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Alcohol dehydrogenase expression as a biomarker of denitrification activity in activated sludge using methanol and glycerol as electron donors

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Summary

Carbon sources such as methanol and glycerol are used for enhancing denitrification at wastewater treatment plants, which are required to meet increasingly stringent effluent nitrogen limits. Consequently, dosing strategies for these compounds could benefit from the development and application of molecular activity biomarkers to infer and distinguish between methanol- or glycerol-based denitrification in activated sludge. In this study, the applicability of genes coding for methanol dehydrogenase (*mdh2* and *mxaF*) and glycerol dehydrogenase (*dhaD*) as potential biomarkers of denitrification activity using these specific substrates was explored and confirmed using a two-pronged approach. First, during short-term spikes of activated sludge biomass with glycerol, the ability of *dhaD* mRNA concentrations to closely track nitrate depletion profiles was demonstrated. Second, a high-degree of correlation of the mRNA concentrations of *mdh2*, *mxaF* and *dhaD* with methanol- and glycerol-based denitrification kinetics during long-term bioreactor operation using these substrates was also shown. Based on these results, expression of *mdh2*, *mxaF* and *dhaD* genes are promising biomarkers of *in situ* denitrification activity on methanol and glycerol, respectively, in mixed-culture engineered wastewater treatment processes.

Introduction

Methanol is commonly used as a carbon source to enhance denitrification in wastewater treatment facilities (Louzeiro *et al*., 2002). However, with the price variability (Masih *et al*., 2010) and safety concerns associated with the low flash-point [49°C (Flick, 1998)] of methanol, alternative carbon sources need to be considered. Glycerol is especially appealing since it is a byproduct of biodiesel production (da Silva *et al*., 2009), and expected to be increasingly available. There are also indications that glycerol can foster higher denitrification kinetics than methanol (Bill *et al*., 2009; Bodík *et al*., 2009).

Currently, denitrification kinetics and activity in activated sludge are commonly monitored using 'extant' specific denitrification assays (for instance, as described previously (Baytshtok *et al*., 2009; Lu and Chandran, 2010). Such extant specific denitrification assays rely upon the interpretation and analysis of nitrate and nitrite depletion profiles to infer denitrification kinetics of the overall denitrifying population in activated sludge. However, these assays cannot be used to infer the contributions of different microbial subgroups degrading different carbon sources to the overall observed nitrogen depletion profiles. Therefore, carbon source-specific biomarkers of denitrification need to be developed. Application of such biomarkers could not only help track carbon-specific denitrification activity but also prove to be a valid screening tool prior to the application of the carbon sources to enhance denitrification in wastewater treatment plants, typically as a final nitrate removal step in Modified Lutzak Ettinger or Bardenpho treatment configurations (Grady *et al*., 1999).

Biomarkers and assays targeting carbon metabolism genes could potentially implicate denitrification activity fostered by a certain carbon source. For instance, alcohol dehydrogenase (ADH) enzymes play a crucial role in the carbon metabolism of many alcohols during denitrification (Bullock, 1990). Methanol dehydrogenase carries out the oxidation of methanol to formaldehyde, a key intermediate, which feeds into both assimilative and dissimilative metabolism in Gram-negative methylotrophic bacteria (McDonald and Murrell, 1997; Kalyuzhnaya *et al*., 2008). In particular, the gene encoding the large subunit of methanol dehydrogenase, *mxaF* is highly conserved among all Gram-negative methylotrophs and has been used as a functional marker to detect methylotrophs in the environment (McDonald and Murrell, 1997; McDonald *et al*., 2008; Chistoserdova *et al*., 2009). However, some

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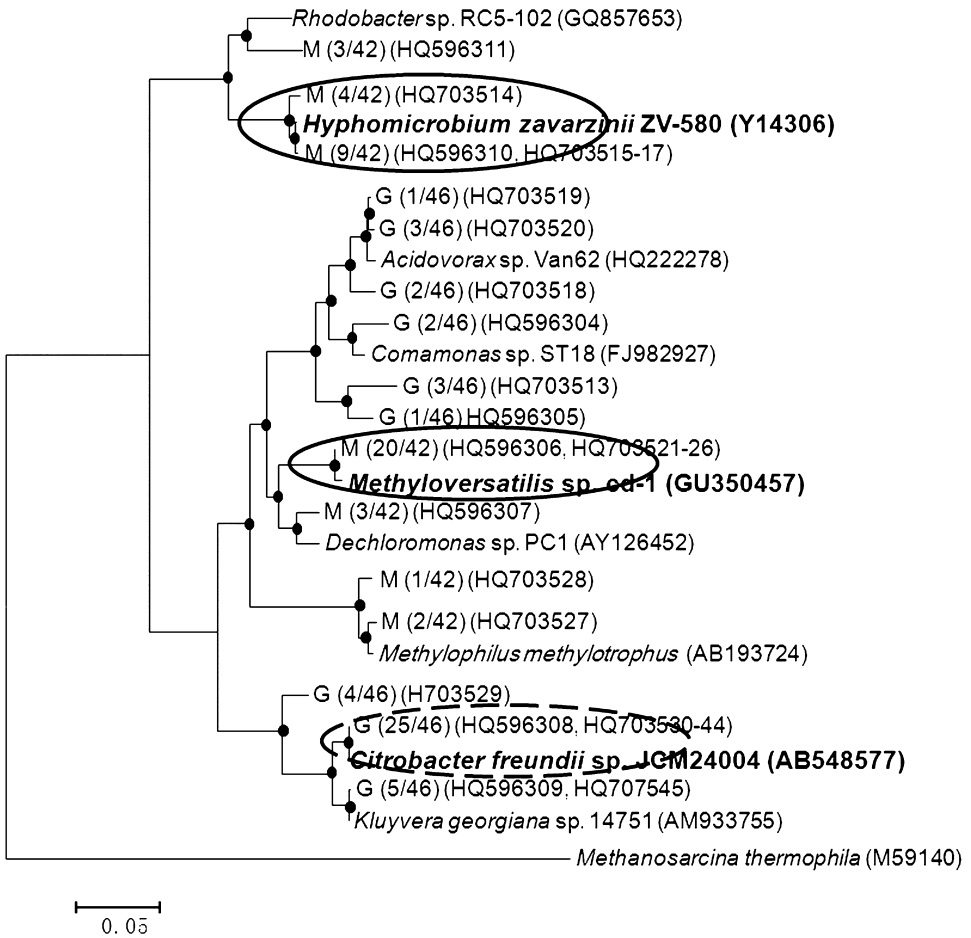

Fig. 1. 16S rRNA-based phylogenetic tree depicting overall populations in the denitrifying reactor with methanol (M) and glycerol (G) as carbon sources. Numbers in parentheses represent fraction of clones most closely associated with a given phylogenetic lineage, and circles indicate dominant species found in the reactor with methanol (◯) and glycerol (◌) as carbon sources.

methylotrophic bacteria lack *mxa* and instead possess other methanol dehydrogenases, such as *mdh2* (Kalyuzhnaya *et al*., 2008).

Glycerol-based denitrification involves glycerol kinase (*glpK*) or glycerol dehydrogenase (*dhaD*) (Forage and Lin, 1982; Austin and Larson, 1991). Thus, either gene could be a valid possible biomarker of glycerol-based denitrification activity. Alternate fermentative glycerol pathways also exist, for example, encoded by glycerol dehydratase (*dhaB*) and 1,3-propanediol dehydrogenase (*dhaT*) as summarized in da Silva and colleagues 2009 and Fig. S1. However, these pathways and genes are not involved in glycerol-based denitrification and are thus not appropriate biomarker candidates for glycerol-based denitrification. Therefore, the expression of the *dhaD* gene, which catalyses the first step of glycerol oxidation during denitrification, was explored as a biomarker of glycerol assimilation activity.

The specific objectives were to:

(i) characterize differences in microbial ecology and kinetics of a sequencing batch reactor (SBR) operated sequentially with methanol and glycerol as electron donors;
(ii) examine the applicability of using *mdh2*, *mxaF* and *dhaD* messenger RNA (mRNA) concentrations as biomarkers of carbon-specific denitrification activity

Results and discussion

Microbial ecology of SBR biomass fed sequentially with methanol and glycerol

Based on 16S rRNA gene clone libraries constructed using biomass samples obtained on day 100 (methanol fed, Phase I) and day 200 (glycerol fed, Phase II), the microbial community supported by these two carbon sources was substantially different (Fig. 1). During Phase I, bacteria closely related to *Methyloversatilis* spp. (20/42)

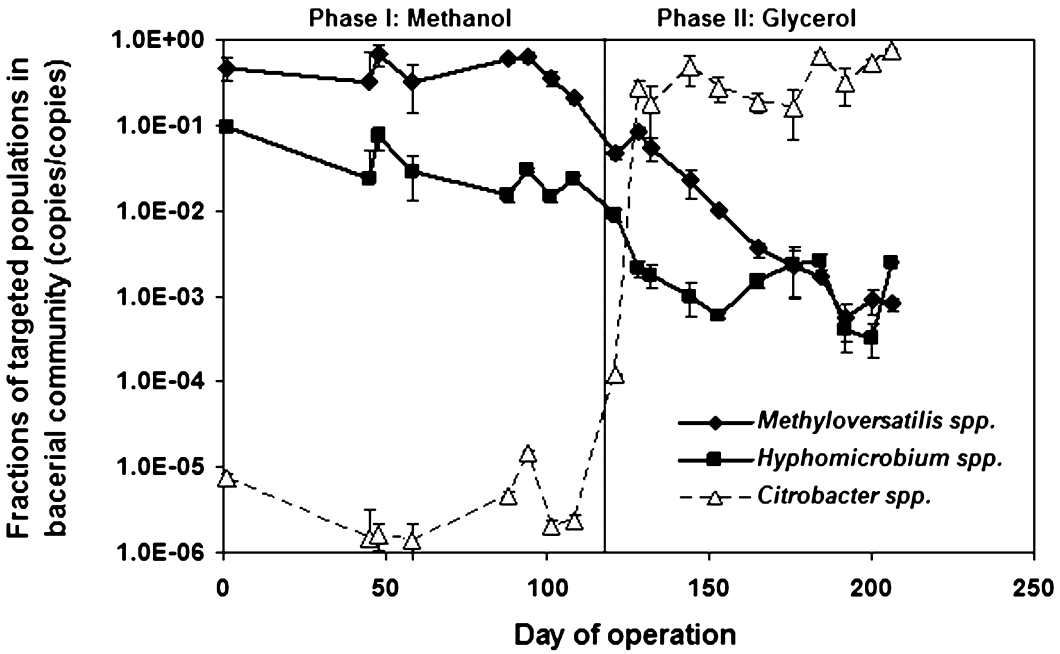

Fig. 2. Fractions of *Methyloversatilis* spp. (—◆—), *Hyphomicrobium* spp. (—■—), *Citrobacter* spp. (—△—) in the overall bacterial community during methanol and glycerol fed phases of SBR operation (population concentrations normalized to total eubacterial concentration). Error bars represent standard deviation from triplicate qPCR assays.

and *Hyphomicrobium* spp. (13/42) were enriched in the SBR as inferred from the clone libraries. These two methylotrophic populations were not found in the clone libraries of Phase II, which were in turn enriched in *Citrobacter* spp.-related bacteria (25/46, Fig. 1). The enrichment of *Methyloversatilis* spp. and *Hyphomicrobium* spp., relative to *Citrobacter* spp. during Phase 1 was confirmed by qPCR (Fig. 2). However, both population fractions decreased even during Phase 1, possibly reflecting the build-up of non-methylotrophic bacteria within the reactor sustained via secondary degradation of the active methylotrophic biomass (Fig. 2). During Phase II, the fractions of *Hyphomicrobium* spp. and *Methyloversatilis* spp. decreased even further, while the fraction of *Citrobacter* spp.-related species rose rapidly subsequently stabilized at approximately 90% of the total bacterial concentration (Fig. 2).

The enrichment of the two methylotrophic populations during Phase I of this present study was in good agreement with a previous study, wherein methanol assimilation in a methanol fed denitrifying SBR was primarily attributed via stable isotope probing (SIP), to *Methyloversatilis* spp. and *Hyphomicrobium* spp. (Baytshtok *et al*., 2008; 2009). A quantitative comparison to the previous study in terms of fractions is precluded by the lack of eubacterial concentrations in the previous study (Baytshtok *et al*., 2008; 2009). Nevertheless, *Methyloversatilis* spp. were more abundant than *Hyphomicrobium* spp. in both Phase I of this present study as well as the previous study referenced (Baytshtok *et al*., 2008; 2009). The higher fraction of *Methyloversatilis* spp. (44.5 $\pm$ 17.3%) relative to *Hyphomicrobium* spp. (3.8 $\pm$ 2.3%, Fig. 2) was likely due to the higher affinity of the former for methanol as also observed and suggested recently (Baytshtok *et al*., 2009). In terms of metabolism, some *Hyphomicrobium* spp. only utilize C1 compounds for energy and biomass synthesis (Harder and Attwood, 1978; Miyata *et al*., 1993) whereas other *Hyphomicrobium* strains can indeed grow on multi-carbon compounds, including glycerol (Garrity *et al*., 2005). *Methyloversatilis* spp. can also grow on methanol as well as multi-carbon sources, including glycerol (Kalyuzhnaya, 2010). However, the significant decrease of both *Hyphomicrobium* spp. and *Methyloversatilis* spp. during Phase II (glycerol fed, Fig. 2) can potentially be attributed either to their out-competition by more rapidly growing glycerol assimilating species such as *Citrobacter* spp., which were enriched during the glycerol feeding phase. *Citrobacter* spp. can indeed grow anaerobically on glycerol as the sole carbon and energy source (Homann *et al*., 1990). *Citrobacter* spp. also possess some metabolic flexibility as demonstrated by their ability to denitrify under both aerobic and anaerobic conditions (Huang and Tseng, 2001). The ecology of glycerol-based denitrification in activated sludge is only just emerging and the ability of *Citrobacter* spp. to denitrify using glycerol has not been demonstrated elsewhere.

Among additionally identified bacteria in the clone libraries of Phase II (Fig. 1), *Comamonadaceae* spp.-

related bacteria, such as *Acidovorax* spp. and *Comamonas* spp., can denitrify using glycerol as the carbon source (Heylen *et al*., 2008; Lu and Chandran, 2010). The *Comamonas* spp. 16S rRNA gene sequences detected in this study were more than 98% similar to those of *C. badia* that were found to assimilate ^{13}C glycerol in a denitrifying biofilm reactor (Lu and Chandran, 2010). Thus, the presence of these additional bacteria in the glycerol fed SBR was also consistent with their reported metabolic capacities. As inferred from their respective genome sequences, *Comamonas testosteroni* KF-1 (http://genome.jgi-psf.org/comte/comte.info.html), *Comamonas testosteronii* CNB-2 (Ma *et al*., 2009) and *Acidovorax* sp. JS42 (Copeland *et al*., 2006) employ the glycerol kinase pathway (encoded by the *glp* system) to transform glycerol instead of the *dha* encoded pathway. Therefore, it is acknowledged that by tracking the mRNA concentrations of *dha*D, the activity of *Comamonadaceae* was likely not included. Nevertheless, based on the positive results obtained herein with the *mxaF, mdh2* and *dhaD* genes, it might be possible to similarly track additional glycerol degradation pathways of *Comamonadaceae* as well.

Impact of varying electron donors on denitrification performance and kinetics

During methanol feed (Phase I), a high degree of nitrate removal (95.4 ± 5.9%, $n = 21$) with minimal nitrite accumulation (< 1 mg N l^{-1}) was achieved. After changing the carbon source from methanol to glycerol (Phase II), transient nitrate accumulation in the range of 20–30 mg N l^{-1} was observed (data shown in Supporting Information, Fig. S2). However, near complete nitrate removal was rapidly achieved subsequently within 6 days and sustained thereafter through the end of the study (97.3 ± 3.5%, $n = 24$) with minimal nitrite accumulation. During Phase I, the methanol specific denitrification rate (sDNR) values were in the range 1078 ± 236 mg N g^{-1} volatile suspended solids (VSS) day^{-1} ($n = 11$). Upon switching to glycerol, the methanol sDNR values decreased significantly (at $\alpha = 0.05$) to 332 ± 198 mg N g^{-1} VSS day^{-1} ($n = 14$) (Fig. 3A). Concurrently, the glycerol sDNR values increased approximately 26-fold, from 36 ± 13 mg N g^{-1} VSS day^{-1} ($n = 11$) to 938 ± 434 mg N g^{-1} VSS day^{-1} ($n = 14$) (Fig. 3B). These inverse trends in sDNR values for methanol and glycerol corresponded well with the washout of known methanol assimilating bacteria in the SBR, and their replacement with a distinct population fostered on glycerol (Fig. 2). From an engineering perspective, the above results also suggest that despite long-term operation with methanol, denitrifying wastewater treatment plants should be able to rapidly adapt to glycerol as a new carbon source. Additionally, minimal re-sizing of anoxic bioreactors for denitrification (which depends on denitrification kinetics) could be expected after the switch to glycerol given the statistically similar ($\alpha = 0.05$) steady-state sDNR values on methanol and glycerol during their respective feeding periods.

mdh2*,* mxaF *and* dhaD *mRNA profiles during extant batch denitrification assays*

Addition of glycerol to SBR biomass samples to initiate the batch sDNR assays during phase II (glycerol fed) significantly stimulated the transcription of *Citrobacter dhaD*, but not *Methyloversatilis mdh2* and *Hyphomicrobium mxaF* (Fig. 4-a2). The rapid increase in *Citrobacter* spp. *dhaD* mRNA concentrations after the addition of glycerol is consistent with previous studies, which showed induction of *dhaD* and other genes of the *dha* regulon in cultures exposed to glycerol or dihydroxyacetone, an intermediate of glycerol oxidation (Ruch *et al*., 1974; Forage and Lin, 1982). Notably, the short-term *dhaD* mRNA profile (which is related to glycerol oxidation *sensu-stricto*) also paralleled the nitrate reduction profile (Fig. 4-a1), thereby reflecting a close metabolic link between nitrate reduction and glycerol oxidation. When the rate of nitrate depletion slowed down presumably in response to glycerol limitation (around $t = 10$ h, Fig. 4-a1) *dhaD* mRNA concentrations also reverted back to their initial level of about 10^{-5} copies/copies (Fig. 4-a2). In contrast the mRNA concentrations of *mdh2* and *mxaF*, were mostly unresponsive to the glycerol spike (Fig. 4-a2), reflecting mutually exclusive and carbon source-specific stimulation of the respective ADH genes.

During the methanol batch tests, *Methyloversatilis* spp. *mdh2* and *Hyphomicrobium* spp. *mxaF* mRNA concentrations increased by factors of 2.2 ± 0.6 and 2.3 ± 0.1, respectively, after the addition of methanol (Fig. 4-b2). The lower increase in *mdh2* and *mxaF* mRNA concentrations (in contrast to the nearly 100-fold increase in *dhaD* mRNA concentrations) were presumably owing to the minimal methanol oxidation capability of the SBR during the glycerol feeding phase, during which these tests were conducted. There was no significant change in *Citrobacter* spp. *dhaD* mRNA concentrations during the entire methanol batch test (Fig. 4-b2). The marginal increase of *mdh2* and *mxaF* expression was paralleled by a gradual decrease in nitrate concentrations during the batch methanol spike to the glycerol enriched biomass (Fig. 4-b1), again underlining the lack of overlap between glycerol oxidation and methanol oxidation capacities in the microbial community within the SBR. This lack of functional overlap is also in parallel with the lack of structural overlap in the microbial communities in the SBR during the two phases of operation (Fig. 1). A similar exclusivity in glycerol- and methanol-based denitrifying bacterial populations in activated sludge using ^{13}C DNA

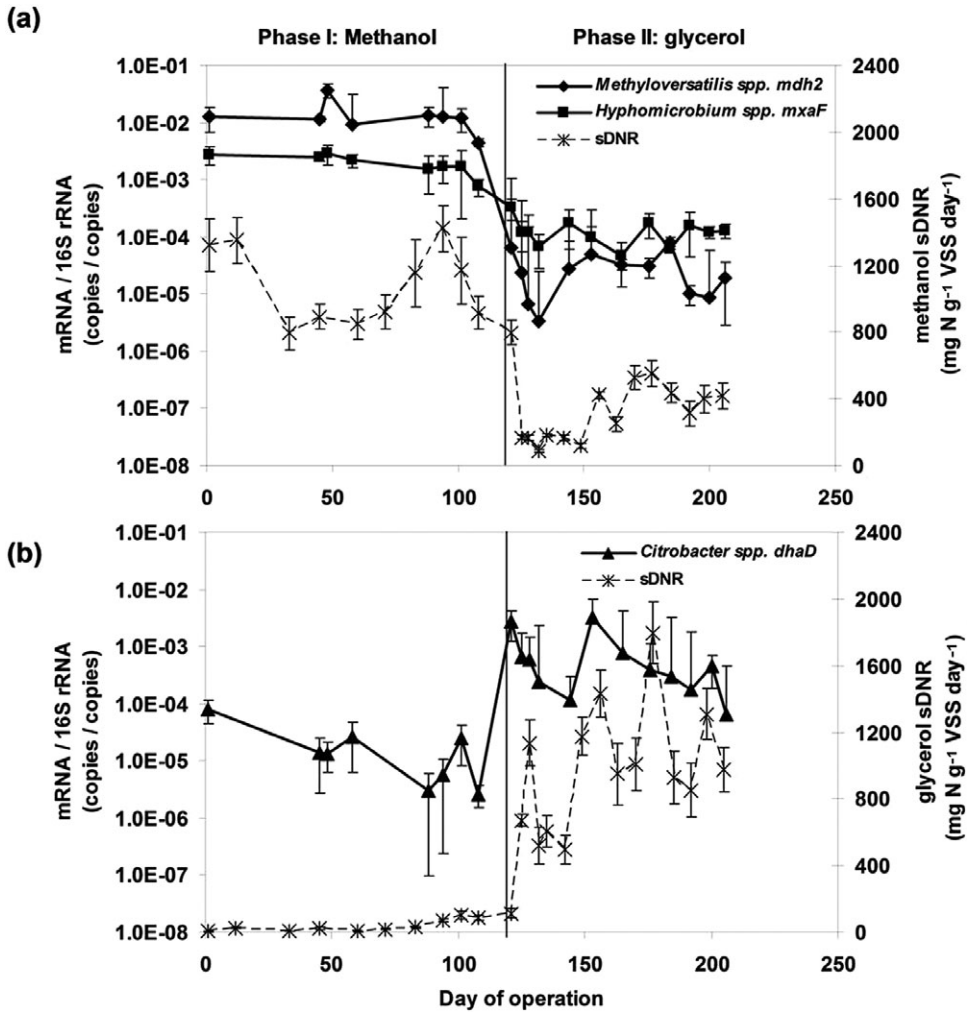

Fig. 3. Time-series profiles during long-term SBR operation of (a) *Methyloversatilis* spp. *mdh2* (—◆—) and *Hyphomicrobium* spp. *mxaF* (—■—) mRNA concentrations and methanol sDNR values (—✳—); (b) *Citrobacter* spp. *dhaD* (—▲—) mRNA concentrations and glycerol sDNR values (—✳—). Error bars represent standard deviation from triplicate qPCR assays.

SIP has also been recently demonstrated (Lu and Chandran, 2010).

Long-term tracking of denitrification activity via alcohol dehydrogenase gene expression

The parallel trends between expression of genes coding for methanol or glycerol oxidation and denitrification activity using these carbon sources, which was observed during short-term biokinetic assays, were repeated over long-term SBR operation as well. During phase I, *Methyloversatilis* spp. *mdh2* and *Hyphomicrobium* spp. *mxaF* mRNA concentrations were higher than that of *Citrobacter* spp. *dhaD* ($\alpha = 0.05$, Fig. 3). During Phase II, the *mdh2* and *mxaF* mRNA concentrations decreased by factors of 525.0 ± 58.7 and 17.3 ± 9.3 respectively (Fig. 3A). In parallel, the methanol sDNR values also decreased significantly except a slight increase around 150–200 days of operation (Fig. 3A). The increase in methanol sDNR and accompanying increase of *Hyphomicrobium mxaF* around 150–200 days (Fig. 3A) could possibly indicate the adaption of methanol dehydrogenase expression to new substrates, such as glycerol or byproducts from glycerol metabolism in the SBR (Schär *et al.*, 1985). In direct contrast, the *Citrobacter* spp. *dhaD* mRNA concentrations increased 37.7 ± 5.3 times and paralleled the trend in glycerol sDNR values (Fig. 3B).

Specific denitrification rates and corresponding weighted biomarker concentrations (calculated based on Eq. S1), showed statistically significant positive correlations at $\alpha = 0.05$ (Table 1). The strong correlations underscore the potential link between functional gene transcription and corresponding carbon specific denitrification activity. Notwithstanding these observations, mechanism-based inferences may not be drawn from these correlations and further studies linking

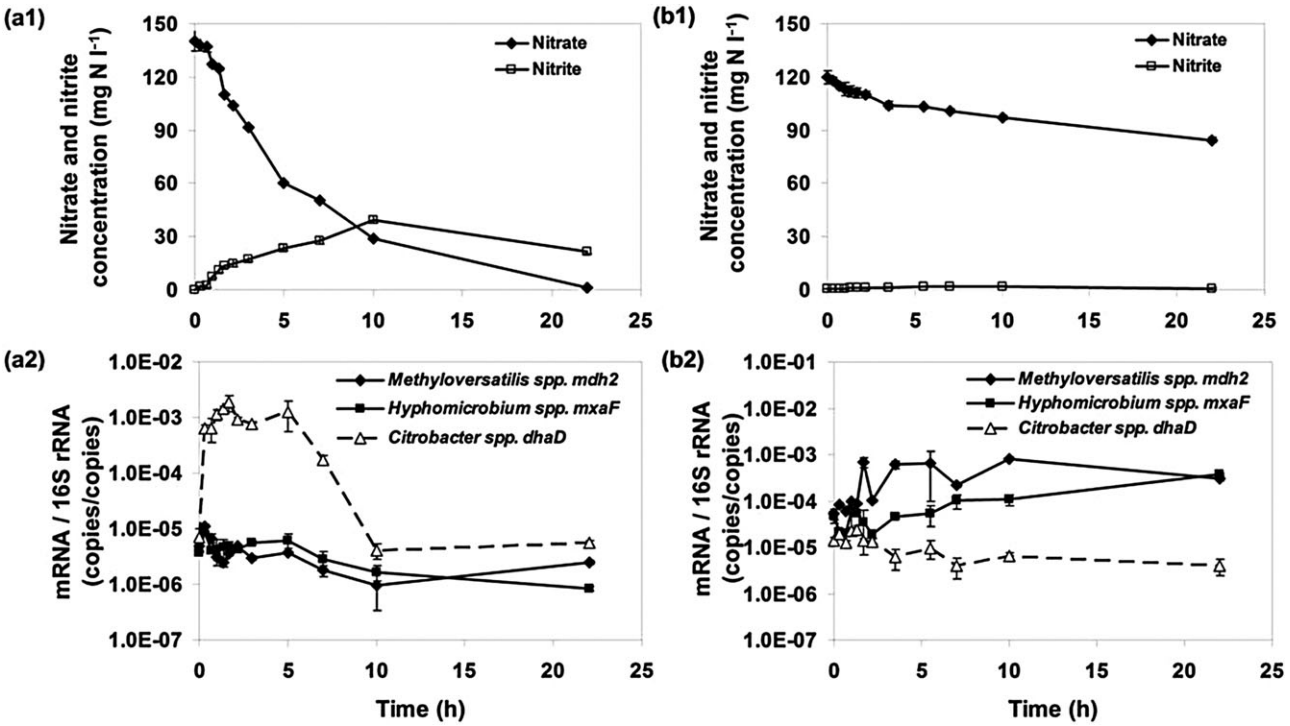

Fig. 4. Time-series profiles of nitrate (—◆—) and nitrite (-□-) concentrations, and *Methyloversatilis* spp. *mdh2* (—◆—), *Hyphomicrobium* spp. *mxaF* (—■—), *Citrobacter* spp. *dhaD* (—△—) mRNA concentrations during a representative extant batch denitrification test with (a) glycerol and (b) methanol as external carbon source. Error bars represent standard deviation from triplicate qPCR assays.

ADH gene transcription and ADH enzyme activity are needed.

These results are significant, since they suggest that the biomarkers developed herein provide a functional activity indicator of the overall SBR population in terms of either methanol or glycerol oxidation activities. This is rather positive also from a wastewater treatment perspective, where the focus is specifically on maximizing microbial functionality (*in casu*, ability to oxidize methanol or glycerol during denitrification and corresponding activity) and determination of specific activity measures to describe the different microbial active fractions (Grady *et al.*, 1999). Use of the biomarkers developed herein could also potentially help to address the impact of intermittent aeration [which is typically employed during wastewater treatment (Grady *et al.*, 1999)] on carbon-specific denitrification rates and ultimately result in better informed bioreactor designs for engineered denitrification. Additionally, despite the presence of downstream steps in the overall process of carbon source oxidation and energy synthesis, it is significant that the genes coding for one of the first steps in this oxidation process (alcohol dehydrogenation to aldehyde) could still correlate well with the rate of electron acceptor (*in casu*, nitrate) reduction.

In sum, this study provides good evidence on the use of select alcohol dehydogenase gene mRNA concentrations as biomarkers of *in situ* denitrification activity fostered by specific carbon sources. The basis for this link is that changes in gene transcription are one of the most fundamental responses of a cell to changes in the extracellular environment such as stress or substrate availability. Similar links between mRNA concentrations, cellular growth rates and metabolic activity have been recently demonstrated in *Nitrosomonas europaea*, an autotrophic ammonia oxidizing bacterium (Chandran and Love, 2008), anaerobic ammonia oxidizing bacterial

Table 1. Bivariate correlation analysis of denitrification activities and weighted potential biomarker concentrations.

	Methanol sDNR and *Hyphomicrobium mxaF*	Methanol sDNR and *Methyloversatilis mdh2*	Glycerol sDNR and *Citrobacter dhaD*
r	0.505	0.609	0.513
P	0.023	0.004	0.021

r, Pearson correlation; *P*, *P*-value (two-tailed); sample size = 20.

mixed-cultures (Park *et al*., 2010) and in the yeast, *Saccharomyces cerevisiae* (Airoldi *et al*., 2009).

Notwithstanding the positive results obtained herein, some limitations must be considered. For example, the prospect of other genes coding for methanol oxidation in some bacteria as yet not detected cannot be overlooked. Additionally, given differences in the structural and functional ecology of different denitrifying wastewater treatment plants, it might be appropriate to develop more broadly targeted biomarkers to detect the concentrations and activity of methanol and glycerol assimilating bacteria therein. Furthermore, methanol dehydrogenase can oxidize a wide range of primary alcohols and aldehydes, albeit at lower rates (de Vries *et al*., 1990). As a result, expression of methanol dehydrogenase could also be correlated with denitrification fostered by other carbon sources, especially in bioreactors or other natural and engineered environments exposed to these alternate substrates. Therefore, further studies directed towards additional biomarker development and application, are recommended, particularly with additional carbon sources and their mixtures.

Experimental procedures

Operation of denitrifying SBR

A methylotrophic enrichment consortium was cultivated at 21°C in a methanol fed denitrifying SBR with a volume of 9.2 l, hydraulic retention time of 1 day, and solids retention time of 10 days. The SBR was seeded with methylotrophic enrichment culture obtained from a previously described identically operated bioreactor (Baytshtok *et al*., 2008; 2009; Lu and Chandran, 2010). Each SBR cycle was 6 h long and 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 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, USA). The pH of the SBR was automatically controlled at 7.5 ± 0.1 using concentrated hydrochloric acid. The SBR was operated for 120 days with methanol (Phase I) and 90 days with glycerol (Phase II) as electron donors using nitrate as the terminal electron acceptor. For both phases, the influent electron donor and acceptor concentrations were 500 mg chemical oxygen demand (COD) per litre and 100 mg N l^{-1} respectively. The SBR feed medium was made up with tap water and also contained (per litre), 0.2 g $MgSO_4 \bullet 7H_2O$, 0.02 g $CaCl_2 \bullet 2H_2O$, 0.087 g K_2HPO_4 and 1 ml trace elements solution (10 mg $Na_2MoO_4 \bullet 2H_2O$, 172 mg $MnCl_2 \bullet 4H_2O$, 10 mg $ZnSO_4 \bullet 7H_2O$, 0.4 mg $CoCl_2 \bullet 6H_2O$ in a total volume made up to 100 ml with distilled water).

Performance and extant denitrification kinetics measurements

SBR performance was determined by measuring effluent nitrate, nitrite and total reactor and effluent COD, all according to standard methods (Eaton *et al*., 2005). Denitrification kinetics were determined via extant batch assays as described previously (Baytshtok *et al*., 2009). Briefly, 500 ml biomass samples were withdrawn from the SBR just prior to the end of the react phase, centrifuged at 5000 *g* for 5 min and re-suspended in nitrate- and carbon-free medium. The initial concentrations of denitrifying biomass in methanol and glycerol batch tests were 0.72 ± 0.12 and 0.68 ± 0.07 g volatile suspended solids (VSS) l^{-1} respectively. The washed biomass samples were rendered anoxic with N_2 gas. The extant batch denitrification assays were initiated by adding sodium nitrate at $t = 0$ min to achieve an initial concentration of 100 mg N l^{-1}. After an initial endogenous period of 20 min, the electron donor (methanol or glycerol) was added at $t = 21$ min at an initial concentration of 250 mg COD l^{-1}, to render the electron donor the ultimate limiting nutrient based on a stoichiometric COD:N requirement of 4:1 for methanol (Tchobanoglous *et al*., 2003) and 5:1 for glycerol (Grabinska-Loniewska, 1991). The initial slope of the nitrate depletion profile (obtained during non-limiting carbon or nitrogen denitrification activity) was normalized to the initial biomass concentrations to obtain the specific denitrification rates (expressed as mg N g^{-1} VSS day^{-1}).

DNA extraction and phylogenetic analysis

Genomic DNA was extracted from 1 ml of SBR biomass (DNeasy Blood and Tissue Kit, Qiagen, Valencia, CA, USA). The microbial community composition on day 100 (methanol fed, Phase I) and day 200 (glycerol fed, Phase II) was inferred via cloning and sequencing by amplifying sample DNA against eubacterial 16S rRNA primers 338f (5′-ACTCCTACGGGAGGCAGC-3′) (Amann *et al*., 1990) and 1387r (5′-GGGCGGWGTGTACAAGGC-3′) (Marchesi *et al*., 1998). Amplicons were cloned (TOPO TA Cloning Kit for Sequencing, Invitrogen, Carlsbad, CA, USA), and 50 colonies for each sample were randomly picked for sequencing (Macrogen USA, Rockville, MD, USA). Phylogenetic trees were generated using MEGA 4.0 with the neighbour-joining method and the substitution model of Jukes-Cantor. *Methanosarcina thermophila* served as the outgroup. The sequence data generated in this study have been deposited in GenBank under accession numbers HQ596303–HQ596311 and HQ703513–HQ703545.

Quantification of dominant microbial populations in the SBR via qPCR

The concentrations of total bacteria, *Methyloversatilis* spp., *Hyphomicrobium* spp. and *Citrobacter* spp. were determined via triplicate qPCR assays performed using a Bio-Rad *iQ5* real-time PCR detection system in 25 μl volumes, containing 12.5 μl iQ SYBR Green Supermix (Bio-Rad, Valencia, CA, USA), 1 μl each of forward and reverse primers (final concentration of 200 nM), 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 qPCR conditions were as follows: 5 min at 95°C, followed by 40 cycles of 94°C for 30 s, 30 s at specific annealing temperature (T_a, Table 2), and 72°C for 45 s and final extension at 72°C for 5 min. Standard curves were con-

Table 2. qPCR primers developed and employed in this study.

Target group	Primer name	Sequence (5′ to 3′)	T_a (°C)	Source
Eubacteria (16S rRNA)	EubF	TCCTACGGGAGGCAGCAGT	58	Nadkarni *et al*. (2002)
	EubR	GGACTACCAGGGTATCTAATCCTGTT		
Methyloversatilis spp. (16S rRNA)	MuF	AAGGCCTACCAAGGCAACGA	55	Baytshtok *et al*. (2008)
	MuR	ACCGTTTCGTTCCTGCCGAA		
Hyphomicrobium spp. (16S rRNA)	HzF	ACAATGGGCAGCAACACAGC	57	Baytshtok *et al*. (2008)
	HzR	ATTCACCGCGCCATGCTGAT		
Citrobacter spp. (16S rRNA)	CfF	AGCGCAACCCTTATCCTTTGTTGC	58	This study
	CfR	TCGCGAGGTCGCTTCTCTTTGTAT		
Methyloversatilis spp. (*mdh2*)	Mu_mdh2	TGCTGTTCGAGTACAAGGATCCGA	58	This study
	Mu_mdh2	TCACGAAGAAGTAGCCATTGCGGT		
Hyphomicrobium spp. (*mxaF*)	Hz_mxaF	TGGACCACAAGGGCAAGGAAATCT	58	This study
	Hz_mxaR	ATTCGTGCAGATGTGGTTGATGCC		
Citrobacter spp. (*dhaD*)	Cf_dhaF	TGGAAGAGATCGAAACCGTGCTGA	56	This study
	Cf_dhaR	TCACCGAGAACGGCATATTGTGGA		

structed with serial decimal dilutions of plasmids containing the target amplicon inserts. PCR product specificity was confirmed by the presence of a single peak during melt curve analysis and a single-band of expected molecular size by agarose gel electrophoresis. The fractions of *Methyloversatilis* spp., *Hyphomicrobium* spp. and *Citrobacter* spp. in the reactor were determined by dividing their respective concentrations by the eubacterial concentrations.

RNA extraction and quantification of ADH mRNA concentrations

Total RNA was isolated from 1 ml of SBR biomass, stabilized with 250 µl RNA Protect Bacteria Reagent (Qiagen) and stored at –80°C using the PureLink RNA Mini Kit (Invitrogen). cDNA synthesis was performed using the QuantiTect Reverse Transcriptase kit (Qiagen). qPCR primers were designed to specifically target the cDNA of *Methyloversatilis* spp. *mdh2* (GenBank: EU548062) (Kalyuzhnaya *et al*., 2008), *Hyphomicrobium* spp. *mxaF* (Fesefeldt and Gliesche, 1997) (GenBank: Y08080), and *Citrobacter* spp. *dhaD* (Daniel *et al*., 1995) (GenBank: U09771) using the PrimerQuest online software from Integrated DNA Technologies (Coralville, LA, USA) (Table 2). BLAST searches using both forward and reverse primers indicated that these biomarkers could not target other methanol or glycerol utilizing bacteria based on currently available genomic data (results shown in Supporting Information). The mRNA concentrations of the three ADH genes were normalized to the corresponding 16S rRNA concentrations. All qPCR assays were performed in triplicate with conditions as described above. The potential of gene expression as a surrogate of denitrification activity was evaluated by tracking and correlating *mdh2*, *mxaF* and *dhaD* mRNA concentrations during several extant batch denitrification tests over Phases I and II of SBR operation (as shown in Eq. S1).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Metabolism of glycerol under oxidative and reductive conditions (adapted from da Silva *et al*., 2009).
Fig. S2. Effluent concentrations of nitrate (–◆–) and nitrite (-□-) from the SBR, measured in duplicate.

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