, M. A.; Martin, F. E.; Jacques, N. A.; Hunter, N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology* **2002**, *148* (1), 257–266.

(30) Eaton, A.; Clesceri, L.; Greenberg, A. *Standard Methods for the Examination of Water and Wastewater*, 21st ed.; APHA, AWWA, and WEF: Washington, DC, 2005.

(31) Grabinska-Loniewska, A. Biocenosis diversity and denitrification efficiency. *Water Res.* **1991**, *25* (12), 1575–1582.

(32) Tago, Y.; Yokota, A. *Comamonas badia* sp. nov., a floc-forming bacterium isolated from activated sludge. *J. Gen. Appl. Microbiol.* **2004**, *50* (5), 243–248.

(33) Tabrez Khan, S.; Hiraishi, A. *Diaphorobacter nitroreducens* gen. nov., sp. nov., a poly(3-hydroxybutyrate)-degrading denitrifying bacterium isolated from activated sludge. *J. Gen. Appl. Microbiol.* **2002**, *48* (6), 299–308.

(34) Khardenavis, A.; Kapley, A.; Purohit, H. Simultaneous nitrification and denitrification by diverse *Diaphorobacter* sp. *Appl. Microbiol. Biotechnol.* **2007**, *77* (2), 403–409.

(35) Sawada, H.; Kuykendall, L. D.; Young, J. M. Changing concepts in the systematics of bacterial nitrogen-fixing legume symbionts. *J. Gen. Appl. Microbiol.* **2003**, *49* (3), 155–79.

(36) Maszenan, A. M.; Rees, G. N. *Tessaracoccus bendigoensis* gen. nov., sp. nov., a Gram-positive coccus occurring in regular packages or tetrads, isolated from activated sludge biomass. *Int. J. Syst. Bacteriol.* **1999**, *49* (2), 459–468.

(37) Allegrucci, M.; Sauer, K. Characterization of colony morphology variants isolated from *Streptococcus pneumoniae* biofilms. *J. Bacteriol.* **2007**, *189* (5), 2030–2038.

(38) Allison, D. G.; Gilbert, P.; Lappin-Scott, H. M.; Wilson, M. *Community Structure and Co-operation in Biofilm*; Cambridge University Press: Cambridge, 2000.

(39) Kirisits, M. J.; Prost, L.; Starkey, M.; Parsek, M. R. Characterization of colony morphology variants isolated from *Pseudomonas aeruginosa* biofilms. *Appl. Environ. Microbiol.* **2005**, *71* (8), 4809–4821.

(40) Davey, M. E.; O'Toole, G. A. Microbial biofilms: from ecology to molecular genetics. *Microbiol. Mol. Biol. Rev.* **2000**, *64* (4), 847–867.

(41) Stewart, P. S.; Franklin, M. J. Physiological heterogeneity in biofilms. *Nat. Rev. Microbiol.* **2008**, *6* (3), 199–210.

(42) Daniel, R. M.; Limmer, A. W.; Steele, K. W.; Smith, I. M. Anaerobic growth, nitrate reduction and denitrification in 46 *Rhizobium* strains. *J. Gen. Microbiol.* **1982**, *128* (8), 1811–1815.

(43) Lu, S.; Ryu, S. H.; Chung, B. S.; Chung, Y. R.; Park, W.; Jeon, C. O. *Simplicispira limi* sp. nov., isolated from activated sludge. *Int. J. Syst. Evol. Microbiol.* **2007**, *57* (1), 31–34.

(44) Su, J. J.; Kafkewitz, D. Toluene and xylene degradation by a denitrifying strain of *Xanthomonas maltophilia* with limited or no oxygen. *Chemosphere* **1996**, *32* (9), 1843–1850.

(45) Akunna, J. C.; Bizeau, C.; Moletta, R. Nitrate and nitrite reductions with anaerobic sludge using various carbon sources: Glucose, glycerol, acetic acid, lactic acid and methanol. *Water Res.* **1993**, *27* (8), 1303–1312.

(46) Miyata, A.; Yoshida, T.; Yamaguchi, K.; Yokoyama, C.; Tanabe, T.; Toh, H.; Mitsunaga, T.; Izumi, Y. Molecular cloning and expression of the gene for serine hydroxymethyltransferase from an obligate methylotroph *Hyphomicrobium methylovorum* GM2. *Eur. J. Biochem.* **1993**, *212* (3), 745–50.

(47) Ichiki, H.; Tanaka, Y.; Mochizuki, K.; Yoshimatsu, K.; Sakurai, T.; Fujiwara, T. Purification, characterization, and genetic analysis of Cu-Containing dissimilatory nitrite reductase from a denitrifying halophilic archaeon *Haloarcula marismortui*. *J. Bacteriol.* **2001**, *183* (14), 4149–4156.

(48) Völkl, P.; Huber, R.; Drobner, E.; Rachel, R.; Burggraf, S.; Trincone, A.; Stetter, K. O. *Pyrobaculum aerophilum* sp. nov., a novel nitrate-reducing hyperthermophilic archaeum. *Appl. Environ. Microbiol.* **1993**, *59* (9), 2918–2926.

(49) Hinojosa, J.; Riffat, R.; Fink, S.; Murthy, S.; Selock, K.; Bott, C.; Takacs, I.; Dold, P.; Wimmer, R. Estimating the kinetics and stoichiometry of heterotrophic denitrifying bacteria with glycerol as an external carbon source In Proceedings of the *Water Environment Federation Technical Exhibition and Conference (WEFTEC)*, Water Environment Federation: Alexandria, VA, Chicago, IL, 2008; pp 274−288.

(50) Perez, J.; Picioreanu, C.; van Loosdrecht, M. Modeling biofilm and floc diffusion processes based on analytical solution of reaction-diffusion equations. *Water Res.* **2005**, *39* (7), 1311–1323.

(51) Boe-Hansen, R.; Albrechtsen, H.-J.; Arvin, E.; Jørgensen, C. Bulk water phase and biofilm growth in drinking water at low nutrient conditions. *Water Res.* **2002**, *36* (18), 4477–4486.

ES102124F