

Investigation Of MicroC™ As An Alternative Carbon Source For Denitrification

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ABSTRACT

The addition of external carbon sources for denitrification becomes necessary for wastewater treatment plants to meet more and more stringent effluent nitrogen limits. The concerns regarding the safety issues and cost of methanol motivated the use of MicroC™ as a non-flammable alternative carbon-source for biological denitrification. The denitrification rates and kinetics obtained by using MicroC™ were determined with both laboratory-acclimated biomass in SBRs system as well as with sludge from full-scale plants. Both low F/M ratio and high F/M ratio tests were performed in this study for comparison. The COD equivalent of MicroC™, the rates and kinetics were used as inputs into the BIOWIN model to simulate and compare the N removal performance with the addition of different external carbons in both pre-denitrification system (MLE) and MLE plus post denitrification system. The temperature effect on performances and the removal of nitrogen was also examined. The ability of a specific carbon-acclimated denitrifying population to instantly utilize other carbon source was also investigated.

KEYWORDS: Denitrification, MicroC™, Biowin, Carbon Sources.

INTRODUCTION

In the last decade, more and more stringent environmental requirements have been imposed on nutrients discharge in surface waters, since excessive nutrients are considered the primary causes of eutrophication. Several sewage treatment plants (STP) are facing the need to be retrofitted and upgraded to accommodate new processes for nutrient removal while counting higher capital and operational costs.

Many of the operating systems achieve the complete degradation of nitrogen and phosphorus species by the use of exogenous carbon sources. Their addition in pre-denitrification anoxic zones increases the denitrification rates and nitrogen removal efficiencies, while addition of carbon to the anaerobic zone of enhanced biological phosphorus removal systems may help improve the process performance and stability. External carbon addition to post-denitrification zone is often required to reach effluent total nitrogen of less than 3 mg/L.

Performances related to long-term experiences with the use of Methanol and Ethanol as sole electron donors and their influence on denitrifying bacterial community have been widely studied and compared (Nyberg et al. 1996, Christensson et al. 1994). In addition to the commonly used external carbons, new alternative compounds have been proposed recently,

including sugar (Gomez et al. 2000, Akunna et al. 1993), glycerol (Akunna et al. 1993), molasses (Quan et al. 2005), corn starch (Lee & Welander 1996), industrial wastewater (Cappai et al. 2004) and others (Tsonis 1997, Akunna et al. 1993, Lee & Welander 1996, Nyberg et al. 1996).

In the US, methanol is the most commonly used electron donor due to lower sludge yield of 0.14 gTSS/gCOD (Christensson et al. 1994) and therefore higher denitrification efficiency as indicated by the relatively lower g methanol/g nitrate ratio; the low cost and the broad availability in the market are supplementary motivations that lead towards this option. The disadvantages of using methanol are the safety issues associated with its transportation, handling and storage as it is considered a reactive and toxic compound. It has been reported that an additional 25 – 31% to the capital construction cost of the 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 start-up process to build the specific methylotrophic population is also relevant (Christensson et al. 1994). Additionally, there have been reports of deteriorated denitrification performance under cold conditions due to the potential wash out of methanol-using denitrifying bacteria from the system as the growth rates decrease at lower temperatures (Mokhayeri et al. 2006). Lastly, prices of methanol have recently tripled, as from a monthly average non-discounted price of 0.75 \$/gal in January 2004 to the 2.5 \$/gal of January 2008 in the U.S. Gulf Coast (Methanex, 2008) and in some cases, shortages have occurred.

The investigation of MicroC™ as an economical alternative carbon source for biological denitrification is of interest and motivated by these concerns. MicroC™ is a proprietary wastewater treatment chemical developed by Environmental Operating Solutions, and designed specifically as a viable, non flammable, agriculturally derived electron donor. Since 2003, MicroC™ has been used throughout the Northeastern United States in 92 facilities plants required to meet stringent effluent nitrogen limits, on the basis of preliminary data (Ledwell, 2006). In general, the plants settings typically used to handle methanol or other carbon sources resulted compatible with MicroC™.

The choice of a carbon source does not only depend on operational features and economy but needs to be also determined by the whole system performance, sludge production and efficiency (Nyberg et al. 1996). Therefore, the objective of this study is to delve into the characteristics and kinetics of MicroC™ to study its potentiality as carbon sources for denitrification process. This goal has been accomplished with a schematic outline, as follow:

- 1) Determine the chemical and biological oxygen demand equivalent of the MicroC™ compound. Since the actual composition of the MicroC™ is not defined, it is not known if all of the available carbon in MicroC™ is in the form of readily biodegradable COD, or if hydrolysis is required before it becomes available for denitrification;
- 2) Develop acclimatized biomass in SBRs system with different carbon sources in order to determine the actual substrate uptake rates and kinetics, including maximum substrate uptake rate and half-saturation constant of denitrification processes, which can be otherwise influenced using un-acclimated WWTP sludge that has varying VSS composition. The substrates tested include MicroC™, methanol and acetate;
- 3) Determine maximum growth rate and observed yield of denitrifying populations grown on MicroC™ and the carbon to nitrogen ratio during denitrification;

- 4) Compare the denitrification rates and kinetics with acclimated microbial populations at different temperatures (10°C and 20°C);
- 5) Investigate the ability of a specific carbon-acclimated denitrifying population to respond when the carbon source is switched to another in terms of denitrification rates and kinetics;
- 6) Compare the nitrogen removal performance with different carbon sources using BIOWIN to simulate different scenarios (MLE, Post denitrification) under various conditions (HRTs, Temperature).

MATERIALS AND METHODS

Chemicals. MicroC™ is a light green liquid compound with a mild alcohol odour containing agricultural products and methanol (5% w/w). Its bulk density and specific gravity are respectively of 9.84 lb/gal and 1.18 while the viscosity is equal to 16.4 cP. The compound presents completely solubility in water and its pH at 25°C is 5.8. MicroC™ presents stability under normal conditions and its freezing point of -20°C avoids storage issues during cold seasons. VOC has not been detected in its composition. It was periodically provided by Environmental Operating Solution; dilutions were freshly prepared for testing and the system feeding, avoiding to compromise the physical and/or performance characteristics of the product. Methanol, Sodium Acetate, Ethanol and Glucose were provided by Fisher Scientific.

Sludge. Full scale activated sludge and laboratory acclimated sludge were tested for comparison.

Full scale activated sludge. MicroC™ acclimated and non-acclimated sludge from WWTPs were provided by:

- the municipal WWTP of Enfield (CT), where the sludge was fully acclimatized with MicroC™, added as the sole carbon source in the post-denitrification stage;
- the municipal WWTP of Wareham (MA), which uses the internal COD as influent to the pre-denitrification stage;

Laboratory acclimated sludge. Different sludges were fully acclimatized with MicroC™, methanol and acetate respectively, as influent substrates, in three laboratory scale SBRs set up at Northeastern University (Boston, MA). The pilot plant was initially seeded with sludge coming from Wareham (MA) WWTP, not specifically acclimated, and it has been continuously fed for one year with the respective substrates of interest.

The influent to the SBRs was a synthetic wastewater diluted in tap water, which contained MicroC™, Methanol, and Acetate respectively as carbon sources with variable concentrations in the range 150-350 mgCOD/l, MgSO₄ · 7H₂O (40 mg/l), CaCl₂ (7.5 mg/l), Fe(SO₄) · 7H₂O (1 mg/l), KH₂PO₄ (22 mg/l), K₂HPO₄ (56 mg/l), NH₄Cl (101 mg/l), yeast extract (30 mg/l), MnSO₄ · 4H₂O (0.2 mg/l), sodium bicarbonate (252 mg/l) and trace minerals.

The system was designed to have a daily influent flow of 9 L/day split into three daily cycles based on a HRT and SRT values of 15 h and 15 days respectively. The cycle includes 2 hours of anoxic phase, 30 minutes of feeding and 4h30' of aerobic period. The influent COD concentration was completely consumed during the anoxic period and therefore, it was not considered an inhibition factor for nitrification in the next phase.

COD, NH₄, NO₃ and NO₂, TSS and VSS were examined on a biweekly basis to monitor the

general functioning of the system. Temperature was in the range 20-23°C, and the oxygen concentration during the aerobic phase was around 7 mg/l. pH was kept in the optimal range of 6.5-7.5.

Typical differences in sludge colours were observed for each SBR, darker brown for Acetate SBR and lighter brown for Methanol and a brown-yellow colour for MicroC™, all with the same TSS concentration.

Analytical measurements. Parameters including N-NO_x, N-NH₃, VSS and TSS were routinely analyzed according to the Standard Methods procedures (APHA, 2001). A Dissolved Oxygen meter YSI 5000 was used to monitor the extent of the aeration while pH and temperature were checked using a Thermo Orion 230 meter.

Dichromate acid digestion was also used to determine the TCOD equivalent to MicroC™ and duplicates of different dilution series have been conducted to obtain statistically confident values. The rbCOD value for a unit amount of MicroC™ was evaluated following 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 determination of the BOD₅ equivalent to MicroC™ occurred accordingly to the Standard Methods (APHA, 2001).

Batch testing

Denitrification tests (low F/M ratio, 0.02-0.05 mgsCOD/mgVSS, short test). A series of denitrification batch tests were conducted to determine the denitrification rates and kinetics of acclimated biomass and full-scale populations with three carbon sources including MicroC™, methanol and acetate. Denitrification rates were determined at various carbon concentrations (0-300 mgsCOD/l) and with an adequate initial nitrate concentration of 20 mg/L accordingly to the method presented by Kujawa et al. (1999). The sludge was kept under a continuous nitrogen gas flow to guarantee anoxic conditions and the pH kept to be constant at 7.5. Samples were taken regularly during the tests to follow the nitrogen uptake rates over time. The denitrification rates as a function of initial COD concentration were then fitted to Monod equation using SPSS 14.0 to estimate the maximum denitrification rate and half saturation constant.

Denitrification tests (high F/M ratio, 2-3 mgO₂/mgVSS, long test). The method proposed by Dold et al. (2005) was applied to estimate the maximum specific growth rate of the specifically cultured denitrifiers and to identify the carbon to nitrogen ratio for each substrate. In these high F/M ratio tests, both electron donor (carbon) and electron acceptor (NO₃) were kept at saturation level to avoid limiting concentrations during the tests. The growth was expected to be exponential and a small initial concentration of biomass (300 mgVSS/l) was loaded into the batch volume. Ammonia and Phosphate were added as nutrients at the beginning of the tests. Kinetic parameters are determined fitting the NO₃ uptake versus time using the equation presented in Dold et al. (2005) in SPSS 14.0:

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

The method applied minimizes the sum of the squares of the residuals by adjusting the three initially estimates parameters: μ_{Hmax} , $X_{\text{N},0}$ and $S_{\text{NO},0}$ by fitting the Y_{HD} and b_{H} to the above equation. Note that the yield Y_{HD} in the model is assumed and has not influence in the μ_{max} of denitrifiers. The decay coefficient b_{H} for all acclimated sludges has been estimated to be 0.1 d^{-1} by low F/M tests run at endogenous conditions, which is agreeable to the values reported in literature (Yuan et al. 2002).

When the accumulation of nitrite occurred during the test the different stoichiometry for the oxidation of carbon using nitrite as electron acceptor was taken into consideration, as equation 2 showed. The coefficient 0.6 takes into consideration the stoichiometry of the denitrification reaction from the ratio between 1.71/2.86, where 1.71 and 2.86 are the oxygen equivalents of nitrite and nitrate respectively (Kujawa & Klapwijk 1999).

$$\text{NO}_x\text{-N}^* = \text{NO}_3\text{-N} + 0.6 \cdot \text{NO}_2\text{-N} \quad (2)$$

The observed Yield of growth for denitrifying (mgVSS/mgCOD) was evaluated from the COD/N ratio measured during the tests, using the equation (3).

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

The μ_{max} at 20°C and 10°C was determined both analytically and experimentally using respectively:

- the low F/M tests at both temperatures;
- an analytical estimation from the K_{dmax} at the same temperature using equation 4 defined by (Naidoo, 1998).

$$\mu_{\text{max}} = Y_{\text{HD}} \cdot \frac{2.86}{(1 - 1.42 \cdot Y_{\text{HD}})} \cdot k_{\text{dmax}} \cdot \frac{24}{f_{\text{HD}} \cdot 1000} \quad (4)$$

Where f_{HD} is the active fraction of the denitrifying VSS.

Biowin simulation

Comparisons of N removal performance using either MicroCTM or methanol as external carbon sources were made by model simulation with Biowin. The kinetics and biodegradability data determined with the batch testing served as input parameters in the simulation model built using the Biowin software. Two different scenarios were analyzed: 1) modified Ludzak-Ettinger (MLE) for predenitrification and, 2) MLE followed with a post-denitrification zone and a final aerobic polishing zone (see Figure 1). The initial design parameters were determined using an in-house Excel spreadsheet. For each scenario, some operational parameters were changed in order to assess their effects of on the steady-state final effluent TN concentration. The parameters that were varied included external carbon dosage, pre-anoxic and post-anoxic volumes (therefore anoxic hydraulic residence time HRT) and temperature. Table 1 summarizes the conditions and operation parameters that were used for the different scenarios analyzed.

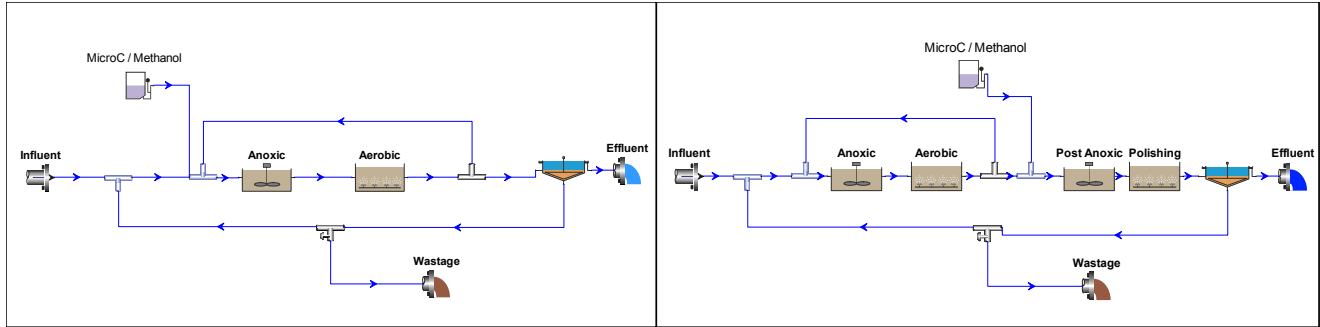


Figure 1. Biowin layouts for the two configuration analyzed: on the left MLE, on the right MLE plus post-denitrification.

Table 1.

Design parameter			Kinetics and stoichiometry		
Configuration	MLE	MLE + post denitrification	Parameter	MicroC™	Methanol
Influent flow rate, Q (MGD)	5	5	μ_{max} [1/d]	3.66	1.25
Temperature (°C)	13 and 20	13 and 20	Ks [mgCOD/L]	20	5
Aerobic SRT (d)	10	10	Aerobic decay [1/d]	0.08	0.06
Influent COD (mg/l)	250	250	Anoxic decay [1/d]	0.08	0.06
V anoxic (MG)	0.25-0.35	0.35	Yield (anoxic) [gCOD/gCOD]	0.52	0.4
V post anoxic (MG)	-	0.3-0.4	Temperature coefficient θ	1.1	1.1
V aerobic (MG)	0.91	0.91	Aerobic growth	yes	no
V polishing (MG)	-	0.1			
Mixed liquor recycle, MLR	3Q	3Q			
RAS	0.5Q	0.5Q			

RESULTS and DISCUSSIONS

Characterization of MicroC™

The TCOD of MicroC™ equal to 662.97 ± 27.23 gCOD/l was found to be close to the value of 672.0 gCOD/l as established by EOS in previous studies (Ledwell, 2006). Approximately, 504 gCOD/l (the 75% of the total COD) was determined to be readily bio-available (rbCOD) by microorganisms, using the method proposed by Mamais et al. (1993) with both laboratory scale and full scale acclimated biomass. The remaining portion of the COD (25%) seems to become bioavailable through hydrolysis; indeed, throughout the study, the COD measured in the effluent from the SBR was consistently below detection limit (5 mg/L), indicating that all COD in MicroC™ is utilized by the biomass. In addition, the BOD₅ was evaluated and resulted in 429 g/l (65% of the total equivalent COD).

Kinetics

Kinetics of MicroC™ and comparison with Methanol and Acetate

SBR-acclimated sludge was used to determine the kinetics of MicroC™ for denitrification processes. Denitrification rates were determined at both 20°C and 10°C, although the latter is more of a transitional rate since the sludges were acclimated at 20°C.

The parallel enrichments of acclimatized biomass specific to methanol, acetate and MicroC™ in SBRS allow also for the comparison of the performances of acclimated sludges grown on different carbon sources in identical boundary conditions.

Maximum specific denitrification rate and half-saturation constant

The maximum nitrate uptake rate found for MicroC™ to be 6.37 mgN/gVSS-h at 20°C; this value is similar to the one obtained with methanol (6.07 mgN/gVSS-h), and therefore showing the feasibility of using MicroC™ as an alternative to methanol as external carbon to enhance denitrification. The use of acetate on the other hand, resulted in higher denitrification rate; the determined value of 13.60 mgN/gVSS-h was in fact nearly twice as high as the others.

A large range of values have been reported in literature for the observed specific denitrification rates for methanol and acetate, ranging from 3.3 mgN/gVSS-h (Nyberg et al. 1996) to 21 mgN/gVSS-h (Foglar et al. 2005) and from 3.09 mgN/gVSS-h (Isaacs & Henze 1995) to 10.6 mgN/gVSS-h (Tam et al. 1994) respectively. The variability of these rates is influenced by the sludge sources (acclimated versus non-acclimated) from full scale or lab-scale systems, type of reactors as well as environmental factors that generally affect biological processes (pH, temperature, etc).

Denitrification rates decrease with declining temperature as demonstrated in previous studies (Mokhayeri et al. 2006, Christensson et al. 1994, Nyberg et al. 1996, Dold *et al.* 2005). At 10°C, k_{dmax} of 2.5 and 2.3 mgN/gVSS-h were obtained for MicroC™ and methanol respectively, correspondent to the 60% and 62% reduction of those evaluated at 20°C.

The maximum specific denitrification rate for methanol at 10°C is comparable to those found by Dold (2005) and a 66% reduction of the nitrate uptake rate was observed for the same compound by Christensson et al. (1994) when the temperature was switched from 25°C to 15°C.

Acetate sludge seemed to be affected the most by temperature drop, with 73% reduction of denitrification rates from 20°C to 10°C. One noteworthy observation regarding the tests conducted with acetate sludge is the accumulation of nitrite during the tests. The incomplete conversion of nitrate to nitrogen gases using acetate as carbon source has been previously reported (Rijn et al., 1996); accumulation of nitrite has been associated with imbalanced activities of nitrate and nitrite reductases, with the inhibition of nitrite reductase by oxygen, nitrate or nitrite and with high COD/N ratio (> 2.5) (Martienssen & Schops 1999).

The accumulation of nitrite for acetate was mainly observed at 10°C, due to the lower growth

rate as well as different temperature sensitivity of nitrite reducer bacteria than those evaluated for the nitrate reducer (Drysdale et al., 1999). Nitrite build up was also obtained at 20°C, but to a much lesser extent.

The half saturation constants for MicroC™, 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 (9 mgCOD/l). The high standard deviation of the results shows the limitation of the NUR methods for the estimation of this parameter. In the anoxic test the choice of substrate to biomass ratio (F/M) is one of the factors which defines the form of the NUR profile; F/M ratios which are too low may result in substrate limitation and indistinguishable breaks, which will lastly translate into errors in the denitrification rates and therefore in the k_s determination.

Half saturation constants are important for N removal capacity and performance at full-scale facilities. This is because in anoxic denitrifying reactors, the in situ specific denitrification rate is substrate-limiting, depending not only on the maximum specific rate, but also on the actual readily bioavailable carbons substrate concentration, and on the half saturation constant. Both maximum denitrification rate and half-saturation constant impact the effluent N concentration. The determination of the k_s is therefore critical; methods in which continuous reactors are run at different solid residence time (SRT) (Grady et al., 2001), are probably a more reliable option, even though they are more complex to operate.

Maximum growth rate of denitrifiers grown on MicroC™, Methanol and Acetate

The maximum growth rate of MicroC™-utilizing denitrifiers at 20°C (3.66 d⁻¹) is comparable to the values previously reported for general heterotrophic denitrifying microorganisms in WWTP (3.2 d⁻¹, Metcalf and Eddy, 2001). Although comparable maximum denitrification rate were observed for methanol and MicroC™, the maximum growth rates estimated at 20°C for the latest was three times greater than the one found for methanol (1.25 d⁻¹); this is due to the much lower yield of methanol-users methylotrophs.

Methylotrophs are specific group of bacteria that are capable of reduced one-carbon (C₁) compounds, such as methanol, methane, and formate, as substrates for biosynthesis and energy requirements. To be able to utilize these carbons they developed a specific pathway, the Serine cycle, where the intermediate formation of formaldehyde occurs. 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-carbons substrates.

A 10°C, the maximum growth rate of MicroC™-acclimated biomass was found to be 1.22 d⁻¹, which is one third of the maximum specific growth rate at 20°C. At the same temperature, methanol-utilizing denitrifiers showed a specific growth rate of 0.34 d⁻¹, which is 3.5 times lower than those grown on MicroC™. The results for methanol are in agreement with values obtained in similar studies (Mokhayeri et al., 2006; Christensson et al., 1994).

A slightly higher value is presented by Metcalf and Eddy (2001) and was obtained with biomass

grown and acclimated at 10°C. In this work, the values measured at 10°C are instead “transitional” kinetics since the sludge was not acclimated at 10°C, therefore this test only simulates instant population response to temperature decrease.

Since the growth rate of a population is directly related to SRT in N removal process, dramatic reduction in the μ_{\max} during cold conditions could potentially lead to the washout of the species of interest from the reactor. In particular, methylotrophs, having the lowest growth rate, will require a minimum anoxic SRT, larger than the one needed to maintain in the system the MicroC™ – utilizing population.

The growth rate of acetate-using denitrifiers at 10°C was not determined due to the partial denitrification and nitrite accumulation occurred during the tests. It seems that the population of bacteria which reduce nitrite to nitrogen gas was more sensitive to temperature change than the nitrate reducers. At 10°C, nitrite consumption was delayed for about 45 hours before the nitrite reducers became active. Partial denitrification was only observed with acetate-acclimated sludge and did not occur in cases with MicroC™ or methanol, indicating a higher sensitivity versus temperature only of nitrite reducing bacteria able to use acetate.

Comparison of COD/N ratio among MicroC™, methanol and acetate

When performing anoxic batch tests is also possible to determine through chemical analysis of both COD and nitrate the, C/N ratio, which can then be used to indirectly estimate the yield based on equation (3). The possibility to obtain multiple data points over a long period of time, and therefore to find a correlation between the depletion of COD and the uptake of nitrate makes the high F/M-type test ideal for this determination.

The C/N ratio was found to be 6.45, 4.82 and 5.74 gCOD/gN, for MicroC™, methanol and acetate, respectively. For compounds of known composition the theoretical COD dose required to reduce a given nitrate load can be determined stoichiometrically; for methanol and acetate the ratio is found to be 4.7 and 3.5 gCOD/gN, respectively (Mokhayeri et al. 2006). No theoretical determination is possible for MicroC™, since its composition is proprietary information and therefore not available. The value determined with methanol resulted close to the theoretical value, however the value obtained for acetate resulted much higher.

The C/N determination from the test 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), the possible activity under anoxic condition of poly-phosphorus accumulating bacteria (PAOs) if present in the sludge (Naidoo et al. 2000), possible aerobic respiration due to oxygen intrusion and the 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 unrelated test in the acetate-fed SBR.

The determination of the correct C/N ratio is crucial for the selection of alternative carbon sources. The COD/N ratio is indeed an indicator of COD usage efficiency for denitrification. The dosage of carbon in post-denitrification systems and the sludge production are both affected by

this parameter. High operational costs, if the carbon source used needs to be purchase, and higher biomass production are the main issues caused by the COD/N overestimation.

Table 2. 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) (d ⁻¹)	μ_{max} (10°C) (d ⁻¹)
MicroC™	6.37 ± 3.6	2.5	38.6 ± 29.2	6.45 ± 3.7	0.39	3.66	1.22
Methanol	6.07 ± 0.7	2.3	15.6 ± 11.2	4.82 ± 1.51	0.29	1.25	0.34
Acetate	13.6 ± 1.86	3.62	38.1 ± 16.2	5.74 ± 1.29	0.35	-	-

Comparison of kinetic coefficients determined using high F/M or low F/M methods

Two different anoxic batch tests were used in this study to evaluate the kinetic coefficients of MicroC™, methanol and acetate.

The low F/M ratio method proposed by Ekama et al. (1986) allows for the direct determination of the specific denitrification rate. The assumptions of this test are that the biomass concentration is constant, and no substrate limiting conditions are present at least at the beginning of the test. The denitrification rates, obtained, were plotted as a function of initial COD concentration and then fitted to Monod-Type equation to estimate the maximum denitrification rate and half saturation constant.

Under these assumptions, the maximum growth rate of denitrifiers can be indirectly determined using equation 4. Besides the maximum denitrification rate the heterotrophic yield under anoxic condition and the fraction of denitrifiers are needed for the calculation. The yield was estimated from the C/N ratio, as already mentioned; the fraction of denitrifiers was estimated considering an active heterotrophic fraction of 55% of the total MLVSS, as suggested in literature (Ni & Yu 2007) and a fraction of denitrifiers among the heterotrophic population of 60% (Metcalf and Eddy, 2001) therefore resulting in a f_{HD} of 0.3.

Maximum growth rate can directly be measured through tests proposed by Dold et al. (2005), as previously described in the materials and methods section. The hypothesis in this case are that the biomass is exponentially growing and no substrate limitation ($S \gg k_s$ and $N \gg k_N$) are present.

In the proposed model μ_{Hmax} , $X_{N,0}$ and $S_{NO,0}$ are estimated. The Y_{HD} and b_H (equation 1) are initially assumed but their values do not influence the μ_{max} of denitrifiers. The estimation of the observed yield can be done afterwards from the measured COD/N using equation 3.

The model was determined to be very sensitive to data sets; a trial and error approach should be therefore implemented to determine the optimal condition (e.g. initial F/M), to obtain a profile, which satisfied the assumption of exponential growth. For example, in the case of methanol at 10°C, the shape of the curve was influenced by the low growth rate of methylotrophs and a linear

trend was observed. Same trend was obtained by Dold et al. (2005) for similar experimental conditions. The selection of the initial seed and the interference of oxygen infiltration could also affect the time profile of nitrate.

In this study, the μ_{\max} at 10°C and 20°C was determined with both the direct (high F/M) and indirect (low F/M) methods and then compared. Results are summarized in Table 3:

Table 3. Comparison of analytical and experimental approach for the maximum specific growth rate or SDNR determination.

	$K_{d\max}$ (20°C) ¹	μ_{\max} (20°C) ¹	μ_{\max} (20°C) ²	$K_{d\max}$ (10°C) ¹	μ_{\max} (10°C) ¹	μ_{\max} (10°C) ²
	mgN/gVSS-h	mgN/gVSS-h	d ⁻¹	mgN/gVSS-h	mgN/gVSS-h	d ⁻¹
MicroC™	6.37 ± 3.6	3.66	1.27	2.5	1.22	0.5
Methanol	6.07 ± 0.7	1.25	0.68	2.3	0.34	0.26
Acetate	13.60 ± 1.86	-	2.17	3.62	-	0.58

¹ direct approach (high F/M)

² indirect approach (low F/M), and assumed f_{HD} .

It can be noted that the maximum specific growth rates at both 10°C and 20°C are underestimated using the indirect methods for all carbon sources tested. The difference in kinetics can be related to assumption of parameters such as the active fraction of biomass in the system and yield of growth for microorganisms. To overcome this problem a method for the direct determination of these parameter was presented (Sozen et al. 1998), where two tests running in parallel measuring one the oxygen uptake rate and the other the NUR are used.

The parameters estimated with the high F/M ratio are in agreement with previous studies using the same carbon source, as already previously discussed. They are therefore considered more reliable for the determination of the specific growth rate; however care must be put carrying out the tests to ensure that the hypothesis, upon which the model is based, are satisfied. Because of these assumption this type of tests cannot, however be used for the evaluation of the maximum specific denitrification rates (changing biomass concentration) and the half saturation constants (no limiting condition).

In conclusion both methods are needed for a complete and more reliable determination of the kinetics and stoichiometric parameters.

Effect of acclimatization

The comparison of denitrification rates obtained with either MicroC™ acclimated or non-acclimated sludge are presented in Table 4. The results indicate that the denitrification rates and kinetics for both full scale sludges are similar (4.72 and 4.34 mgN/gVSS-h) even though the population originated in a pre-denitrification stage has never been exposed to MicroC™. This suggests that the denitrifying microbial population capable of using the compound is active in typical WWTPs and therefore acclimatization to MicroC™ may not be needed. The higher rates

observed with the SBR acclimated sludge compared to the acclimated-full-scale biomass is likely due to the enrichment in population in the SBR sludge, and to the higher amounts of inert solids in the biomass at WWTPs.

The maximum growth rate of denitrifiers was only evaluated for acclimated biomass; hence comparisons cannot be made in respect of the un-acclimatized population. Both the SBR mixed culture and the full scale sludge generate similar μ_{\max} at 20°C.

Similar results for the COD/N ratio were determined in both cases of acclimatization, while a lower value was found when the sludge from the un-acclimated system was used. This can be related to the lower SRT, thus the COD/N, characteristic of pre-denitrification stage configurations compared to plants that operate with post-denitrification. Consequently, also the calculated yield of growth resulted lower.

Table 4. Kinetic coefficients for MicroC™ tested on acclimated and not acclimated biomass.

	$K_{d\max}$ (20°C) mgN/gVSS-h	$K_{d\max}$ (10°C) mgN/gVSS-h	K_s mgsCOD/l	COD/N mgsCOD/mgN	Yield _{obs} mgVSS/mgCOD	μ_{\max} (20°C) d ⁻¹	μ_{\max} (10°C) d ⁻¹
Acclimatized SBR	6.37 ± 3.6	2.5	38.6 ± 29.2	6.45 ± 3.7	0.39	3.66	1.22
Acclimatized WWTP	4.72 ± 0.48	-	28 ± 11.48	6.98 ± 1.4	0.415	3.9	-
Not- acclimatized WWTP	4.34 ± 0.52	-	49.74 ± 18.8	3.98	0.20	-	-

Ability of acclimated sludge to use other carbon sources

It has been demonstrated that the quantity, quality (Lee & Welander 1996) and combined use of external carbon sources can have various effects on the respiratory denitrification. The addition of combined external carbons in post-denitrification zones can either enhance the removal of nitrogen (Cho et al. 2004) or affect the metabolic properties of the established population with the resulted decreasing in rates. Moreover, supplemental carbon addition can reduce the capacity of denitrifiers to utilize internal carbon in pre-denitrification systems (Hallin & Pell 1998). The ability and acclimatization time required of a specific population to utilize other carbons sources also have practical implications, affecting the easiness and adaptation time a WWTP would require when changing the carbon from one to another.

In this study, we evaluated the ability of specific carbon-acclimated biomass to instantly utilize other carbon sources. The initial denitrification rates upon addition to various carbons are determined with low F/M ratio tests and compared. One data set that fully reflects the general trends is herein presented (Table 5), although some variations from the absolute rates have also been noticed.

A review of the biochemical pathways for anoxic metabolism of these carbons is presented in Table 6, and encloses the factors that influence the response of each combination sludge-carbon source tested.

Methanol acclimated biomass could readily use all of the substrate for denitrification with high rates besides glucose, which was poorly utilized. From a metabolic point of view we can summarize the following observations:

- 1) Acetate and Ethanol could easily enter the methylotrophics pathway directly in the Tricarboxylic Acid Cycle (TCA) skipping the Serine cycle characteristic of one carbon compounds. In the case of Ethanol, the immediate response was probably due to the presence of *alcohol dehydrogenase* enzymes that catalyzed the conversion to acetate, and the consequent transformation into Acetyl CoA.
- 2) Interesting was the slightly higher affinity that methanol acclimated sludge had with MicroC™, showing how it can simply be utilized by a highly specialized community. The presence of methanol in MicroC™ formulation (5%) probably allows for the development of the enzymes characteristic of the Serine pathway. MicroC™ was easily consumed by methylotrophs, demonstrating that its components can either enter directly the TCA cycle or be easily converted by the existing enzymatic activity to Acetyl CoA.
- 3) The denitrification efficiency of methanol-utilizing bacteria seems to be affected when glucose was used as substrate, as previously showed Mohseni-Bandpi et al. (1998). Glycolysis is a multistep pathway that microorganisms utilized to obtain energy from glucose before entering the citric acid cycle. The high specificity of biocatalyst, maybe not specifically developed by methylotrophs and the complex chain reactions can be the reasons behind the reduced denitrification activity when multi carbon compounds became available. Similar results and conclusions were found in the study of Hallin and colleagues (1998). Dold et al. (2005) showed low denitrification rates using glucose in combination with mixed liquor from nitrification stage, demonstrating that only specific microorganisms in a community are able to metabolize sugars.

Table 5. SDNR for different combination sludge-electron donor

	MicroC™ Sludge (mgN/gVSS-h)	Methanol Sludge (mgN/gVSS-h)	Acetate Sludge (mgN/gVSS-h)
MicroC™	17.11	12.65	0.92
Methanol	5.36	11.32	1.14
Acetate	15.75	8.64	12.9
Ethanol	7.77	13.44	-
Glucose	8.92	0.4	0.23

Acetate sludge could only use acetate efficiently; a slight (methanol) or totally absent (MicroC™, ethanol and glucose) response was obtained with the other carbons. Since the same behavior was observed for all the experimented substrates, two main factors have been considered responsible for the reduced biological activity:

- 1) the acclimatization to acetate either enrich only the populations that exclusively use acetate, or simply turn off all the genes for those up-stream enzymes, important for the other pathways, since they are not needed. Therefore, the acclimated population which is used to enter directly the TriCarboxylic Acid Cycle, lack the enzymatic activity able to convert more complex multi-carbon compounds into acetate. Additionally, methanol, as single carbon, must follow a

reduction process to form tri-carbons or four carbons intermediates before entering the TCA cycle. Nyberg et al. (1996) for systems previously acclimated with methanol or ethanol also confirmed that un-specialized bacteria have difficulty in degrading methanol
 2) microorganisms prefer to utilize internally stored compounds that enter directly the tri-carboxylic acid (TCA) cycle for the conversion of organic substrate as sources of energy and carbon.

Table 6. Biochemical pathways involved in the utilization of the tested carbons

Carbon source	Microorganism capable of C utilization	Biochemical pathways involved	Key steps of metabolism	Enzymes involved
Methanol	Methylotrophs	Serine Pathway (Type II)	$CH_3OH \rightarrow$ Formaldehyde \downarrow Serine Pathway \rightarrow Acetyl CoA \downarrow TriCarboxylic Acid Cycle	<i>Serin transhydroxymethylase, α-ketoglutarate dehydrogenase, enzymes characteristic of TCA</i>
Acetate	Diverse community	TriCarboxylic Acid Cycle	$CH_3COO^- \rightarrow$ TriCarboxylic Acid Cycle	<i>Citrate synthase, isocitrate dehydrogenase, Succinyl-CoA synthetase, etc</i>
Ethanol	Diverse community	TriCarboxylic Acid Cycle	$C_2H_5OH \rightarrow$ Acetaldehyde \rightarrow Acetate \downarrow TriCarboxylic Acid Cycle	<i>alcohol dehydrogenase, acetaldehyde dehydrogenase, etc</i>
Glucose	Diverse community	Glycolysis + TCA	Glucose \rightarrow Glyceraldehyde-3-P \downarrow Pyruvate \rightarrow Acetyl CoA \downarrow TriCarboxylic Acid Cycle	<i>Hexokinase, Glyceraldehyde-3-P dehydrogenase, Pyruvate kinase, etc</i>
MicroC™	Diverse community	TCA cycle, Serine Pathway and Glycolysis	Diverse	Diverse

MicroC™ sludge responded with different rates with all the tested carbons: the highest rate for **MicroC™** as carbon source was the effect of the previous acclimatization (17.11 mgN/gVSS-h). The lowest denitrification rate value was measured for Methanol instead (5.36 mgN/gVSS-h) demonstrating that this compound can only be degraded by a specific class of microorganisms able to enter the Serine pathway. In this case the presence of methylotrophs was expected for the content of methanol in **MicroC™**. The results indicate that the **MicroC™** acclimated biomass contains a relatively large diversity of microorganisms, and possibly with different metabolic pathways, due to the complexity of the **MicroC™** composition as well as its high degradability. Furthermore, it can be seen how methanol can be used by **MicroC™** sludge with high rates and how **MicroC™** can be used as a start up compounds for those plants which utilize Methanol as external carbon in post denitrification, in order to avoid the high lag time needed by methylotrophs.

Implication of kinetics on full scale practice

From the results of the simulation with Biowin it can be concluded that when same amount of COD as either **MicroC™** or as methanol is added to enhance pre-denitrification, **MicroC™** gives

slightly better performance than methanol at 20°C. Also, HRT of anoxic zone affects denitrification performance and therefore both COD dosing and HRT should be considered when adding an external carbon for enhancing denitrification.

Considering the higher yields of MicroC™-utilizing bacteria compared to methylotrophs, it is important to verify the effect of both carbon additions to the sludge production. The difference in yield did not however translate in a very significant difference in sludge production: using MicroC the sludge production was 1% higher than with methanol for post denitrification scenario, whereas no noteworthy differences were found for the pre-denitrification scenario.

Temperature affects denitrification rates and kinetics of both methylotrophs and MicroC™-utilizer bacteria. The denitrification in the system with MicroC™ supplement seems to be less sensitive to temperature drop compared to with methanol supplement. The results of the simulation (Figure 2 and 3) indicate that at 13°C, for equivalent COD added, MicroC™ performs slightly better than methanol, especially in the post-denitrification scenario. Comparison of the impact of temperature on denitrification between using MicroC™ and using methanol is more clearly demonstrated when the temperature drops to be <5°C. At this very low temperature the minimal SRT required to prevent methylotrophs from washing out from the suspended post denitrification reactor is about 4.5 days. In contrast, the minimal SRT required for keeping the MicroC™-utilizers is 1.5 days. This implies that larger anoxic reactor volume is required for methanol compared to MicroC™ at extremely low temperature conditions.

Following a good characterization, which include the determination of biodegradability (COD fractions), kinetics and stoichiometric parameters, the use of simulator as BIOWIN, can help in the selection, for an existing facility, of the most effective external carbon source, as well as the selection of optimal operational condition (carbon dosage). For new plant the operational temperature and the selection of the necessary anoxic volume are critical for achieving good nitrogen removal.

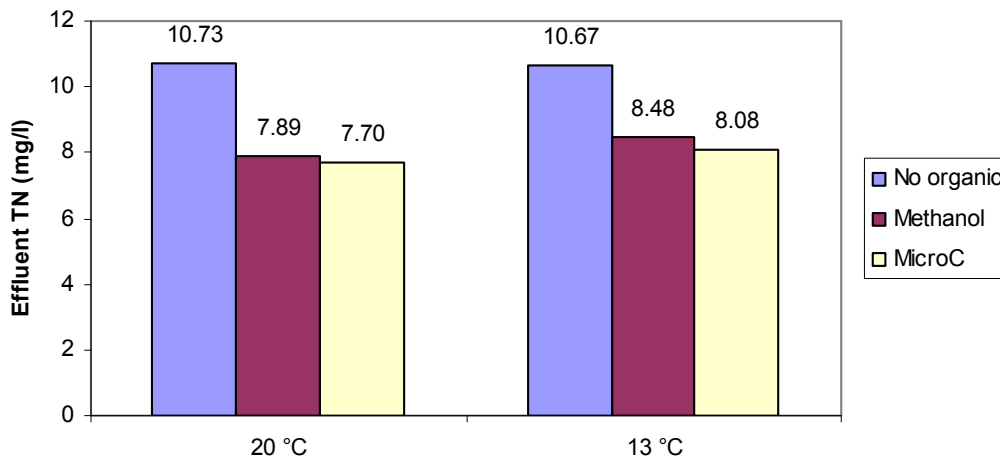


Figure 2: Biowin steady state results for MLE configuration without and with external addition at two different temperatures.

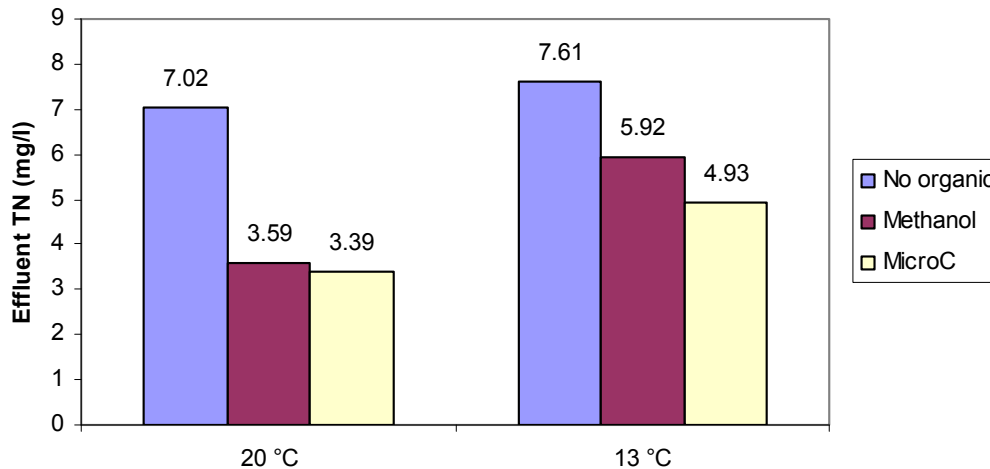


Figure 3: Biowin steady state results for MLE configuration plus post denitrification without and with external addition (480 kgCOD/d) at two different temperatures.

CONCLUSIONS

In conclusion we can state that:

1. MicroC™ can effectively support denitrification with both non-acclimated WWTP sludge and MicroC™ acclimated WWTP and SBR biomass.
2. The maximum specific denitrification rates with MicroC™ as carbon source were higher with laboratory grown sludge than those obtained with full-scale communities likely due to the higher active denitrifying fraction of biomass in the SBR.
3. Comparison of maximum specific denitrification rates in SBR acclimated sludges that were acclimated with MicroC™, methanol and acetate as sole carbon source showed that acetate-fed reactor has the highest rate, followed by MicroC™ and then methanol.
4. The maximum growth rates estimated at 20°C with SBRs-acclimated sludges yield the highest value for MicroC™-fed sludge (3.66 d⁻¹) and the lowest with methanol (1.21d⁻¹).
5. When the SBR biomass that was acclimated at 20°C was tested at 10°C for “transitional” denitrification rates and kinetics, MicroC™ acclimated sludge had higher maximum specific denitrification rate and maximum growth rate than the methanol-fed one. This indicates that MicroC™ sludge likely provides more stable denitrification under conditions that experiences temperature fluctuation. Under very low temperature conditions where methanol-using denitrifiers have much lower denitrification rates and maximum growth rate, it could potentially lead to washed out of the methylotrophs from the system if the design SRT is not sufficient.
6. Acetate-fed sludge was most sensitive to temperature change and nitrite accumulated as a result of partial denitrification. The nitrite-reducing denitrifying microorganisms seem to be more sensitive to temperature decrease than the nitrate-reducing denitrifying populations and therefore lead to incomplete denitrification.
7. Using BIOWIN simulations, with equivalent amount of COD as either MicroC™ or methanol being added to a MLE or a MLE post-denitrification process, a slightly better performance was shown with MicroC™ than methanol, especially for the post-denitrification process and under low temperature conditions.

8. The series of tests conducted to evaluate the immediate response of a specific carbon-acclimated sludge to utilize other alternative carbon sources showed that MicroC™-sludge can readily utilize all the tested substrates. Methanol-fed sludge can immediately utilize MicroC™, acetate, ethanol, but not glucose. Acetate-fed sludge can utilize ethanol and glucose at much slower rates and could not readily utilize MicroC™ or methanol.

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