Implication of Using Different Carbon Sources for Denitrification in Wastewater Treatments

Carla Cherchi, Annalisa Onnis-Hayden, Ibrahim El-Shawabkeh, April Z. Gu*

ABSTRACT: Application of external carbon sources for denitrification becomes necessary for wastewater treatment plants that have to meet very stringent effluent nitrogen limits (e.g., 3 to 5 mgTN/L). In this study, we evaluated and compared three carbon sources—MicroC^{TM} (Environmental Operating Solutions, Bourne, Massachusetts), methanol, and acetate—in terms of their denitrification rates and kinetics, effect on overall nitrogen removal performance, and microbial community structure of carbon-specific denitrifying enrichments. Denitrification rates and kinetics were determined with both acclimated and non-acclimated biomass, obtained from laboratory-scale sequencing batch reactor systems or full-scale plants. The results demonstrate the feasibility of the use of MicroC^{TM} for denitrification processes, with maximum denitrification rates (k_{dmax}) of 6.4 mgN/gVSS·h and an observed yield of 0.36 mgVSS/mgCOD. Comparable maximum nitrate uptake rates were found with methanol, while acetate showed a maximum denitrification rate nearly twice as high as the others. The maximum growth rates measured at 20°C for MicroC^{TM} and methanol were 3.7 and 1.2 day^{-1}, respectively. The implications resulting from the differences in the denitrification rates and kinetics of different carbon sources on the full-scale nitrogen removal performance, under various configurations and operational conditions, were assessed using Biowin (EnviroSim Associates, Ltd., Flamborough, Ontario, Canada) simulations for both pre- and post-denitrification systems. Examination of microbial population structures using Automated Ribosomal Intergenic Spacer Analysis (ARISA) throughout the study period showed dynamic temporal changes and distinct microbial community structures of different carbon-specific denitrifying cultures. The ability of a specific carbon-acclimated denitrifying population to instantly use other carbon source also was investigated, and the chemical-structure-associated behavior patterns observed suggested that the complex biochemical pathways/enzymes involved in the denitrification process depended on the carbon sources used. *Water Environ. Res.*, **81,** 788 (2009).

KEYWORDS: denitrification, nitrogen removal, biological nutrient removal, MicroC^{TM}, Biowin modeling, carbon sources, Automated Ribosomal Intergenic Spacer Analysis, and community structure.

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Introduction

In the last decade, increasingly stringent environmental requirements have been imposed on nutrients discharge in surface waters, because excessive nutrients are considered the primary causes of eutrophication. The biological nutrient removal (BNR) process remains the most common practice for achieving nitrogen and phosphorus removal. Many wastewater treatment plants (WWTPs) are facing challenges to achieve lower effluent nutrient levels with current technology limits and available resources (Water Environment Research Foundation, 2007). The addition of external carbon sources often becomes necessary for achieving high-efficiency BNR, especially for facilities with weak influent biochemical oxygen demand (BOD) and/or those facing strict effluent limits. The addition of extra carbon in pre-denitrification anoxic zones can increase the denitrification rates and nitrogen-removal efficiencies, while external carbon addition to the post-denitrification zone often is required to reach an effluent total nitrogen concentration of less than 3 to 5 mg/L.

In the United States, methanol is the most commonly used electron donor, as a result of the higher denitrification efficiency, as indicated by the relatively lower methanol-to-nitrate ratio, lower cost, and broad availability in the market. The main disadvantage of using methanol is the safety issues associated with its transportation, handling, and storage, because it is a reactive and toxic compound. It has been estimated that an additional 25 to 31% of the capital construction cost for methanol storage, pumping, and delivery systems is required to meet the safety standards over the use of a non-flammable, non-hazardous product (CDM, 2007). The long adaptation periods required in the startup process to build the specific methanol-using denitrifying bacteria (methylotrophs) also is relevant (Christensson et al., 1994). Additionally, there have been reports of deteriorated denitrification performance under cold conditions, as a result of the potential washout of methanol-using denitrifying bacteria from the system, as the growth rates decrease at lower temperatures (Mokhayeri et al., 2006). Lastly, the prices of methanol recently have been volatile (Methanex, 2008), and, in some cases, shortages have occurred. The above concerns have motivated the investigation of other economical alternative carbon sources for denitrification.

Performances related to long-term experiences with the use of methanol and ethanol as sole electron donors and their influence on the denitrifying bacterial community have been studied and compared (Christensson et al., 1994; Nyberg et al., 1996). Alternative compounds that have been investigated for supporting denitrification are sugar (Akunna et al., 1993; Gomez et al., 2000), glycerol (Akunna et al., 1993), molasses (Quan et al., 2005), corn starch (Lee and Welander, 1996), industrial wastewater (Cappai et al., 2004), and others (Akunna et al., 1993; Lee and Welander,

Department of Civil and Environmental Engineering, Northeastern University, Boston, Massachusetts.

* Dept. of Civil and Environmental Engineering, Northeastern University, 360 Huntington Ave., Boston, MA 02115; e-mail: april@coe.neu.edu.

1996; Nyberg et al., 1996; Tsonis, 1997). MicroC™ is a proprietary product with an undisclosed composition, developed by Environmental Operating Solutions (EOS), and designed specifically as a nonflammable, agriculturally derived carbon source. Since 2003, MicroC™ has been distributed throughout the northeastern United States in over 200 facilities required to meet stringent effluent nitrogen limits, and its price has remained stable in the past 3 years at approximately $0.48/L ($1.81/gal). In general, plant infrastructures typically used to handle methanol or other carbon sources are compatible with MicroC™. Selection of a carbon source for denitrification must consider many aspects, including nitrogen-removal performance, cost, operational requirements and features, and possible effect on effluent quality and sludge production (Nyberg et al., 1996). The objective of this study is to evaluate the denitrification kinetics and potential of MicroC™ as a carbon source for the denitrification process and compare them with the most commonly used carbon source (methanol) and widely studied carbon source (acetate), although practical use of the latter has been limited, as a result of its higher cost. Specific goals include the following:

(1) Determine and compare the denitrification rates, kinetics, and growth rates among MicroC™, methanol, and acetate, with both acclimatized biomass and non-acclimated biomass from either laboratory-scale sequencing batch reactors (SBRs) or full-scale WWTPs.
(2) Investigate the implications and effects of using different external carbon sources on nitrogen-removal performance at full-scale facilities using Biowin model simulations. The effect of both denitrification kinetics and operational conditions are evaluated.
(3) Assess the ability and response of a specific carbon-acclimated denitrifying population to immediately use various other carbon sources.
(4) Investigate the microbial population structures and dynamics associated with various carbon-source-specific denitrifying enrichment cultures and reveal the biochemical fundamentals underlying the different denitrification behaviors observed.

Materials and Methods

Chemicals. MicroC™ is a light green liquid compound with a mild alcohol odor containing agricultural products and methanol (5% w/w). Its bulk density, specific gravity, and viscosity are 1.18 g/cm^3, 1.18, and 0.0164 kg/m-s, respectively. The compound is soluble in water, and its pH at 25°C is 5.8. MicroC™ is stable under normal conditions and has a freezing point of −20°C, which avoids storage issues during cold seasons. Volatile organic compounds have not been detected in its composition (EOS, 2008). Stock chemicals were provided by EOS and dilutions were prepared freshly for testing and the system feeding. Methanol, sodium acetate, ethanol, and glucose were from Fisher Scientific (Springfield, New Jersey).

Denitrifying Biomass (Sludge). Both acclimated and non-acclimated denitrifying biomass, from laboratory-scale SBR reactors or full-scale activated sludge WWTPs, were tested for comparison.

Full-Scale Activated Sludge. MicroC™-acclimated sludge was provided by the municipal WWTP of Enfield, Connecticut, where the sludge had been acclimatized fully with MicroC™ added as the sole carbon source in the post-denitrification stage for 9 months; the non-acclimated sludge was from the municipal WWTP of Wareham, Massachusetts. The Wareham facility is a Modified Ludzack-Ettinger (MLE) system, with secondary clarification followed by Leopold downflow denitrification sand filters and UV disinfection. The plant did not use any external carbon source, and the sludge samples were taken from the pre-anoxic zone.

Laboratory-Acclimated Sludge. Three carbon-source-specific denitrifying biomasses were acclimatized fully for over 3 months before the beginning of batch testing in laboratory-scale SBRs with MicroC™, methanol, and acetate, respectively, using the same seeding sludge from the Wareham, Massachusetts, WWTP. The synthetic wastewater for the SBR feed contained MicroC™, methanol, or acetate (150 to 350 mgCOD/L), dissolved in media containing $MgSO_4 \cdot 7H_20$ (40 mg/L), $CaCl_2$ (7.5 mg/L), $Fe(SO_4) \cdot 7H_2O$ (1 mg/L), KH_2PO_4 (22 mg/L), K_2HPO_4 (56 mg/L), NH_4Cl (101 mg/L), yeast extract (30 mg/L), $MnSO_4 \cdot 4H_20$ (0.2 mg/L), sodium bicarbonate (252 mg/L), and trace minerals. The SBR system had an influent flow of 9 L/d and was operated with an SRT of 15 days and 3 daily cycles, which included a 2-hours anoxic phase, 30 minutes of feeding, and 4.5-hour aerobic period. The chemical oxygen demand (COD), ammonia (NH_4), nitrate (NO_3), and nitrite (NO_2) were examined on a weekly basis to monitor the general functioning of the system. Total and volatile suspended solids (TSS and VSS, respectively) were maintained at concentrations between 700 and 1100 mg/L. The temperature was in the range 20 to 23°C, and the oxygen concentration during the aerobic phase was approximately 5 to 7 mg/L. The pH was kept in the optimal range of 6.5 to 7.5.

Analytical Measurements. Nitrate, nitrite, ammonia, volatile and total suspended solids were analyzed according to *Standard Methods* (APHA et al., 2001). A YSI 5000 dissolved oxygen meter (YSI Inc., Yellow Springs, Ohio) was used to monitor the extent of aeration, while the pH and temperature were checked using a Thermo Orion 230 meter (Thermo Fisher Scientific, Waltham, Massachusetts). Dichromate acid digestion was used to determine the total COD equivalent to MicroC™, and duplicates of different dilution series were conducted to obtain statistically confident values. The readily bio-available COD (rbCOD) value for MicroC™ was evaluated using the method proposed by Mamais et al. (1993), based on the filtered and flocculated COD (ffCOD) measurement. The principle is to determine the rbCOD as the difference between the ffCOD of the influent wastewater and the ffCOD of the final effluent in a specific treatment process. The 5-day BOD (BOD_5) of MicroC™ was analyzed based on *Standard Methods* (APHA et al., 2001).

Denitrification Rates and Kinetics. *Denitrification Batch Tests (Low Food-to-Microorganism Ratio, 0.02 to 0.05 mgCOD/mgVSS, Short Test).* A series of denitrification batch tests was conducted to determine the denitrification rates and kinetics with biomass that was acclimated with three carbon sources—MicroC™, methanol, and acetate, respectively. Denitrification rates were determined at various carbon concentrations (0 to 300 mg sCOD/L) and with an adequate initial nitrate concentration (20 to 40 mg/L), according to the method presented by Kujawa and Klapwijk (1999). The sludge was kept under a continuous nitrogen gas flow, to guarantee anoxic conditions, and the pH remained constant at 7.5. Samples were taken at intervals of 10 to 15 minutes. The denitrification rates, as a function of initial COD concentration, were then fitted to the Monod equation

using SPSS 14.0 (SPSS Inc., Chicago, Illinois) to estimate the maximum denitrification rate and half-saturation constant.

Denitrification Tests (High Food-to-Microorganism Ratio, 2 to 3 mgCOD/mgVSS, Long Test). The method proposed by Dold et al. (2005) was applied to estimate the maximum specific growth rates of each carbon-specific denitrifying culture and to estimate the carbon-to-nitrogen ratio (C/N) during denitrification. The tests were all run in duplicate. In these high F/M ratio tests, the biomass growth was expected to be exponential, with both the electron donor (carbon) and electron acceptor (NO_3) being kept at the saturation level (non-limiting condition). Kinetic parameters were determined by fitting the nitrate uptake rate versus time using the equation presented in Dold et al. (2005) using statistical software SPSS 14.0, as follows:

$$S_{NOx,t} = S_{NOx,0} - \frac{1 - Y_{HD}}{2.86} \cdot \frac{\mu_{\max} \cdot X_0}{Y_{HD} \cdot (\mu_{\max} - b_H)} \cdot \left(e^{(\mu_{\max} - b_H)t} - 1 \right) \quad (1)$$

The method applied minimizes the sum of the squares of the residuals by adjusting the three initially estimated parameters (μ_{Hmax}, $X_{\mathrm{N},0}$, and $S_{\mathrm{NO},0}$), and by fitting the assumed Y_{HD} and b_{H} to the above equation. Note that, although the yield Y_{HD} in the model is assumed, it has no influence on the $\mu_{\max}$ of denitrifiers (Dold et al., 2005). The decay coefficient b_{H} for acclimated sludge was estimated to be 0.1 day^{-1} by low F/M tests run at endogenous conditions, which is agreeable with the values reported in literature (Yuan et al., 2002). In cases where the accumulation of nitrite occurred during the test, the coefficient 0.6 takes into consideration the stoichiometry of the denitrification reaction from the ratio 1.71/2.86, where 1.71 and 2.86 are the oxygen equivalents of nitrite and nitrate, respectively (Kujawa and Klapwijk 1999), as shown in eq 2.

$$\mathrm{NOx\text{-}N} = \mathrm{NO_3\text{-}N} + 0.6 \times \mathrm{NO_2\text{-}N} \quad (2)$$

The observed growth yield for denitrifying (mgVSS/mgCOD) was estimated from the COD/N ratio measured during the tests, using eq 3.

$$Y_{HD} = \left(1 - \frac{2.86}{(COD/N)} \right) / 1.42 \quad (3)$$

The maximum growth rate ($\mu_{\max}$) at 20 and 10°C was determined experimentally with the high F/M method, as described above.

Molecular Characterization of Carbon-Source-Specific Denitrifying Cultures. Automated Ribosomal Intergenic Spacer Analysis (ARISA) was used in this study, to assess the dynamics of bacterial composition over time in SBR-enriched cultures. This new ecological approach differentiates the bacterial species based on the length in the intergenic region between the 16S and 23S ribosomal RNA, at a high level of resolution (Jones et al., 2007). The method was found to be highly reproducible and reliable compared with other commonly used molecular techniques, such as Terminal Restriction Fragment Length Polymorphism (T-RFLP) (Fisher and Triplett, 1999). The bacterial community composition and dynamics then were observed and compared, to demonstrate the specificity of the community when acclimated with different carbon sources, such as MicroC™, methanol, or acetate.

Samples from the SBRs were collected every month, and DNA was extracted from activated sludge using the Ultraclean Soil DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, California). DNA presence in the samples was checked using gel electrophoresis in 1% agarose Tris/Borate/EDTA (TBE) gel stained with ethidium bromide; visualization of the nucleotides fragments was done under UV light using a Bio-Rad Gel-Doc XR imaging system (Bio-Rad Laboratories, Hercules, California). Polymerase chain reaction (PCR) was performed in a Biorad IQ5 thermocycler to amplify 1 μL of extracted DNA using two primers—6FAM-labeled universal forward primer 1406F (5′-TGYACACACCGCCCGT -3′) and 23Sr bacterial specific (5′-GGGTTBCCCCATTCRG -3′) as reverse primer. The PCR consists of a preliminary DNA denaturation step for 2 minutes at 94°C followed by 30 cycles of denaturing (94°C for 35 seconds), annealing (55°C for 45 seconds), and elongation (72°C for 2 minutes) steps, ending with 2 minutes of final extension at 72°C. The ARISA was performed according to the method proposed by Fisher and Triplett (1999) using an ABI 3730 genetic analyzer. The profiles obtained by the labeled primer were analyzed using two different internal sized standards (Bioventures Inc., Murfreesboro, Tennessee)—ROX 2500 GeneScan custom sized with fragments of 50 bps in the range 100 to 2000 bps and the ROX fragile with less fragmentation within the same range. The ARISA fragments were determined using Peak Scanner software version 1.0 provided by Applied Biosystems Inc. (Foster City, California). A threshold of 100 fluorescence units was set to eliminate the background noise, and only sizes between 300 and 1550 bps were evaluated. The ARISA generally produces one peak for each bacterial isolate (Danovaro et al., 2006), and the relative abundance of each identified genotype was determined by normalizing the peak height by the total height (fluorescence units) characteristic of each electropherogram.

Biowin Simulations. Effects on overall nitrogen-removal performance using MicroC™ or methanol as an external carbon source were assessed by model simulation with Biowin, using the kinetics and biodegradability data determined by the batch tests. The following two commonly practiced denitrification configurations were analyzed: (1) MLE for pre-denitrification, and (2) MLE followed with a post-denitrification zone and a final aerobic polishing zone (see Figure 1). Different scenarios were simulated and compared under variant operational parameters, such as carbon dosage, anoxic hydraulic retention time (HRT), and temperature. For each scenario, effects on the final effluent total nitrogen concentration and on sludge production were the main evaluation parameters. Table 1 summarizes the conditions and operation parameters that were used for the different scenarios analyzed as well as the kinetics and stoichiometric parameters used in Biowin. The values of maximum specific growth rates and yields were the one determined in the batch tests, however for the values of the half saturation constants the default values were selected; this is because of the high standard deviation found in the batch testing for this parameter, as well as from full scale data considerations. MicroC™ utilizers are considered to growth both under aerobic and anoxic conditions due to the complexity of MicroC™ composition, whereas considered the limited availability of single-C compound in the aerated zone, the growth of methylotrophs was assumed to be limited to anoxic zones only where methanol is supplemented (default setting for Biowin).

Results and Discussion

Characterization of MicroC™. The total COD of MicroC™ was found to be 663 ± 27.2 gCOD/L, which is similar to the value of 672 gCOD/L, as established by EOS in previous studies

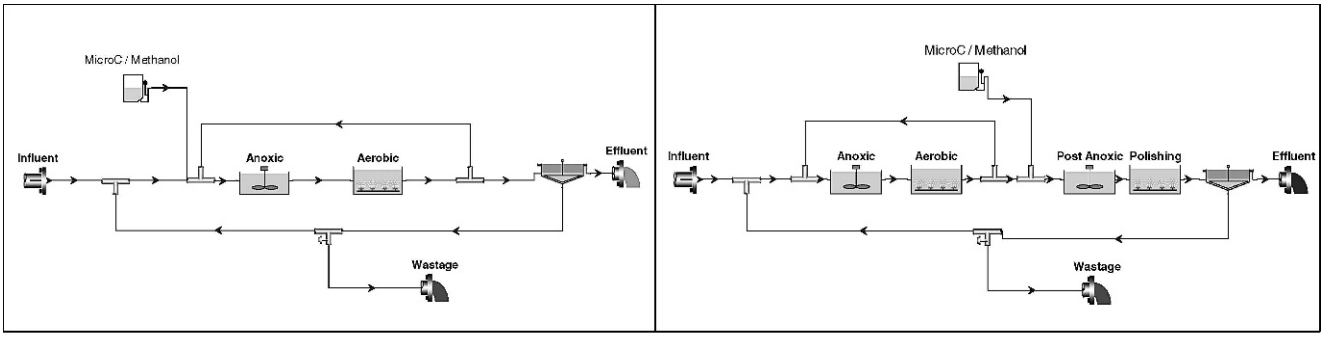

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Figure 1—Process schematic for Biowin simulations: (a) MLE configuration, and (b) MLE plus post-denitrification.

(Ledwell, 2006). Approximately 504 gCOD/L (75% of the total COD) was determined to be rbCOD. The remaining portion of the COD (25%) seems bioavailable through hydrolysis. Soluble COD measured in the effluent from the SBR was consistently below the detection limit (5 mg/L), indicating that nearly all COD in the MicroC^{TM} is used by the biomass. In addition, BOD_5 was evaluated and resulted in 429 g/L (65% of the total equivalent COD).

Comparison of Denitrification Kinetics among MicroC^{TM}, Methanol, and Acetate. Table 2 summarizes the denitrification rates and kinetics obtained for the three carbons sources (MicroC^{TM}, methanol, and acetate) using laboratory-scale SBR-acclimated sludge and full-scale WWTP sludge. The denitrification rates were determined and compared at two different temperatures (20 and 10°C), although the latter is more of a transitional rate, because the sludge was acclimated at 20°C. The maximum nitrate uptake rate found for MicroC^{TM} (6.4 mgN/gVSS·h) is comparable with that obtained with methanol (6.1 mgN/gVSS·h), and therefore shows the feasibility of using MicroC^{TM} as an alternative external carbon source to methanol for enhancing denitrification. The use of acetate resulted in a much higher denitrification rate (13.6 mgN/gVSS·h) than both MicroC^{TM} and methanol. A wide range of values has been reported in literature for the observed specific denitrification rates for methanol, ranging from 3.3 mgN/gVSS·h (Nyberg et al., 1996) to 21 mgN/gVSS·h (Foglar et al., 2005), and, for acetate, ranging from 3.09 mgN/gVSS·h (Isaacs and Henze, 1995) to 10.6 mgN/gVSS·h (Tam et al., 1994), respectively. The variability of these rates likely was influenced by the sludge sources (acclimated versus non-acclimated) from full- or laboratory-scale systems, type of reactors, and environmental factors that generally affect biological processes (pH, temperature, etc.).

The average half-saturation constants for MicroC^{TM}, methanol, and acetate were found to be 38.6, 15.6 and 38.1 mgCOD/L, respectively. These values are higher than the typical value reported by Metcalf & Eddy (2003) (9 mg biodegradable COD/L). The high standard deviation of the results possibly is related mainly to the use of the soluble COD (sCOD) measurement for the estimation of this parameter. In general, for carbon sources that have a low half-saturation value, the direct measurement (e.g., gas chromatography) of the substrate, rather than the use of soluble COD measurements, might be better to estimate the half-saturation constant (K_s). However for a carbon source with an unknown composition, such as MicroC^{TM}, soluble COD would be the only measurable parameter. Half-saturation constants are important for nitrogen-removal capacity and performance at full-scale facilities, because the *in situ* specific denitrification rate in reactors typically is substrate-limiting, depending not only on the maximum specific rate, but also on the actual readily bioavailable carbon substrate concentration and the half-saturation constant.

The maximum growth rate of MicroC^{TM}-using denitrifiers at 20°C (3.7 day^{-1}) is comparable with the values previously reported for general heterotrophic denitrifying microorganisms in WWTPs (3.2 day^{-1}, Metcalf & Eddy, 2003), and it was nearly

Table 1—Design and operational parameters input to Biowin simulations.

Design parameter			Kinetics and stoichiometry		
Configuration	MLE	MLE + post denitrification	Parameter	MicroC^{TM}	Methanol
Influent flow rate, Q (m^3/d)	18 930	18 930	μ_{max} (1/day)	3.66	1.25
Temperature (°C)	13 and 20	13 and 20	K_s (mgCOD/L)	20	5
Aerobic SRT (days)	10	10	Aerobic decay (1/day)	0.08	0.06
Influent COD (mg/L)	250	250	Anoxic decay (1/day)	0.08	0.06
Anoxic volume(m^3)	950 to 1325	1325	Yield (anoxic) (gCOD/gCOD)	0.52	0.4
Post-anoxic volume(m^3)	-	1140 to 1515			
Aerobic volume (m^3)	3445	3445	Temperature coefficient (θ)	1.1	1.1
Polishing volume (m^3)	-	378			
Mixed-liquor recycle, MLR	3Q	3Q	Aerobic growth	yes	no
RAS	0.5Q	0.5Q			

Table 2—Denitrification kinetic coefficients for different carbon sources tested on laboratory-acclimated biomass in SBR systems.

	K_{dmax} (20°C) (mgN/gVSS·h)	K_{dmax} (10°C) (mgN/gVSS·h)	K_s (mg sCOD/L)	COD/N (mg sCOD/mgN)	$Yield_{obs}$ (mgVSS/mgCOD)	μ_{max} (20°C) (day^{-1})	μ_{max} (10°C) (day^{-1})
MicroC™	6.4 ± 3.6	2.5	38.6 ± 29.2	6.5 ± 3.7	0.39	3.7	1.2
Methanol	6.1 ± 0.7	2.3	15.6 ± 11.2	4.8 ± 1.5	0.29	1.3	0.3
Acetate	13.6 ± 1.9	3.6	38.1 ± 16.2	5.7 ± 1.3	0.35	-	-

three times greater than that found for methanol (1.3 day^{-1}). Figure 2 shows the typical nitrate-consumption curve versus time obtained in a high F/M ratio batch test, which is used to estimate the maximum growth rate, as previously described. The maximum specific growth rate of methylotrophs reported ranged from 1 to 6.3 day^{-1} (Onnis-Hayden and Gu, 2008). The most recent investigations have shown that the rate of methanol utilization under anoxic conditions may be very much slower than believed before, therefore also the default value of process simulation models such as Biowin, have been modified in the latest version. Methanol enriches for methylotrophs, a specific group of bacteria that are capable of using one-carbon (C_1) compounds, such as methanol, methane, and formate, as substrates for biosynthesis and energy requirements. They developed specific pathways, such as the Serine cycle, where the intermediate formation of formaldehyde occurs (Madigan and Martinko, 2006). Based on the exchange of free energy between electron donor and acceptor, the amount of biomass produced per unit of substrate removed for methylotrophs is relatively low, with respect to microorganisms grown on multi-carbon substrates (Rittmann and McCarty, 2000); therefore, it leads to a lower anoxic yield.

Effect of Temperature on Denitrification Rates and Kinetics. Denitrification rates decrease with declining temperature, as demonstrated in previous studies (Christensson et al., 1994; Dold et al., 2005; Mokhayeri et al., 2006; Nyberg et al., 1996). In our study, a decrease in temperature from 20 to 10°C resulted in a significant reduction in maximum denitrification rates and growth rates (Table 2) for both MicroC™- and methanol-enriched biomass. Approximately 60 and 62% decreases in the denitrification rates, and 67 and 73% decreases in the growth rates were observed at 20 and 10°C, for MicroC™ and methanol, respectively. The maximum specific denitrification rate for methanol at 10°C is comparable with those found by Dold et al. (2005). A 66% reduction of the nitrate uptake rate also was observed by Christensson et al. (1994), when the temperature was changed from 25 to 15°C. Acetate sludge seemed to be affected the most by the temperature drop, with a 73% reduction of denitrification rates from 20 to 10°C.

It is worth mentioning that significant nitrite accumulation occurred during the tests with acetate at both 20 and 10°C. Figure 3 shows the extent of nitrite accumulation with acetate-enriched biomass at 10°C, where, after 21 hours, nearly 80% of the nitrate was converted to nitrite, and the reduction of nitrite did not begin until 1.7 days later. No nitrite accumulation was observed with methanol. For MicroC™, the presence of nitrite was minimal (less than 10% of the total inorganic nitrogen) and limited to the first part of the low F/M test (when the COD/N was relatively high). The incomplete conversion of nitrate to nitrogen gas using acetate as a carbon source has been reported previously (VanRijn et al., 1996). The accumulation of nitrite has been associated with imbalanced activities of *nitrate* and *nitrite reductases*, with the inhibition of nitrite reductase by oxygen, inhibition by nitrate or nitrite, and inhibition at high COD/N ratios (>2.5) (Martienssen and Schops, 1999). The accumulation of nitrite for acetate was more pronounced at 10°C, as a result of the different temperature sensitivity of nitrite-reducer bacteria and nitrate reducers (Drysdale et al., 1999).

Note that the values measured at 10°C are ''transitional'' kinetics, because the sludge was not acclimated at 10°C; therefore, this test only simulates the instant population response to the temperature decrease. Because the growth rate of a population is directly related to SRT in the nitrogen-removal process, a dramatic reduction in the growth rate (μ_{max}) during cold

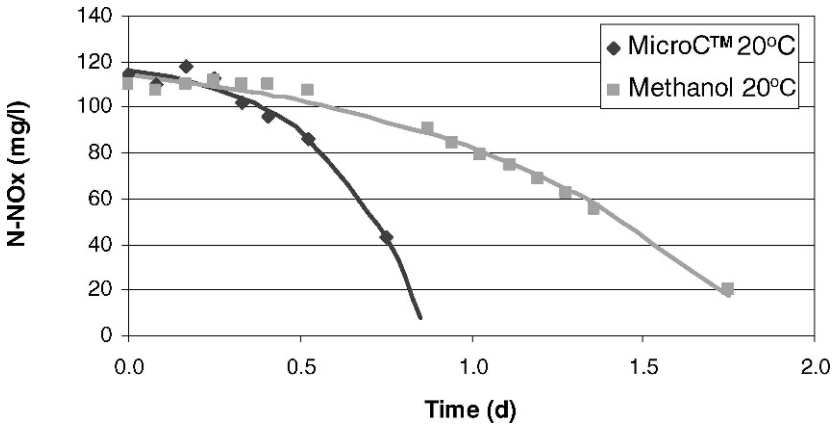

Figure 2—Depletion of NOx during high F/M denitrification kinetic test at 20°C with MicroC™ or methanol as a carbon source.

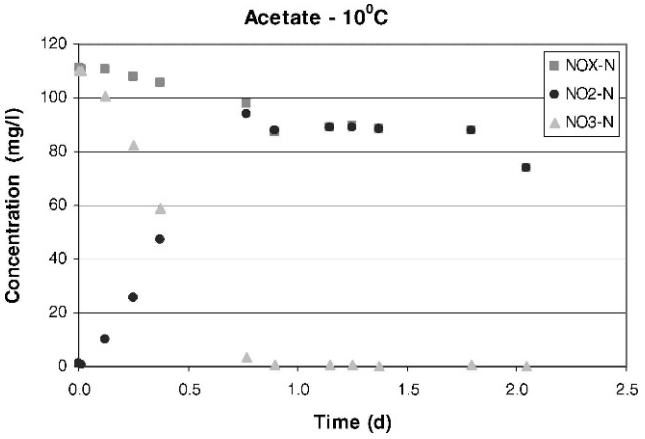

Figure 3—Accumulation of nitrite during high F/M denitrification kinetic test with acetate as a carbon source at 10°C.

conditions potentially could lead to the washout of the species of interest from the reactor.

Comparison of COD/N Ratio Among MicroC™, Methanol, and Acetate. The carbon-use-to-nitrate-consumption ratio (COD/N ratio) indicates the carbon-use efficiency for denitrification, and the values were estimated based on the depletion of COD and uptake of nitrate during the denitrification batch tests. The C/N ratio was found to be 6.5, 4.8, and 5.7 gCOD/gN, for MicroC™, methanol, and acetate, respectively. The C/N value depends not only on the theoretical yield, but also the conditional parameters (SRT, k_d) as shown in eq 3. For methanol and acetate, the theoretical ratio was determined to be 4.7 and 3.5 gCOD/gN, respectively (Mokhayeri et al., 2006). The observed C/N can be affected by several factors, including the possible interference of storage phenomena (luxury uptake), which can take place when a considerable amount of organic substrate is put in contact with the biomass (Majone et al., 1998); possible activity under an anoxic condition of polyphosphorus-accumulating bacteria (PAOs) if present in the sludge (Naidoo et al., 2000); possible aerobic respiration resulting from oxygen intrusion; and reliability of the COD and nitrate measurement itself. For example, the high value obtained for acetate could be associated with the abundant presence of PAOs, which was observed during an unrelated test in the acetate-fed SBR.

The determination of the correct C/N ratio is crucial for the selection of alternative carbon sources, because it is an indicator of COD usage efficiency for denitrification. High operational costs and higher biomass production can be caused by COD/N overestimation.

Effect of Acclimation. The comparison of denitrification rates obtained with either MicroC™-acclimated or non-acclimated sludge are presented in Table 3 and Figure 4. The results indicate that the denitrification rates and kinetics were similar for the acclimated and non-acclimated full-scale sludge (4.7 and 4.3 mgN/gVSS·h). This suggests that the denitrifying microbial population capable of using MicroC™ likely is active in typical WWTPs; therefore, acclimatization to MicroC™ may not be needed. The higher rates observed with the SBR-acclimated sludge compared with the acclimated full-scale biomass likely is the result of the higher enrichment of the denitrifying population in the SBR sludge and the higher amounts of inert solids in the biomass at WWTPs.

Comparison of Microbial Community Structures of Denitrifying Cultures Enriched with Different Carbon Sources. The microbial community composition of each carbon-specific denitrifying culture was monitored and compared using a molecular method (ARISA) from the startup for a period of 9 months. Figure 5 summarizes the relative abundance (assumed to be proportional to fluorescence signal intensity) of diverse bacterial species (represented by distinct peaks) present in the overall microbial community during the test period. The relative abundance of each community member identified is quantified as a percentage of total fluorescence and is shown in gray-scale for easy observation. Peaks with an abundance level of less than 1% are not shown. Figure 6 shows an example of the ARISA profile for each different denitrifying culture.

The ARISA profiles clearly show the distinct community structures of the three denitrifying cultures as the result of enrichment with three different carbon sources. Approximately 17% more ARISA peaks were found in the MicroC™-enriched culture (67) than that identified in methanol- (57) or acetate (57)-acclimated cultures, indicating larger diversity in the former culture than the other two. The community compositions are rather dynamic, and no ''stable'' structures were achieved, even after 9 months of acclimation. However, there were consistent trends that could be observed with some of the peaks (members) in the community.

For MicroC™-enrichment, peaks at 507 bps, 523 bps, 634 bps, 688 bps, 759 bps, 802 bps, 816 bps, 823 bps, 918 bps and 1253 bps were found recurrently, and, for some, the abundance increased over time, indicating their enrichment and potential role in the MicroC™ metabolism. For methanol-enriched culture, peaks at 483, 548, 554, 725, 758, 797 bps, 810 bps, 858 bps, 890 bps, 919 bps, 965 bps and 1262 bps were found to increase in their abundance over time, especially peaks at 548, 554, and 965 bps, which were the most dominant members in the community. However, it is not clear why peaks at 548 and 554 bps disappeared at the end of the 9th month. These predominant peaks likely represent the methanol-enriched methylotrophic organisms, which are capable of using methanol. More distinctive transition trends were observed with the acetate-enriched culture, in which only seven species with a relative abundance higher than 1% were identified, and the majority of the community (56%) consisted of

Table 3—Denitrification kinetic coefficients for MicroC™ tested on acclimated and un-acclimated biomass.

	K_{dmax} (20°C) mgN/gVSS·h	K_{dmax} (10°C) mgN/gVSS·h	K_s mg sCOD/L	COD/N mg sCOD/mgN	$Yield_{obs}$ gVSS/gCOD	μ_{max} (20°C) day^{-1}	μ_{max} (10°C) day^{-1}
Acclimatized SBR	6.4 ± 3.6	2.5	38.6 ± 29.2	6.5 ± 3.7	0.39	3.7	1.2
Acclimatized WWTP	4.7 ± 0.5	-	28 ± 11.5	7.0 ± 1.4	0.42	3.9	-
Unacclimatized WWTP	4.3 ± 0.5	-	49.7 ± 18.8	4.0	0.20	-	-

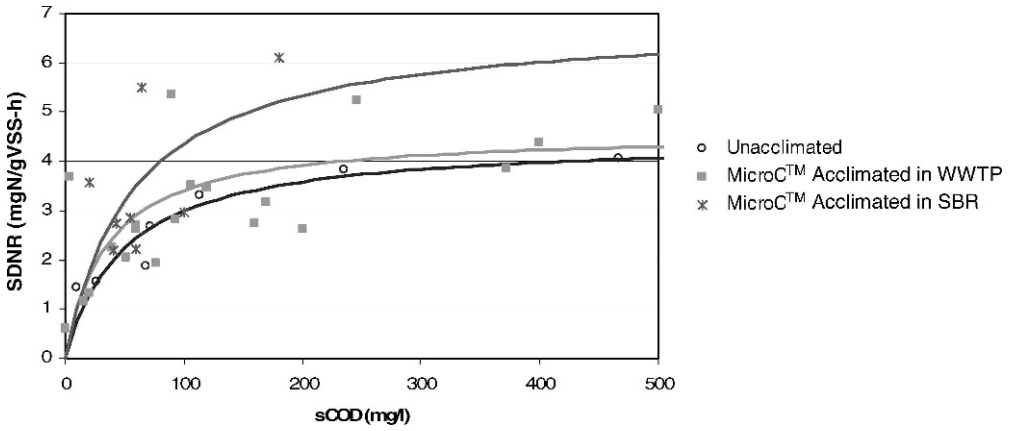

Figure 4—Specific denitrification rates (SDNRs) at different initial soluble COD concentrations with acclimated and non-acclimated biomass.

bacteria with intergenic space of 759 bps after 9 months of incubation.

Ability of Specific Carbon-Acclimated Sludge to Use Other Carbon Sources. It has been demonstrated that the quantity, quality (Lee and Welander, 1996) and combined use of external carbon sources can have various effects on denitrification. The addition of combined external carbons in post-denitrification zones can enhance the removal of nitrogen (Cho et al., 2004) or affect the metabolic properties of the established population, resulting in decreased rates. Moreover, supplemental carbon addition can reduce the capacity of denitrifiers to use internal carbon in pre-denitrification systems (Hallin and Pell, 1998). The ability and acclimatization time required of a specific population to use other carbons sources also have practical implications, affecting the easiness and adaptation time a WWTP would require when changing from one carbon source to another. In this study, we evaluated the ability of specific carbon-acclimated biomass to instantly use other carbon sources. Table 4 summarizes the response of each carbon-specific-acclimated biomass upon addition of various carbon sources. In addition, a review of the biochemical pathways for anoxic metabolism of these carbons is presented in Table 5, which highlights the key enzymes/pathways involved in the use of each specific carbon compound, and they are discussed below.

MicroC™-acclimated biomass was able to use all the carbon sources tested, including methanol, although at a relatively lower rate than the methanol-enriched biomass (data not shown). This was expected, because MicroC™ contains 5% methanol, as disclosed by EOS. The ARISA spectra indicated that the MicroC™-acclimated biomass contains a relatively large diversity of microorganisms, possibly with different metabolic pathways, as a result of the relative complexity of the MicroC™ composition. The practical implication is that, for facilities that change from using methanol as a carbon source to using MicroC™, or vice versa, lag time (acclimatization) likely is not needed. In addition, a microbial community with larger diversity typically provides more stability in the system.

Methanol-acclimated biomass could readily use all of the substrate tested for denitrification, except for glucose. Acetate is readily used by the methanol sludge, because acetate easily could enter the tricarboxylic acid (TCA)/glyoxylate cycle. Moreover, methylotrophs can use the enzymes characteristic of the glycine regeneration in the serine-glyoxylate pathway to activate the anaplerotic glyoxylate bypass of the TCA cycle when acetate is used as a carbon source. This diversion is used by organisms grown on acetate or fatty acids to provide the cells with 4- and then 3- carbons intermediates for biosynthesis. In the case of ethanol, the immediate response probably was the result of the presence of *Alcohol Dehydrogenase* enzymes, which catalyzed the conversion to acetate, and the consequent transformation into acetyl CoenzymeA. The denitrification efficiency of methanol-using bacteria seems to be affected when glucose was used as substrate, as shown previously (Mohseni-Bandpi and Elliot, 1998). Glycolysis is a multistep pathway that microorganisms use to obtain energy from glucose before entering the citric acid cycle. The high specificity of biocatalyst, perhaps not developed specifically by methylotrophs, and the complex chain reactions may be the reasons behind the reduced denitrification activity. Similar results and conclusions were found in the study of Hallin and Pell (1998). Dold et al. (2005) showed low denitrification rates using glucose in combination with mixed liquor from the nitrification stage, demonstrating that specific microorganisms and/or enzymatic systems required for glucose metabolism may not be present in methylotrophic culture.

Acetate-acclimated biomass could use only acetate efficiently, and only marginal denitrification rates were obtained with the other carbons. The specificity of carbon use seemed to be consistent with the highly selective community, which had few dominant members, as shown by the ARISA results earlier. Microorganisms grown on acetate as the only carbon and energy source require the operation of a particular anaplerotic pathway known as the *glyoxylate bypass* concomitantly with TCA. Therefore, it is likely that the acclimatization to acetate either enriches only the populations that exclusively use acetate, or simply turns off all the genes for those upstream enzymes, and therefore lacks the enzymatic activities to convert more complex multicarbon compounds into acetate (Cozzone, 1998). Specifically, metabolizing methanol must follow a reduction process to form tri-carbons or four-carbon intermediates before entering the TCA cycle. Nyberg et al. (1996) confirmed that un-specialized bacteria have difficulty in degrading methanol in systems previously acclimated with other carbons (e.g., ethanol).

Implication of Using Various Carbon Sources for Denitrification for Full-Scale Practice. Biowin simulations were used to compare and evaluate the effects of using different carbons

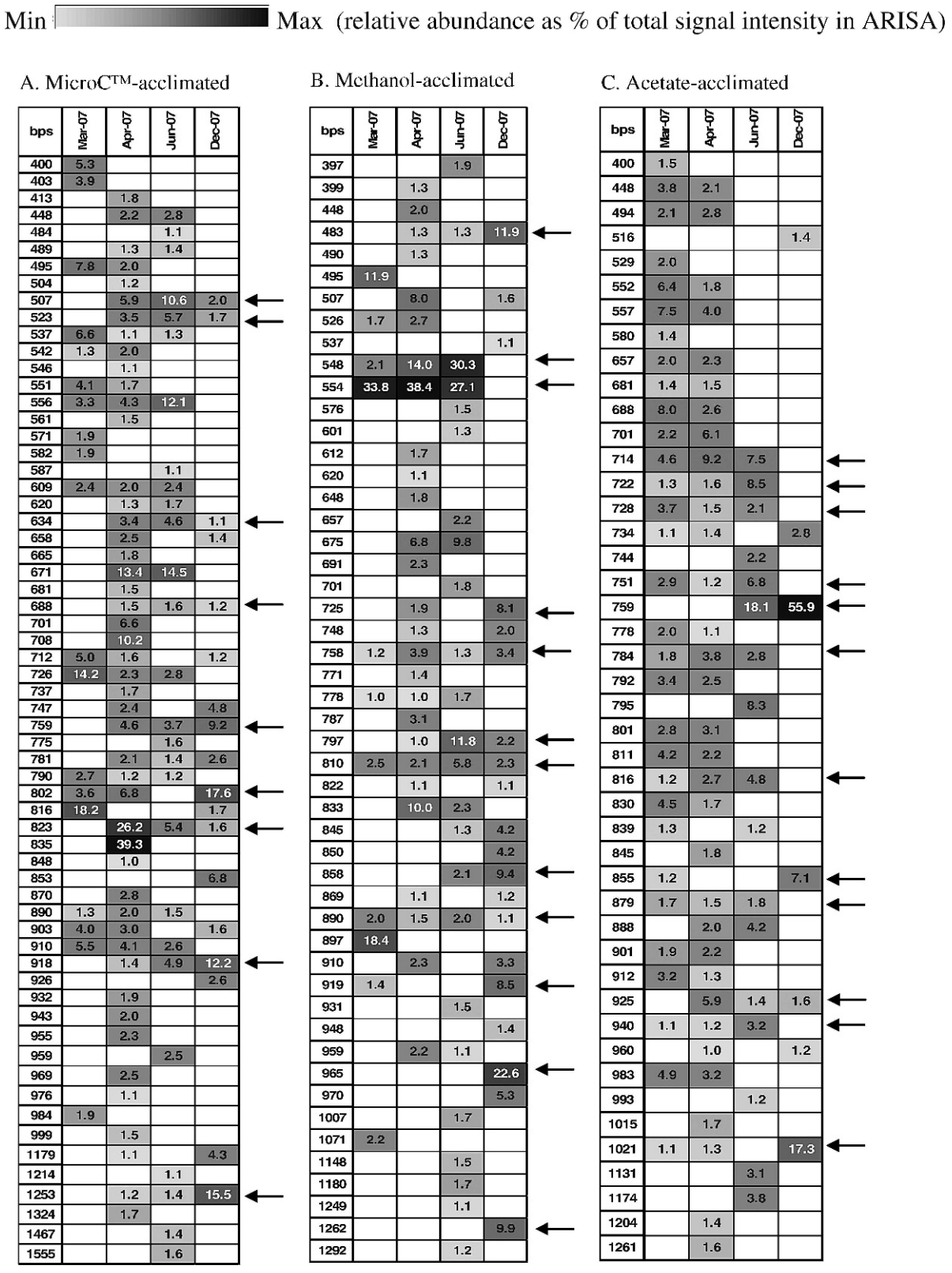

Figure 5—Relative abundance of microbial community members (represented by ARISA peaks at different bps lengths) in each specific carbon-acclimated denitrifying culture in SBRs.

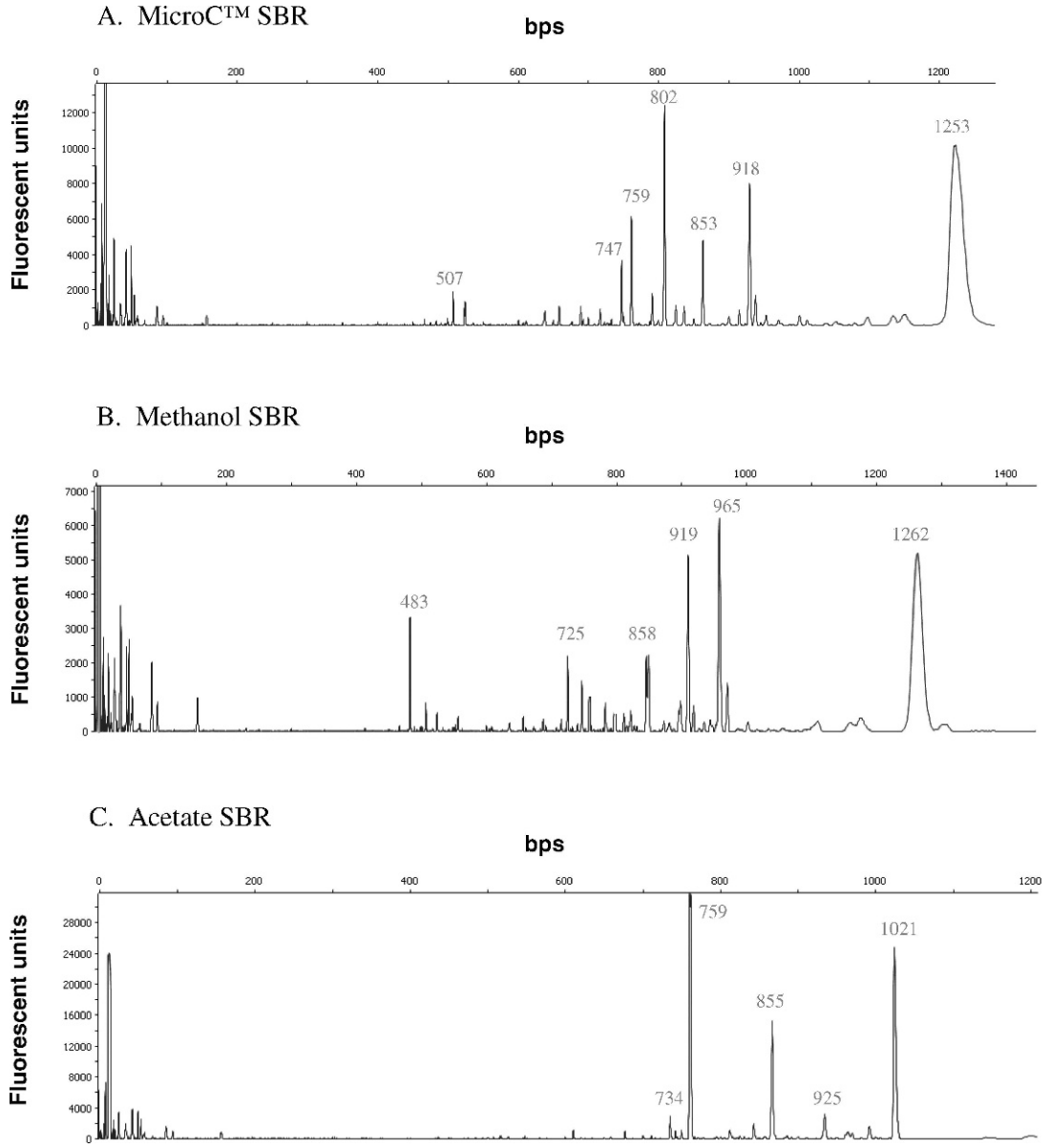

Figure 6—Electropherograms from ARISA, characteristic of (a) MicroC™-, (b) methanol-, and (c) acetate-acclimated denitrifying bacterial community in each SBR (sample taken in December 2007).

sources for enhancing denitrification at the full-scale level. The effects of process configuration, dosage concentrations, anoxic HRT, and temperature were assessed. Note that, in the Biowin model, MicroC™-using microorganisms are considered to be general heterotrophs, which can grow under both aerobic and anoxic conditions. This is supported by our previous observation, that MicroC™ can be consumed easily by sludges from acclimated WWTPs. Methanol use requires a special group of microorganisms, namely methylotrophs, which are capable of using single-carbon compounds for their growth. Because it remains unclear whether methylotrophs can use other COD in the aerobic zones, and considering the limited availability of single-carbon compounds in the aerated zone, the growth of methylotrophs was assumed to be limited to anoxic zones only where methanol is supplemented (default setting for Biowin). Figure 7 shows an example of the simulations results with MLE configuration with carbon addition to the pre-denitrification anoxic zone. Figure 8 shows the comparison of effluent total nitrogen with different external carbon source addition to the post-denitrification anoxic zone in a Bardenpho (4-stage) system. The results show that, for both configurations, when MicroC™, methanol, or acetate were dosed at the same concentrations (as COD), application of MicroC™ led to slightly better performance than methanol at both 13 and 20°C.

Temperature affects denitrification rates and kinetics and therefore the nitrogen-removal performance. As the temperature decreased from 20 to 10°C, the slower reaction rates led to slightly elevated effluent total nitrogen in the MLE configuration. The low temperature effect was more pronounced with MLE post-denitrification configuration, in which there was a 7% increase in the effluent total nitrogen using methanol, compared with 5% using MicroC™, as the simulation temperature dropped from 20

Table 4—Short-term response of each specific carbon-acclimated biomass to use various carbon sources.*

	MicroC™-acclimated sludge	Methanol-acclimated sludge	Acetate-acclimated sludge
MicroC™	+	+	−
Methanol	+	+	−
Acetate	+	+	+
Ethanol	+	+	−
Glucose	+	−	−

* Note: "+" = positive, able to use; "−" = negative, unable to use.

to 13°C. The denitrification in the system supplemented with MicroC™ seemed to be less sensitive to temperature drops compared with methanol supplementation. A more pronounced effect of temperature on denitrification, with MicroC™ or methanol as a carbon source, also can be illustrated by calculating and comparing the minimal SRT required to prevent washing-out at a very low temperature (5°C). The maximum growth rates at 5°C for both MicroC™ culture and methanol culture were determined based on the rates previously measured at 20°C and the temperature correction coefficient ($\theta = 1.1$). At this low temperature, the minimal SRT required to maintain methylotrophs in the post-denitrification reactor was approximately 4.5 days. In contrast, the minimal SRT required for keeping the MicroC™-users in the system was only 1.5 days. This implies that a larger anoxic reactor volume is required for methanol compared with MicroC™ at extremely low temperature conditions. In addition, the advantage of applying a fixed-film process instead of a suspended activated sludge process for denitrification, especially at lower temperatures, is implied.

Simulations with varying anoxic HRTs show that HRT also affects the denitrification performance (data not shown); therefore, both COD dosing and HRT should be considered when adding external carbon for enhanced denitrification. Effects on sludge production with different external carbon sources also were evaluated, because the yield associated with each carbon-specific denitrifying culture affects the final sludge production from external carbon addition. Figure 9 shows the comparison of the overall sludge production in the system and the specific portion of sludge produced from MicroC™ or methanol addition. Although a higher amount of sludge was produced for the MicroC™-using biomass than for the methanol-using biomass, as a result of the higher yield, the overall sludge production at the plant level was not significantly different; the total sludge production when adding external MicroC™ was only approximately 1.5% higher than that when adding methanol for the post-denitrification scenario. The reason for the relatively small effect on overall sludge production, despite the difference in the yield values, is that the externally added COD is only a small percentage of the total amount COD fed to the system, including the raw influent COD (only $<10\%$). The difference in the yield affects only the sludge produced from the externally added carbon, which is relatively a small percentage of the overall wastage of the plant.

The results of the simulation also show that, despite the higher COD/N for MicroC™, at equivalent COD dosages, the nitrogen removal obtained with MicroC™ addition was similar to that with methanol addition. This is because the nitrogen removal at a full-scale plant, unlike that in batch tests, depends on many other factors besides the COD/N ratio, such as HRT, limiting conditions in the anoxic zone, and abundance of specific denitrifiers in the system. In the case of MicroC™ simulations, for example, the abundance of MicroC™-users at steady state was higher than the amount of methylotrophs for equivalent COD added, as a result of higher yield, therefore resulting in a similar amount of nitrogen removal despite the higher COD/N. With adequate kinetics and stoichiometric parameters as input, the use of a simulator, such as Biowin, can help in the selection, for an existing facility, of the most effective external carbon source and selection of the optimal operational condition

Table 5—Known biochemical pathways involved in the use of the tested carbons.

Carbon source	Microorganism capable of C utilization	Biochemical pathways involved	Key steps of metabolism	Enzymes involved
MicroC™	Diverse community	Unknown	Diverse	Diverse
Methanol	Methylotrophs	Serine Pathway (Type II)	$CH_3OH \rightarrow$ Formaldehyde ↓ Serine/Glyoxylate Pathway → AcetylCoA ↓ TCA cycle	*Serine transhydroxymethylase, α–ketoglutarate dehydrogenase isocitrate lyase, enzymes characteristic of TCA*
Acetate	Diverse community	TriCarboxylic Acid Cycle (TCA cycle)	Acetate (CH_3COO^-) ↓ TCA cycle/ Glyoxylate Bypass	*Citrate synthase, isocitrate dehydrogenase, isocitrate Lyase, Malate synthase, Succinyl-CoA synthetase, etc*
Ethanol	Diverse community	Oxidation + TCA cycle	$C_2H_5OH \rightarrow$ Acetaldehyde → Acetate ↓ TCA cycle	*alcohol dehydrogenase, acetaldehyde dehydrogenase, etc*
Glucose	Diverse community	Glycolysis + TCA cycle	Glucose → Glyceraldehyde-3-P ↓ $Pyruvate^- \rightarrow$Acetyl CoA ↓ TCA cycle	*Hexokinase, Glyceraldehyde-3-P-dehydrogenase, Pyruvate kinase, etc*

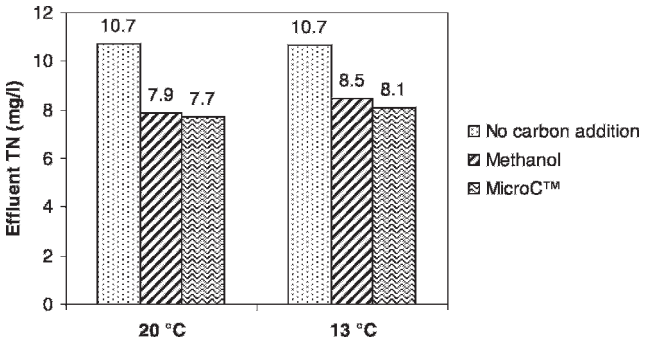

Figure 7—Comparison of nitrogen removal in MLE configuration with methanol or MicroC™ added as an external carbon source (60 mg/L COD) at two different temperatures, using Biowin simulations.

(carbon dosage). For a new plant, the selection of process configuration and type (suspended versus fixed-film) and anoxic volume are critical for expected nitrogen removal.

Conclusions

Our study results have led to the following conclusions:

(1) The maximum specific denitrification rates obtained with MicroC™, at both 20 and 10°C, were comparable with those obtained with methanol, indicating that MicroC™ can effectively support denitrification. A much higher denitrification rate was observed with acetate at 20°C; however, at 10°C, significant nitrite accumulation occurred, resulting in only partial denitrification.

(2) Comparison of denitrification rates obtained with MicroC™-acclimated or non-acclimated biomass yielded similar rates, suggesting that the denitrifying microbial population capable of using MicroC™ is present in typical WWTPs; therefore, acclimatization to MicroC™ may not be needed.

(3) The maximum growth rates (μ_{max}) estimated for MicroC™-acclimated culture was nearly three times greater than the one found for methanol at both 20 and 10°C. This implies that a longer anoxic SRT and larger post-denitrification reactor volume would be required using methanol than that using MicroC™, to prevent the slow-growing populations from washing-out from the system, especially at colder temperatures.

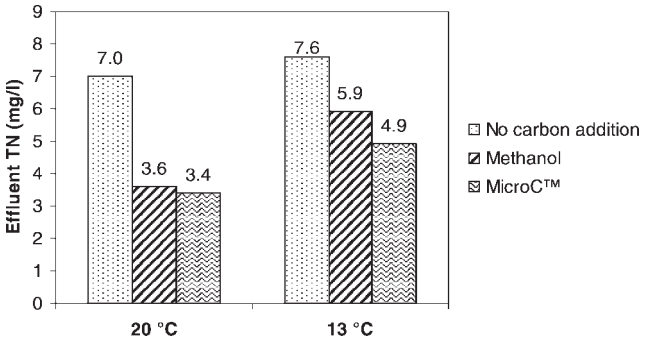

Figure 8—Comparison of nitrogen removal in MLE + post denitrification (Bardenpho 4-stage) configuration with either methanol or MicroC™ as an external carbon source (25.4 mg/L COD) at two different temperatures, using Biowin simulations.

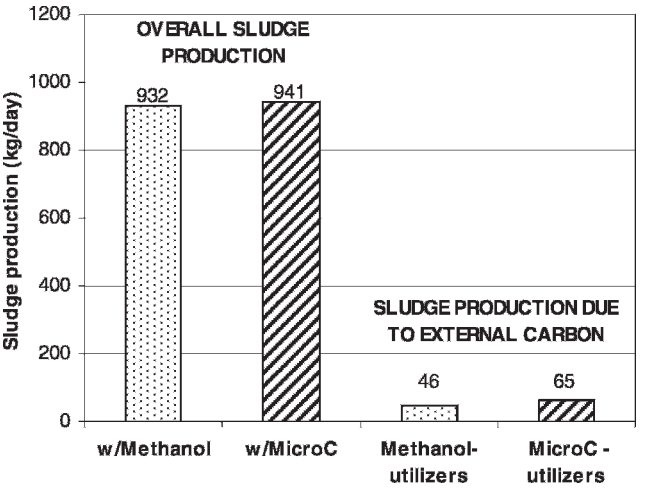

Figure 9—Comparison of sludge production in MLE + post denitrification (Bardenpho 4-stage) configuration with methanol or MicroC™ as an external carbon source (60 mg/L COD), using Biowin simulations.

(4) The microbial community analysis using ARISA profiles clearly shows the distinct community structures of the three denitrifying cultures, as a result of enrichment with three different carbon sources. Although the community compositions are rather dynamic over the period of acclimation, there were consistent trends that could be observed with some of the predominant members (peaks) in the community. The MicroC™ enrichment seemed to have larger diversity than methanol or acetate enrichment.

(5) Evaluation of the capability of a specific carbon-acclimated sludge to instantly use other carbon sources showed that MicroC™ sludge can readily use all the substrates tested, including MicroC™, methanol, acetate, ethanol, and glucose. Methanol-fed sludge can immediately use MicroC™, acetate, and ethanol, but not glucose. Acetate-fed sludge can only use acetate and could not use other carbons readily.

(6) Effect assessment of using different external carbon sources on the nitrogen-removal performance with typical full-scale denitrification process configurations was conducted using Biowin simulations. The results indicated that, with equivalent COD dosage, application of MicroC™ leads to slightly better performance than methanol, especially for the post-denitrification process and under lower temperature conditions. However, the results also showed that the difference in yield did not translate into a significant difference in sludge production.

Credits

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References

Akunna, J. C.; Bizeau, C.; Moletta, R. (1993) Nitrate and Nitrite Reductions with Anaerobic Sludge Using Various Carbon-Sources—Glucose, Glycerol, Acetic-Acid, Lactic-Acid and Methanol. *Water Res*., **27** (8), 1303–1312.

American Public Health Association; American Water Works Association; Water Environment Federation (2001) *Standards Methods for the Examination of Water and Wastewater*, 20th ed.; American Public Health Association: Washington, D.C.

Cappai, G.; Carucci, A.; Onnis, A. (2004) Use of Industrial Wastewaters for the Optimization and Control of Nitrogen Removal Processes. *Water Sci. Technol*., **50** (6), 17–24.

CDM (2007) Evaluation of Methanol Feed, Storage and Handling Costs at Municipal Wastewater Treatment Facilities. CDM: Cambridge, Massachusetts.

Cho, E. S.; Ahn, K. H.; Molof, A. H. (2004) Comparison of Sequentially Combined Carbon with Sole Carbon in Denitrification and Biological Phosphorus Removal. *Water Sci. Technol*., **49** (5–6), 251–256.

Christensson, M.; Lie, E.; Welander, T. (1994) A Comparison Between Ethanol and Methanol as Carbon-Sources for Denitrification. *Water Sci. Technol*., **30** (6), 83–90.

Cozzone, A. J. (1998) Regulation of Acetate Metabolism by Protein Phosphorylation in Enteric Bacteria. *Ann. Rev. Microbiol*., **52,** 127–154.

Danovaro, R.; Luna, G. M.; Dell'Anno, A.; Pietrangeli, B. (2006) Comparison of Two Fingerprinting Techniques, Terminal Restriction Fragment Length Polymorphism and Automated Ribosomal Intergenic Spacer Analysis, for Determination of Bacterial Diversity in Aquatic Environments. *Appl. Environ. Microbiol*., **72** (9), 5982–5989.

Dold, P.; Murthy, S.; Takacs, I.; Bye, C. (2005) Batch Test Method for Measuring Methanol Utilizer Maximum Specific Growth Rate. *Proceedings of the 78th Annual Water Environment Federation Technical Exposition and Conference*, Washington, D.C., Oct 29–Nov 2; Water Environment Federation: Alexandria, Virginia.

Drysdale, G. D.; Kasan, H. C.; Bux, F. (1999) Denitrification by Heterotrophic Bacteria During Activated Sludge Treatment. *Water SA*, **25** (3), 357–362.

Environmental Operating Solutions (2008) Material Safety Data Sheet—EOS MicroC^{TMTM}. Environmental Operating Solutions: Bourne, Massachusetts.

Fisher, M. M.; Triplett, E. W. (1999) Automated Approach for Ribosomal Intergenic Spacer Analysis of Microbial Diversity and its Application to Freshwater Bacterial Communities. *Appl. Environ. Microbiol*., **65** (10), 4630–4636.

Foglar, L.; Briski, F.; Sipos, L.; Vukovic, M. (2005) High Nitrate Removal from Synthetic Wastewater with the Mixed Bacterial Culture. *Bioresour. Technol*., **96** (8), 879–888.

Gomez, M. A.; Gonzalez-Lopez, J.; Hontoria-Garcia, E. (2000) Influence of Carbon Source on Nitrate Removal of Contaminated Groundwater in a Denitrifying Submerged Filter. *J. Hazard. Mater*., **80** (1–3), 69–80.

Hallin, S.; Pell, M. (1998) Metabolic Properties of Denitrifying Bacteria Adapting to Methanol and Ethanol in Activated Sludge. *Water Res*., **32** (1), 13–18.

Isaacs, S. H.; Henze, M. (1995) Controlled Carbon Source Addition to an Alternating Nitrification Denitrification Waste-Water Treatment Process Including Biological P-Removal. *Water Res*., **29** (1), 77–89.

Jones, S. E.; Shade, A. L.; McMahon, K. D.; Kent, A. D. (2007) Comparison of Primer Sets for Use in Automated Ribosomal Intergenic Spacer Analysis of Aquatic Bacterial Communities: An Ecological Perspective. *Appl. Environ. Microbiol*., **73** (2), 659–662.

Kujawa, K.; Klapwijk, B. (1999) A Method to Estimate Denitrification Potential for Predenitrification Systems Using NUR Batch Test. *Water Res*., **33** (10), 2291–2300.

Ledwell, S. (2006) Comparison of Commercially Available Electron Donors and a Non-Flammable Proprietary Carbon Source MicroC^{TMTM} for Biological Nitrogen Removal by Denitrification in the Onsite and Decentralized Industries: Report. Environmental Operating Solutions: Bourne, Massachusetts.

Lee, N. M.; Welander, T. (1996) The Effect of Different Carbon Sources on Respiratory Denitrification in Biological Wastewater Treatment. *J. Ferment. Bioeng*., **82** (3), 277–285.

Madigan, M. T.; Martinko, J. M. (2006) *Brock Biology of Microorganisms*, 11/E. Pearson Education: Upper Saddle River, New Jersey.

Majone, M.; Massanisso, P.; Ramadori, R. (1998) Comparison of Carbon Storage Under Aerobic and Anoxic Conditions. *Water Sci. Technol*., **38** (8–9), 77–84.

Mamais, D.; Jenkins, D.; Pitt, P. (1993) A Rapid Physical-Chemical Method for the Determination of Readily Biodegradable Soluble Cod in Municipal Waste-Water. *Water Res*., **27** (1), 195–197.

Martienssen, M.; Schops, R. (1999) Population Dynamics of Denitrifying Bacteria in a Model Biocommunity. *Water Res*., **33** (3), 639–646.

Metcalf & Eddy (2003) *Wastewater Engineering: Treatment and Reuse*, 4th ed.; McGraw-Hill: New York.

Methanex (2008) Methanex Monthly Average Regional Posted Contract Price History. Methanex: Vancouver, British Columbia.

Mohseni-Bandpi, A.; Elliott, D. J. (1998) Groundwater Denitrification with Alternative Carbon Sources. *Water Sci. Technol*., **38** (6), 237–243.

Mokhayeri, Y.; Nichols, A.; Murthy, S.; Riffat, R.; Dold, P.; Takacs, I. (2006) Examining the Influence of Substrates and Temperature on Maximum Specific Growth Rate of Denitrifiers. *Water Sci. Technol*., **54** (8), 155–162.

Naidoo, V.; Urbain, V.; Ginestet, P.; Buckley, C. A. (2000) Reliability of the Anoxic Respirometric Technique for Wastewater Characterization Using EBPR and Non-EBPR Sludge. *Water Institute for Southern Africa Biennial Conference*, Sun City, South Africa, May 28–June 1; Water Institute for Southern Africa: Midrand, South Africa.

Nyberg, U.; Andersson, B.; Aspegren, H. (1996) Long-Term Experiences with External Carbon Sources for Nitrogen Removal. *Water Sci. Technol*., **33** (12), 109–116.

Onnis-Hayden, A.; Gu, A. Z. (2008) Comparisons of Organic Sources for Denitrification: Biodegradability, Denitrification Rates, Kinetic Constants and Practical Implication for Their Application in WWTP. *Proceedings of the 81st Annual Water Environment Federation Technical Exposition and Conference*, Chicago, Illinois, Oct. 18–22; Water Environment Federation: Alexandria, Virginia.

Quan, Z. X.; Jin, Y. S.; Yin, C. R.; Lee, J. J.; Lee, S. T. (2005) Hydrolyzed Molasses as an External Carbon Source in Biological Nitrogen Removal. *Bioresour. Technol*., **96** (15), 1690–1695.

Rittmann, B. E.; McCarty, P. L. (2000) *Environmental Biotechnology: Principles and Applications*; McGraw-Hill, New York.

Tam, N. F. Y.; Leung, G. L. W.; Wong, Y. S. (1994) The Effects of External Carbon Loading on Nitrogen Removal in Sequencing Batch Reactors. *Water Sci. Technol*., **30** (6), 73–81.

Tsonis, S. P. (1997) Olive Oil Mill Wastewater as Carbon Source in Post Anoxic Denitrification. *Water Sci. Technol*., **36** (2–3), 53–60.

VanRijn, J.; Tal, Y.; Barak, Y. (1996) Influence of Volatile Fatty Acids on Nitrite Accumulation by a Pseudomonas Stutzeri Strain Isolated from a Denitrifying Fluidized Bed Reactor. *Appl. Environ. Microbiol*., **62** (7), 2615–2620.

Water Environment Research Foundation (2007) Working Together to Solve the Challenge of Nutrient Removal. *Nutrient Research Stakeholder Workshop*, Baltimore, Maryland, March 7–8; Water Environment Research Foundation: Alexandria, Virginia.

Yuan, Z. G.; Oehmen, A.; Ingildsen, P. (2002) Control of Nitrate Recirculation Flow in Predenitrification Systems. *Water Sci. Technol*., **45** (4–5), 29–36.