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A Study of Aerobic Methanol Addition in Denitrifying Sequencing Batch Reactors

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Thesis submitted to the faculty of the University of Cincinnati, District of Columbia Water and Sewer Authority, and the Virginia Military Institute in partial fulfillment of the requirements for the degree of

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ABSTRACT

The Blue Plains Advanced Wastewater Treatment Plant (AWTP) in Washington, DC has a high rate activated sludge system followed by a suspended growth nitrification-denitrification system. Methanol is used for post-denitrification, because it is the least expensive synthetic compound available that does not leave a residual biochemical oxygen demand (BOD) in the process effluent. This process achieves low total nitrogen values in summer, while during winter, the process is more difficult to operate due to stressed conditions. This suggests kinetic limitations of methylotrophic bacteria.

The objective of this project was to evaluate methods for possibly overcoming limitations of methanol addition for full-scale facilities that have limited available anoxic solids retention time (SRT). There is considerable uncertainty associated with the denitrification potential of aerobically grown methylphilic bacteria and its effect on full-scale plant design/operation. Gujer and Irene (1999) suggested that strict anoxic methylotrophs may have somewhat different kinetic capability compared to facultatively-grown methanol utilizing bacteria. It was hypothesized that methanol addition under aerobic conditions (bleeding a small amount of methanol into the aerobic zone of a BNR process) could alleviate anoxic SRT limitations in a full-scale plant by increasing the active methylotrophic biomass content in the process. It is critical to understand denitrification kinetics under these conditions. To perform this research, 3.5 liter sequencing batch reactors were operated to observe methanol acclimation. Data was collected by monitoring the SBRs to determine the specific denitrification rate (SDNR). Once the SBRs stabilized, High Food to Microorganism tests were performed to determine the maximum anoxic growth rate ($\mu_{\text{max}}$) of the methanol denitrifiers.
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CHAPTER 1: Introduction to Denitrification

1.1 Introduction to Water Quality

Over the last century, treatment objectives for wastewater treatment plants (WWTP) have changed dramatically, and will continue to change with new technology and regulations driving them. From the start of wastewater treatment and up into the early 1970s, the focus was on preliminary and primary treatment with the removal of floatable, suspended, and colloidal materials (Tchobanoglous et al, 2003). In 1972 the clean water act (CWA) was implemented to address impaired waters and to achieve swimmable and fishable waters. The focus of treatment for the CWA was a higher level of treatment of biochemical oxygen demand (BOD), total suspended solids (TSS), and pathogenic organisms.

The need for nitrification became evident as stricter regulations were imposed after recognizing the need for nutrient removal to prevent eutrophication and the toxicity of ammonia to aquatic and marine organisms. Biological nitrification, which is an aerobic two step oxidation process, is the most common method for ammonia conversion in a WWTP.

\[ \text{Nitrification} \quad \text{NH}_4 \rightarrow \text{NO}_2 \rightarrow \text{NO}_3 \]

More advanced WWTPs have begun phosphorus removal as nutrient limits have become more stringent in critical, typically freshwater bodies. Excess loading of phosphorus has also been linked to eutrophication. Phosphorus can be removed by methods such as enhanced biological phosphorus removal (EBPR), chemical precipitation, and other emerging techniques. The next step in overall removal of nitrogen is denitrification. Denitrification is conjoined with nitrification in WWTPs that
must meet stricter total nitrogen (TN) effluent limits. Biological denitrification is the
anoxic reduction of the nitrate produced by nitrification to nitrogen gas.

\[
\text{Denitrification} \quad \text{NO}_3 \rightarrow \text{NO}_2 \rightarrow \text{NO} \rightarrow \text{N}_2 \text{(g)}
\]

-- (g) denotes gaseous species

Denitrification within a typical WWTP can occur in either preanoxic or post
anoxic processes, with the difference being the carbon source used as the electron donor.
In a preanoxic reactor an internal carbon source is used to drive denitrification, such as
the influent BOD, as where a post anoxic reactor often requires an external carbon source
be used to enhance microbial growth kinetics.

1.2 Biological Denitrification

The ability for heterotrophic bacteria to accomplish denitrification is distributed
over a broad range of physiological and taxonomic groups of more than 130 bacterial
species and 50 genera. Denitrification is somewhat more characteristic of the alpha and
beta classes of proteobacteria, and is most commonly done by facultative heterotrophic
bacteria (Zumft, 1992). Denitrification is performed through nitrate respiration, nitrite
respiration combined with NO reduction, and N2O respiration. The process occurs under
conditions where oxygen is not present, and instead nitrate is used as the terminal
electron acceptor (Zumft, 1992).

These heterotrophic denitrifiers are able to obtain their energy and carbon
requirements by breaking down organic substrates. The organic carbon serves as the
electron donor, and the electrons removed are transferred through multiple stages to the
terminal electron acceptor. Chemical energy is released in the form of adenosine
triphosphate (ATP) during this process (Buckley et al, 1999).

This anoxic respiration is broken down into 4 stages
1. Complex organics molecules are hydrolyzed to simpler ones.

2. The end products from stage one are degraded further to form acetyl-Coenzyme A (acetyl Co-A) and CO₂.

3. End products from stage 2 (Acetyl Co-A) enter the tri-carboxylic (TCA) cycle and are oxidized.

4. The electrons and protons produced in stages 2 and 3 pass via electron and proton carrier enzymes to a final electron acceptor. (Buckley et al, 1999).

Denitrifying organisms are divided into two categories when looking at the overall denitrification process. There are those that are capable of complete nitrate reduction (nitrate to dinitrogen gas) and those microorganisms that carryout only one or more of the reaction steps (nitrate to nitrite or nitrite to nitrous oxide, etc.) (Henze, 1992). These microorganisms that are incapable of complete denitrification are called partial denitrifiers. It is estimated that the majority of heterotrophs are partial denitrifiers only capable of nitrate reduction (Lemmer et al, 1997).

**1.2.1 Denitrification with Methanol at a Full-Scale WWTP**

When evaluating external carbon sources for denitrification it is important to consider all impacts that they may have on an operating system. Changes in carbon sources impact the specific denitrification rates (SDNR) and the maximum growth rates of the microorganisms. Other important factors include the sludge characteristics such as the production rates and settling properties. Also, the economics and availability of the carbon source are of significant concern.

The use of methanol to drive post denitrification was investigated in this study since it is the most commonly used external carbon source for biological denitrification, and is
used at the Blue Plains AWTP (Foglar et al, 2003). The overall reaction when using methanol as carbon source for denitrification is shown below (Tchobanoglous et al, 2003)

$$5\text{CH}_3\text{OH} + 6\text{NO}_3^- \rightarrow 3\text{N}_2 + 5\text{CO}_2 + 7\text{H}_2\text{O} + 6\text{OH}^-$$

From the above equation, it is calculated that 1.90 grams MeOH is required per gram of NO$_3$-N removed (2.86 gCOD/ gNO$_3$-N). This value does not take into account cell growth required for biological denitrification. Therefore, the ratio required will be greater depending on the actual yield of the denitrifying biomass. The typical yields for denitrifying biomass are further discussed in Section 1.2.6.

Some of the advantages of methanol include the relatively low cost and sludge production rate (low yield) as compared to acetate and other organic compounds (Purtschert et al, 1996). Despite its advantages, there are some disadvantages of using methanol. These include a lower SDNR and a longer acclimation period than other carbon sources. These lower rates are most likely due to the fact that methanol, a single carbon compound, must undergo a reduction process to form 3-C or 4-C intermediates before entering the TCA cycle (see 4 stages of anoxic respiration, section 1.2), whereas acetate is able to directly enter the TCA cycle (Tam et al, 1992).

**1.2.2 Suspected Methanol Denitrifiers**

When using methanol to supplement denitrification at a WWTP it is important to consider the microorganisms present in a methylophilic denitrifying biomass, and the sensitivity that might be associated with them. Aerobic high-rate BOD removal and heterotrophic denitrification using influent BOD or a wide range of other external carbon sources in a WWTP can be performed by a very large array of microorganisms, but this is not the case for some nutrient removal processes. Biological nitrification selects
autotrophic nitrifiers that convert ammonia to nitrate. In much the same way, these denitrifying methanol-utilizing bacteria are thought to be a very specific group of microorganisms, and thus makes the treatment process more susceptible (Heylen et al, 2005).

Due to the initial low concentration of methylotrophs in activated sludge, a lag period is observed when first adding methanol for denitrification. This acclimation period, likely associated with the growth and establishment of a new population of methylotrophic organisms, can last between a few days several to many weeks depending on the situation and conditions (e.g. temperature) in the reactor (Carrera et al, 2003). Therefore, methanol dosing must be kept constant to maintain the specific biomass after acclimation. This population shift has been visualized with molecular tools in other papers such as Hallin et al (2006) and Ginige et al (2003).

The environmental conditions present in a WWTP tend to select for specific microorganisms. Methylotrophic bacteria can be divided into three subgroups: the obligate methylotrophs that can use only single-carbon compounds; the restricted facultative methylotrophs that also grow on a limited range of more complex organic compounds (ethanol, propanol); and typical facultative methylotrophs that grow on a wider range of polycarbon compounds (acetate, glucose) (Doronina et al, 2005).

Microorganisms that have been reported to be responsible for denitrification in activated sludge are varied. This could be due to the limitations of bacterial culture techniques from biological treatment process samples, the molecular methods used for identification or from the simple differences in reactor conditions. Magnusson et al (1998) reported that all of the denitrifying methanol-utilizing strains were Gram negative
proteobacteria, facultative aerobic motile rods. Multiple culture-dependent techniques have suggested that Hyphomicrobium spp. and/or Parococcus spp. were the dominant species associated with methanol-driven denitrification and therefore the likely methylotroph present in post-denitrification processes (Timmermans and Haute, 1983; Neef et al, 1996; Foglar and Briski, 2003; Lee and Welander, 1996; Lemmer et al, 1997).

The weakness of using culture based methods is that they can fail to accurately represent the microbial community structure since function cannot be directly linked to identity (Wagner et al, 1993). Using methods such as stable-isotope probing (SIP) can help to overcome some of these limitations. SIP uses $^{13}$C substrates to biologically label the DNA of microorganisms responsible for using that substrate (Radajewski et al, 2000).

The use of SIP has suggested that methylotrophs that were not previously known as denitrifiers in activated sludge could be selected in the process. Ginige et al (2004) and Osaka et al (2006) have suggested microorganisms closely related to obligate methylotrophs Methylobacillus and Methylophilus in the order of methylophilales of beta-proteobacteria are responsible for denitrification with methanol. Ginige et al (2004) also reported that there was no correlation between denitrification rates and hyphomicrobium abundance.

There are limitations of SIP that must be recognized. First, there is the chance of microorganisms not responsible for denitrification being mislabeled because intermediates and products can become incorporated into the DNA of microbial communities when primary consumers metabolize the original substrates and excrete labeled metabolites (Osaka et al, 2006). There will be continued studies on the specific
microorganisms responsible for denitrification, because it remains unclear whether the most important or efficient denitrifiers in activated sludge have been correctly identified.

1.2.3 Factors Affecting Denitrification

There are many factors that are capable of affecting the operation of a post-denitrification process. Some of these factors are difficult to establish, due to varying conditions of influent wastewater such as shock loads that may often go unnoticed in a wastewater treatment plant. Other conditions such as temperature, pH, and dissolved oxygen are more easily monitored.

Methanol-utilizing denitrification systems are usually biomass limited due to reported slow growth rates. Therefore, it is important to look at factors that may impact these growth rates.

Almost all denitrifiers are thought to be mesophilic bacteria (Zumft, 1992), traditionally between 20 and 45°C. Therefore, when water temperature drops during the colder months of the year it can have adverse impact since denitrification rate is proportional to bacterial growth rate. The lower temperatures can result in significantly slower growth rates. It was observed that a change in temperature from 20 to 10°C will decrease the denitrification rate by approximately 70% in a methanol-utilizing biomass (Timmermans and Haute, 1983). Tchobanoglous et al (2003) reports an Arrhenius coefficient of 1.026 for a denitrifying biomass using endogenous carbon sources. More recently Dold et al (2005) suggests an Arrhenius coefficient of 1.09 for a methanol-utilizing denitrifying biomass. This coefficient implies a doubling rate for an 8°C increase in temperature. This greater value for the specific methanol-utilizing biomass
may suggest that their growth rates are more sensitive to temperature than a general denitrifying biomass.

Denitrification can occur over a reported wide range of pH values that most commonly fall between 6.5 and 8.5. Dodd and Bone (1975) reported that a pH of 7.5 had the greatest denitrification rate after testing a range of values between 7 and 8.5. It has also been suggested that by maintaining a pH above 7.3 it will ensure the end product of denitrification is dinitrogen gas (Christensen and Harremoes, 1977). These optimal values for pH may differ with reactor conditions and the microorganisms responsible for denitrification.

It is assumed that denitrification occurs in absence of dissolved oxygen (DO), since the presence of DO acts as a strong inhibitor of denitrification as it prevents the necessary expression of the enzymes for electron transfer (Kornaros and Lyberatos, 1998). Wastewater treatment design suggests that a DO of less than 0.5 mg/L is necessary to prevent oxygen from being used as the electron acceptor, and that an increase in DO will decrease the denitrification rates. The impacts of low concentrations of DO in a reactor are difficult to evaluate, since the actual DO concentrations inside the flocs are unknown. Contrary to traditional thought there are some microorganisms, aerobic denitrifiers, which are able to use oxygen and nitrate simultaneously as an electron acceptor (Lemmer et al, 1997). Most process simulation models use a switching function to simply reduce the growth rate of denitrifying organisms as the bulk liquid DO increases (Ko values for denitrification are process specific and typically range from 0.1 to 0.2 mg/L, (Tchobanoglous et al, 2003)).
1.2.4 Performance Parameters for Denitrification

Two of the most commonly used values when evaluating the performance of a system are the denitrification rates (and maximum specific growth rate) and the carbon efficiency (directly related to the organism yield). There is much reported literature on both of these tests due to the relative ease and accuracy in which they can be performed.

1.2.5 Denitrification Rates

The denitrification rate in a reactor is the rate in which nitrate is converted to dinitrogen gas (assuming complete denitrification) normalized to the MLVSS concentration. Values for the SDNR will vary depending on the design of the post denitrification process since the MLVSS term is a lump sum of all of the microorganisms present in the treatment system. Therefore, a treatment process with a separate denitrification stage will have a higher percentage of methanol utilizing denitrifiers in the MLVSS term. Units for an SDNR are most often reported as mg NO₃-N/ g MLVSS/ hour.

All the reported values in this paper were taken from studies that were done with methanol on either denitrifying only mixed liquor or a second stage nitrification/denitrification sludge. These values ranged from 2.5 to 60 mg NO₃-N/g VSS/hr. This wide range is due to the cultivation method (the kind of microorganism selected), treatment technology used (active fraction of denitrifiers in biomass), and experimental temperature. Below in Table 1 are literature values.
Table 1: Reported specific denitrification rates (SDNR) in literature (mgNO₃-N/gVSS/hr)

<table>
<thead>
<tr>
<th>Technology</th>
<th>Process</th>
<th>T (°C)</th>
<th>SDNR</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Nitrification-Denitrification</td>
<td>Continuous</td>
<td>10</td>
<td>2.5</td>
<td>Nyberg et al, 1992</td>
</tr>
<tr>
<td>Nitrification-Denitrification</td>
<td>Continuous</td>
<td>30</td>
<td>5.8</td>
<td>Teichgraber; Stein, 1994</td>
</tr>
<tr>
<td>Nitrification-Denitrification</td>
<td>Continuous</td>
<td>-</td>
<td>6</td>
<td>Bailey et al, 1998</td>
</tr>
<tr>
<td>Nitrification-Denitrification</td>
<td>Continuous</td>
<td>20</td>
<td>7.1 – 20</td>
<td>Henze, 1991</td>
</tr>
<tr>
<td>Denitrification sludge only</td>
<td>Continuous</td>
<td>25</td>
<td>7.5</td>
<td>Carrerra et al, 2003</td>
</tr>
<tr>
<td>Denitrification sludge only</td>
<td>Batch</td>
<td>22</td>
<td>12</td>
<td>Lee; Welander, 1996</td>
</tr>
<tr>
<td>Denitrification sludge only</td>
<td>Batch</td>
<td>25</td>
<td>21</td>
<td>Foglar et al, 2005</td>
</tr>
<tr>
<td>Denitrification sludge only</td>
<td>Continuous</td>
<td>20</td>
<td>25</td>
<td>Timmermans; Haute, 1983</td>
</tr>
<tr>
<td>Denitrification sludge only</td>
<td>Continuous</td>
<td>20</td>
<td>29</td>
<td>Lee; Welander, 1996</td>
</tr>
<tr>
<td>Denitrification sludge only</td>
<td>Continuous</td>
<td>22</td>
<td>60</td>
<td>Ginige et al, 2004</td>
</tr>
</tbody>
</table>

Directly correlated with the rate at which a microorganism is able denitrify is its maximum specific growth rate, \( \mu_{\text{max}} \). Reported values for growth rates of methanol utilizing denitrifiers were few in literature due to the limited methods of testing and the accuracy of these tests. These values are included below in table 2.

Table 2: Reported maximum growth rates of methanol-utilizing denitrifiers (d\(^{-1}\))

<table>
<thead>
<tr>
<th>( \mu_{\text{max}} ) (d(^{-1}))</th>
<th>T (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.40</td>
<td>25</td>
<td>Foglar et al, 2005</td>
</tr>
<tr>
<td>0.50</td>
<td>13</td>
<td>Mokhayeri et al, 2006</td>
</tr>
<tr>
<td>0.77</td>
<td>15</td>
<td>Lee; Welander 1996</td>
</tr>
<tr>
<td>1.00</td>
<td>19</td>
<td>Mokhayeri et al, 2006</td>
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</table>

1.2.6 Denitrification Efficiency, MeOH/N Ratio

The methanol dose required to denitrify a given nitrate load for a WWTP can vary greatly depending on the actual reactor conditions. A well designed anoxic basin may be very efficient and approach experimental values for g MeOH used per g of NO₃-N eliminated. If a reactor basin is poorly designed, methanol may be wasted due to inefficient mixing and hydraulic short circuiting or DO input from other zones causing
some of the methanol to be used aerobically. An example of this is shown by Purtschert et al (1996) by observing a MeOH/N 3.67 for full scale operation and a MeOH/N ratio of 3.2 in laboratory batch experiments for the same mixed liquor.

This amount of methanol added also effects the denitrification performance. If too little of methanol is added, the reactor will be carbon limited and unable to completely denitrify, and if too much methanol is added not only is this wasting resource, it also must be degraded aerobically to prevent high effluent COD. Reported literature values on the optimum MeOH/NO3-N ratio for denitrification vary. Measured values for complete denitrification have varied from as low as 2.5 gMeOH/ gNO3-N (3.75 COD/N) (McCarty et al, 1969; Neef et al, 1996; Timmermans and Haute, 1983; Nyberg et al, 1996) to upward values of 4 gMeOH/ gNO3-N (6.0 COD/N) (Bailey et al, 1998). More common values for this ratio fall around 3 gMeOH/ gNO3-N. In turn, by using the COD/N ratio, the yield can now be calculated for the methanol-utilizing heterotrophs (Ym). These biomass yield values ranged from 0.24 to 0.52 as estimated from the formula below (Dold et al, 2005).

\[
Y_m = 1 - \frac{2.86}{\text{COD/N}}
\]

1.3 Blue Plains Advanced Wastewater Treatment Plant

As regulations become more stringent there is an increasing demand for large WWTPs to meet limit of technology (LOT) treatment for nitrogen removal year round (Mokhayeri, 2006). This situation is very evident at the Blue Plains Advanced wastewater treatment plant (AWTP) in Washington, DC (shown in Figure 1). The Chesapeake Bay watershed is the largest estuary in the United States with the Potomac
River making up a significant portion of its flow. The Blue Plains AWTP discharges into the Potomac River with average flow of 370 million gallons per day (MGD) and peak flows of up to 1 billion gallons per day. These large effluent discharges make Blue Plains AWTP the single largest point source of nitrogen load to the Chesapeake Bay (Bailey et al, 1998).

**Figure 1**: Blue Plains Advance Wastewater Treatment Plant in Washington, DC

In 2004, Blue Plains AWTP had an annual average effluent total nitrogen (TN) of 7.5 mg/L, but is striving to decrease this to 3 to 5 mg/L in the near future. Their goal for lower TN is in accordance with a commitment to Chesapeake Bay Agreement (CBA) (Carroll et al, 2005).

The Blue Plains AWTP has a high rate activated sludge system targeting BOD removal only, followed by a suspended growth nitrification-denitrification system, as shown in Figure 2. When denitrification was implemented at Blue Plains AWTP post denitrification with methanol was chosen. This process was selected due to the configuration and limited amount of space available on the site at Blue Plains. Large capital costs were avoided by using the existing reactor space in the nitrification stage.
Figure 2: Schematic of Blue Plains AWTP suspended growth biological treatment process

The nitrification-denitrification process consists of 5 zones. The first two zones in the treatment are kept aerobic for nitrification to occur. The third zone is unaerated which allows the dissolved oxygen to be consumed prior to the addition of methanol in the fourth zone. This fourth zone is the anoxic zone for denitrification. The fifth zone in the treatment process is reaerated prior to the clarifier. The schematic for this process is shown below in Figure 3.

Figure 3: Schematic of Blue Plains AWTP nitrification-denitrification process

The Blue Plains nitrification-denitrification treatment process achieves low total nitrogen values during summer warm weather conditions, but during prolonged cold weather, particularly with peak flows and snow melt conditions, denitrification performance is not sufficient. The reason for this problem is somewhat unclear, but is
likely linked to the decrease in temperature and wet weather flow associated with winter conditions (Figures 4 and 5).

![Figure 4 and 5](image)

**Figure 4:** Relationship between temperature and total nitrogen for Blue Plain’s effluent (2004 data – 30 day rolling average)

**Figure 5:** Total nitrogen for Blue Plains effluent (2004 data)

The data in Figures 4 and 5 suggest kinetic limitations of methylotrophic bacteria that have been corroborated recently by other research focusing on the measurement of anoxic maximum specific growth rates using methanol as well as full-scale plant simulation model calibration efforts (Mokhayeri et al, 2006). Due to these limitations on methylotrophic biomass, it suggests that Blue Plains AWTP is likely limited by anoxic SRT, and that methylotrophs are washed out of the process under stressed conditions. In attempt to resolve the problem additional methanol was added in the anoxic reactor. This extra anoxic methanol didn’t seem to boost denitrification due to the restrictions on anoxic HRT. Therefore, it is necessary to evaluate other methods to aid the denitrifying biomass.

**1.3 Research Project Overview and Hypothesis**

The objective of this project was to evaluate methods for possibly overcoming limitations of methanol addition for full-scale facilities that have limited available anoxic
solids retention time (SRT). There is considerable uncertainty associated with the
denitrification potential of aerobically grown methylotrophic bacteria and its effect on
full-scale plant design/operation. Yuan, Keller, and Lant (2003) suggested that a
complete evaluation of different carbon sources for denitrification and operational
strategies for their overall performance is largely missing.

Gujer and Irene (1999) suggested that strict anoxic methylotrophs may have
somewhat different kinetic capability compared to facultatively-grown methanol-utilizing
bacteria. They represented these two different populations of methylotrophic denitrifiers
by X1 and X2. Both of these microorganisms are able to use methanol aerobically and
anoxically, but their kinetics differ depending on their cultivation method. It is suspected
that X1 has a high aerobic growth rate and a high yield. The X1 biomass is expected to
dominate if substantial methanol degradation occurs under aerobic conditions. The X2
microorganism has a lower aerobic growth rate, but a smaller yield and a higher anoxic
growth rate. This X2 biomass will dominate if all methanol degradation takes place
under anoxic conditions. Since X1 has a higher yield it was predicted it would have a
MeOH/N ratio of 4.5 while the predicted value of X2 is 3.4 MeOH/N. Therefore, if
Gujer and Irene’s theory is correct, it would make the cultivation of the X2 organism
more favorable to a WWTP due to its higher anoxic growth rate and more efficient
denitrification.

For this research project, it was hypothesized that methanol addition under
aerobic conditions (bleeding a small amount of methanol into the aerobic zone of a BNR
process) could ease anoxic SRT limitations in a full-scale plant by increasing the active
methylotrophic biomass content in the process. To date, there is little work
demonstrating that aerobically-grown methylotrophs have the potential to denitrify (i.e. increase the anoxic mass fraction) in single sludge BNR processes that use post-denitrification with methanol addition. It is also critical to evaluate the denitrification kinetics under these conditions to see if there is a difference in growth rates or efficiency between aerobically and anoxically growth methylotrophs.
CHAPTER 2: Materials and Methods

2.1 Introduction to the Operation Sequencing Batch Reactors

Two sequencing batch reactors (SBRs) were operated at the Blue Plains AWTP to assess methylotroph kinetics under varying conditions. The conditions evaluated in this project were aerobic vs. anoxic growth, with measurements of SDNR in the SBRs and ex situ measurement of \( \mu_{\text{max}} \) by a modification of the “High F/M” method developed to measure \( \mu_{\text{max}} \) for nitrifying bacteria (Melcer et al, 2003; Dold et al, 2005; Mokhayeri et al, 2006).

The SBRs had a working volume of 3.5 liters and were operated over a period of 80 days. This reactor set-up was used to simulate, as closely as possible, the Blue Plains AWTP nitrification/denitrification system, with aerobic and post-anoxic reaction periods. Nitrification of the synthetic wastewater was not attempted, because of complexity of maintaining a nitrification-denitrification process.

Both of the reactors were operated with a total solids retention time (SRT) of 12 days (6 day anoxic SRT, with half of the reaction period aerobic), and a hydraulic retention time (HRT) of 12 hours. The temperature was maintained at a constant 20 °C using an incubator to house the SBRs, and the pH was maintained at 7.5 with the use of pH controllers. The SBR influent consisted of 40 mg/L NO3-N in tap water, supplemented with ammonia and phosphate to meet nutrient requirements. Sodium sulfite was also added to the feed for dechlorination of the tap water. Each SBR cycle duration was 6 hours, consisting of 2.25 hours of aerobic, 15 minutes dissolved oxygen (DO) stripping with nitrogen gas, 2.25 hours of anoxic with constant N\(_2\) purging, 15 minutes reaeration, and 1.0 hour for settling and decanting. For both reactors A and B,
methanol was added at 90 mg/L COD (based on the full reactor volume) at the beginning of the anoxic period. For reactor B only, methanol was added at 45 mg/L COD at the beginning of the aerobic period. Below in table 3 is the SBR timer program.

<table>
<thead>
<tr>
<th>SBR Program Setup</th>
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<tbody>
<tr>
<td>Name</td>
</tr>
<tr>
<td>Air</td>
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<td>Mixers</td>
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<td>Mixers</td>
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<td>Decant Pump</td>
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<td>Decant Pump</td>
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</table>

After reaching steady state (approx. 3 SRTs per SDNR and MLSS data), mixed liquor samples were removed from the reactors to measure anoxic maximum specific growth rates for methylotrophic bacteria using the High F/M method reported by Dold et al (2005) and later used by Mokhayeri et al (2006).
2.2 Sequencing Batch Reactor Sampling, Design, and Maintenance

2.2.1 Sample Source

The reactors were seeded with sludge from the Washington Suburban Sanitary Commission Piscataway WWTP. This plant was chosen because of its configuration, which includes a step feed BNR process with no external carbon source addition. This allowed an unbiased acclimation to methanol in the SBRs.

2.2.2 Sample Method

(a) Sampling for one SBR cycle was performed bi-weekly initially, and then once a week after day 30 of SBR operation. To get usable data during the sampling of an SBR cycle, sampling times were optimized in the anoxic period to get 6 data points prior to depletion of COD or NO3-N. From this data, the Specific Denitrification Rate of the SBRs was calculated (mgNO3-N/gVSS/hr). The SBR samples were collected using a 20ml disposable syringe and filtered immediately with a 0.45 µm syringe filters.

(b) 2.2.3 Sample Analysis

Samples taken were refrigerated until analyzed. The time between sampling and analysis varied from immediately up to 4 hours. Monitoring of the SBRs included NO3-N, NO2-N, NH4-N, COD, MLSS, MLVSS, and effluent TSS. Specific denitrification rates (SDNR) were calculated from NO3-N profiles over the course of an SBR cycle. Samples for molecular analysis were also collected weekly in a 50 ml tubes, centrifuged, and stored in a freezer (pellet only) until shipped to Columbia University for further analysis.
2.2.4 External Carbon

Reactors A and B were both fed the same methanol dose at the beginning of the anoxic cycle, but reactor B was fed addition methanol at the beginning of the aerobic cycle (half of the dose added anoxically). The concentration of the stock methanol solution was 31150 mg COD/L. A total volume of 10 mls was added anoxically to each cycle, for each of the four cycles per day. This gave a starting reactor concentration of 90 mg COD/L. For reactor B, an additional volume of 5 mls from the same stock solution was added at the beginning of the aerobic period. The pump used to feed the anoxic methanol was an 8-channel peristaltic pump that was set to run at approximately 5 mls/minute feeding from a one liter container. The weight of each one of these containers was recorded daily to ensure an accurate, consistent carbon addition to each reactor. Note: This pump required a few minor adjustments to achieve an accurate feed of 10 mls (plus or minus 0.1 ml).

2.2.5 Feed

The feed for both of the reactors was the same. The original feed solution used Blue Plains plant effluent, but this process was determined to be too time consuming and unfeasible if the reactors were to be moved to George Washington University at a later date. Instead, tap water was used to make the feed for this experiment with the addition of sodium nitrate, potassium phosphate, and sodium sulfite. Ammonia was supplied from a concentrated stock solution at the same time methanol was fed anoxically. It was assumed that the tap water contained sufficient micro-nutrients and no external addition was needed. The NO$_3$-N concentration in the feed was approximately 40 mg N/L. The
volume of feed added prior to the start of each new cycle was 1.75 liters, which is half of the 3.5L of operating volume. This, gave a 12 HRT.

2.2.6 Wastage of Sludge

The mean cell residence time or solids residence time (SRT) was set at a target of 12 days after factoring in the effluent TSS. This SRT was maintained by wasting 60mls per cycle with four cycles every 24hrs. Wasting was performed at the end of the anoxic cycle prior to settling while the reactor was still completely mixed. The waste from each reactor was kept separated and collected in small containers in order to accurately measure the actual waste volume. This wastage data along with the effluent TSS was recorded throughout the duration of the SBR operation.

2.2.7 Decant

Following the final aerobic polishing step, the wastewater settled for 45 minutes. At this time, 1.75 liters was decanted from both reactors. The SBR effluent was collected on sampling days to measure the TSS effluent. After the SBRs were “stabilized”, effluent was collected to conduct high F/M experiments.

2.2.8 pH

pH controllers were used on the SBRs to maintain a target pH of 7.5, with 3.6 N sulfuric acid addition only during the anoxic period. The pH controllers had a set point of 7.6 with a 90 second on delay for the pumps. The off point was set at 7.45 with no delay. The typical change in pH during the anoxic cycle varied between 7.40 and 7.63, with the aerobic pH never dropping below 7.10.
2.2.9 Temperature

The temperatures of the reactors were maintained at a constant 20 °C (plus or minus 1 degree). The incubators used were large enough to hold the SBRs and the mixers. The rest of the SBR components such as the pumps, timers, carbon sources, feed, and nitrogen/air regulators were kept outside of the incubator at lab room temperature with a supply running through the back of the unit. Since the incubator temperature and the lab temperature were relatively close to each other, it was not necessary to store the 50-liter feed container in a separate incubator.

2.2.10 Air

Aerobic conditions were maintained in the SBRs during the initial 2.25 hours of the cycle, and once again at the end of the anoxic phase to strip nitrogen gas and improve settling. The DO in the reactors was always quite high do to the lack of COD in the aerobic phase, except for reactor B with the aerobic carbon addition that consumed O\textsubscript{2} for approximately the 25 minutes prior to the COD being expended.

2.2.11 Nitrogen gas

The nitrogen gas feed was controlled by a solenoid valve immediately following the aerobic phase. This period was used to strip the oxygen from the reactors prior to adding the methanol. Initially (first day of operation), the SBRs were not truly anoxic when the methanol was added, with a DO concentration of 1.0 mg O\textsubscript{2}/L or greater. To offset this problem extra time was allotted for DO stripping with the N\textsubscript{2} gas. Also, it was necessary to provide each reactor with a floating foam cover to improve the nitrogen purging efficiency and prevent O\textsubscript{2} entrainment. After just one cycle with the foam cover it proved to solve the stripping problem with DO concentrations of less than 0.4 mg O\textsubscript{2}/L.
prior to methanol addition. The nitrogen gas was kept on to ensure zero DO and proper mixing throughout the anoxic period.

During the first week of operation, the DO was measured frequently with a complete DO profile aerobically and anoxically to ensure proper denitrification conditions were occurring. Denitrification is typically thought to occur when the DO is less than 0.5 mg/L. The typical DO for the anoxic phase was less than 0.4 mg/L prior to carbon addition and non-detectable thereafter for the duration of the anoxic period.

Note: The difference in measurements between the LDO HACH probe versus the YSI DO probe used for BOD measurements was significant when the DO concentration was less than 1.0 mg/L. The YSI DO probe would show DO of 0.1 mg/L throughout entire the anoxic period, where as the HACH LDO probe showed zero DO throughout the anoxic cycle.

2.2.12 Mixing

For the SBRs, 100 RPM mechanical paddle mixers were used. The depth of the paddles was 25% of the total volume for the reactor (i.e. at 0.875 L for a 3.5L reactor liquid volume). These mixers were kept on throughout the entire SBR cycle of aerobic/anoxic with exception being the settling and decanting times. The paddles used were flat paddles taken from a jar testing apparatus with the shafts lengthened 5 inches. With the lengthened shaft it allowed more clearance for maintenance of the reactors and allowed use of the existing racks in the incubator to mount the mixer motors.
2.2.13 Timing

The operation of all the pumps, mixers, and solenoid valves for the gases was controlled by two programmable timing units. These allowed timing adjustments to be made as needed.

2.2.14 Reactor Maintenance

Daily reactor maintenance consisted of washing the walls of the reactor with a syringe using reactor contents, and then further cleaning each reactor with a separate spatula to keep the reactor walls free of build up. When the reactor was decanted, prior to starting a new cycle, the same as above would be performed to clean the lower half of the reactor and the submerged tubing. Aside from the daily cleaning of the reactors, the SBRs were relatively maintenance free once operational.

2.3 High Food to Microorganism Batch Test (High F/M)

This ex-situ batch test was performed for two reasons:

1. To observe the anoxic growth rates in a batch test by adding a small amount of mixed-liquor to a reactor and monitoring the disappearance of the NO3-N overtime.

2. To test the theory of facultative methylotrophs by running duplicate reactors with the same amount of mixed-liquor added to each. The only variation in the two reactors would then be the anoxic vs. aerobic/anoxic operation.

2.3.1 Anoxic High F/M

This test was designed to measure the maximum growth rate of the methanol utilizing organisms in activated sludge for denitrification. The experimental procedure was based off past experiments outlined by Dold and Takacs (2005) and off the High
F/M test method for estimating the maximum specific growth rate of nitrifiers (Melcer et al, 2003). The maximum specific growth rate of the denitrifiers in the batch test was estimated by non-linear curve fitting using Solver functions available in Microsoft Excel. A template was supplied by Dold and Takacs, and is described in greater detail in Dold et al (2005). These separate batch tests were set up using effluent from the parent reactor that had been filtered using 0.45 µm filters. In this reactor, methanol and nitrate were added at 600 mg/L COD and 100 mg/L NO3-N, respectively. Additional nutrients such as P and NH3 were added accordingly to the predicted nutrient requirements for growth.

The high F/M reactors were air tight with a gasket seal around the lid for pH, ATC, ORP probes, sampling tube, nitrogen gas, CO2 gas, and the off pressure water trap line. The line going to the water trap was used as a visual indicator to ensure the reactor was air tight by observing the water bubbling in the flask. The contents of the reactor were mixed with a magnetic stir bar and nitrogen gas purge.

Prior to starting an experiment, the treated effluent was purged with N2 for 12 hours to ensure fully anoxic conditions. During this purge time, nitrogen gas flow was increased. Prior to the start of the experiment, the N2 was decreased to a minimum flow to ensure the conditions remained anoxic.

At time zero, a small amount of mixed-liquor was added from the parent SBR. This sludge was taken from the end of the anoxic period. All other nutrients needed for growth were added to the high F/M reactors with the filtered effluent prior to the start of the experiment. After mixed liquor addition, the reactor contents were mixed for 15 minutes before the first sample was taken for analysis. With the first sample, 100 mls were also collected for the duplicate analysis of TSS/VSS.
The sampling procedure consists wasting the first 1.5 ml from the sampling tube since there is a 1.2ml difference between the reactors’s working volume and the top of the sampling port. This was done to ensure the sample volume analyzed was taken from the completely mixed area of the reactor. Samples were then collected every 4 to 6 hours, and analyzed shortly there after. For these samples, NO$_3$-N, NO$_2$-N, NH$_3$-N, and COD were tested.

The pH was maintained between 7.4 and 7.6 with a mixture of nitrogen and CO$_2$ gas that was adjusted as needed. ORP was constantly monitored for each reactor during the duration of the experiment. At the start of high F/M experiments the ORP was always less than 100 mV and by the end of the experiment, which lasted 3 to 5 days, the ORP values were less than –100 mV. The temperature in the incubator was maintained at a constant 20 °C and this was verified by the ATC probes in the reactor.

**2.3.2 Aerobic/Anoxic High F/M**

The high F/M tests were strictly anoxic except for one set of experiments. In this case, the only difference was an initial aerobic period in one of the reactors. The original high F/M experiment would start the same as an anoxic high F/M experiments, but the second reactor was aerated for the first 24 to 36 hours of the experiment, after which the air flow as shut off and the nitrogen gas was turned on.

**2.4 Mini Reactor Ex-situ Batch Experiments**

Ex-situ batch tests were performed for two reasons.

1. To get an initial estimate of aerobic methanol utilization rate to later design the aerobic/anoxic High F/M.
2. To perform aerobic and anoxic stable-isotope probing (SIP)

2.4.1 Aerobic Methanol Utilization

This test was used to give an idea of whether reactor A was able to use methanol as readily as reactor B under aerobic conditions (reactor B is fed methanol aerobically and anoxically, reactor A is fed methanol only anoxically). The results of this aerobic batch test were used to design future experiments, with the one test being the aerobic SIP. This aerobic test was also used to calculate the methanol usage for aerobic/anoxic high F/Ms.

These ex-situ experiments were limited in volume due to the amount of SBR wastage available per day. Over the course of 4 cycles per day the SBRs produce a total wastage of 240 mls. When conducting these ex-situ these experiments, it was important to avoid any possible upsets in the parent SBRs. This disruption in SBR operation could be caused if a large volume (greater than daily wastage) of mixed liquor were taken from a parent SBR and not replaced immediately. A condition like this, could possibly select towards a different organisms in the SBRs by having an excess of COD at the end of the cycle, and therefore eliminating the design difference between reactors A and B.

Immediately after 240 mls of mixed liquor were removed from the parent SBR, 240 mls from the previous day of wastage was used to replace the depleted volume.

After sample collection, the mini-reactor was setup to mimic the parent SBR with a few exceptions. The mixed-liquor was added to a 500 ml beaker with a sealed lid and stirred with a magnetic stir bar. The nutrients added to the ex-situ experiments were in the same ratio as the parent SBRs.
2.4.2 Stable-Isotope Probing

Stable-Isotope probing (SIP) was performed as a side experiment for this study, and does not directly pertain to any of the results or discussions in this paper. The biomass samples from these experiments were centrifuged down into a pellet and frozen until they were shipped to Columbia University for further analysis.

The mini-reactors used for the stable-isotope probing are very similar to the mini-reactors used for the aerobic methanol utilization test. The only differences between the two experiments being the reactor setup, pH control, and substrate. The reactor was sealed with holes in the lid for the pH probe, nitrogen gas, CO₂ gas, sampling tube, and the water trap line. The hose going to the water trap was used as a visual indicator to ensure the reactor was air tight. After the sludge sample was added, it purged with high nitrogen gas flow for approximately one hour or until the DO was less than 0.1 mg/L (the nitrogen gas used for all batch tests was scrubbed to remove trace O₂ with BIP nitrogen tanks). At this point the external carbon source was added, and the nitrogen gas was turned down to maintain a minimum flow through the water trap.

For the first sample during a SIP experiment, the NO₃-N, NO₂-N, and COD were analyzed. COD sampling thereafter was performed only periodically due to the small reactor volume, volume required for COD measurements, and the cost for the heavy methanol. The NO₃-N was monitored throughout the experiment for quality control to ensure denitrification rates were similar to those of the parent SBR. The substrate for the SIP experiments had the same ratios for COD to N and nutrients as the parent SBRs with the only exception being that they were increased to accommodate a longer experiment (see details for test one and test two below).
SIP Test 1

The first SIP experiment (anoxic) was performed with a starting concentration of 40 mg NO$_3$-N/L with all the other nutrients increased in direct proportion. The COD concentration was added at 200 mg COD/L per cycle (a cycle was defined as near depletion of NO$_3$-N). Samples for molecular work were collected at 12 hours, 18 hours, and again at 24 hours.

SIP Test 2

The second batch SIP experiment (anoxic) was slightly different from the first experiment. The initial COD concentration was 600 mg/L with a NO$_3$-N of 120 mg/L. Another factor that was considered was the difference of almost twice the MLSS concentration in reactor B than in reactor A. To account for this difference, reactor B mixed liquor was diluted with its own effluent until reactor A and reactor B had an equivalent MLSS concentration. With similar MLSS concentrations, it enabled both of the experiments to be designed to run for the same duration of time since the SDNRs of the two were equivalent in the parent SBRs. Samples for molecular work were taken at 12 hours and 24 hours.

SIP Test 3

The third SIP experiment (aerobic) was designed based on the initial aerobic methanol utilization experiment. The MLSS of reactor B was diluted to match MLSS concentration of reactor A, as in SIP Test 2. Initial COD concentration was 600 mg/L as methanol, and with the aerobic growth rates the COD was depleted in just in a little over 5 hours. The dissolved oxygen in the mini reactors was maintained at 5 mg/L or greater.
The only analysis in these experiments was the NH$_3$-N and COD. Samples for molecular work were taken at the end of the experiment.
CHAPTER 3: Experimental Results

3.1 Sequencing Batch Reactor Results

3.1.1 Nitrate Profiles and Acclimation

When using methanol to drive denitrification, it is known that a long acclimation periods are required before seeing significant nitrate reduction, with up to 50 to 100 days before maximum denitrification rates are achieved (Nyberg et al, 1992). In this study, the acclimation period was monitored in the SBRs since the sludge used to seed the reactors was taken from a WWTP that used the influent BOD as the electron donor in denitrification instead of an external carbon source (step feed BNR process). When looking at the initial condition in the SBR, as shown below in Figure 6, it is clear that the seed sludge was unacclimated to methanol. The small change in NO$_3$-N cycle profile for both the reactors goes to show the number of methylotrophs initially present was quite low.

![Figure 6](image-url):
Figure 6: Nitrate profile for reactors A and B on day two of reactor operation.
By day 7 reactor B was fully denitrifying down from 20 mg NO$_3$-N/L, while in the same time period (exact same HRT) reactor A nitrate was reduced from 31 mg/L to 23 mg NO$_3$-N/L by the end of the cycle, as shown in Figure 7. It is important to reiterate the differences between reactors A and B when viewing these figures. Their design parameters are exactly the same with the only difference being reactor B receives additional methanol aerobically. As stated in section 2.2.4 the aerobic concentration of COD as methanol in reactor B is 45 mg/L. This distinct difference between reactors A and B in Figure 7 shows the advantage of adding excess methanol aerobically for the time required for acclimation in the SBRs. (This same advantage of reactor B over reactor A was also demonstrated in a short trial run of the SBRs used to help develop the hypothesis, prior to restarting the reactors and generating the data used in this manuscript).
Figure 7: Nitrate profile for reactors A and B on day seven of reactor operation. A representative NO$_3$-N profile over a 6-hour SBR cycle for the acclimated SBR is shown in Figure 8 (profile from day 33). The same distinct advantage of reactor B versus reactor A is displayed when comparing the nitrate profiles for day 7 and day 33, with the only difference being the increase in the slopes of both reactors. Considering the cause of the differences in the slopes between reactors A and B, it is important to remember that both reactors started out with the same initial MLSS concentration at day 1 of operation. The difference in slopes will be discussed further in section 3.1.2, but one would assume it could come from one of two things:

1. The there is simply a greater concentration of denitrifying biomass in reactor B than A (active fraction would be equal between the two reactors once they are stabilized)

2. There is a possible difference in microorganisms that have been selected, with reactor B having selected a microorganism with a higher denitrification rate.
**Figure 8:** Typical nitrate profile for reactors A and B taken at day 33 of reactor operation.

Figures 9 and 10 are shown below to better visual the individual increase in denitrification rates for each reactor. Presumably, as time increases, the number of methylotrophic bacteria also increased and many of the non-methanol utilizing heterotrophs were washed out. The nitrate cycle profiles are shown in Figure 9 from day 2 to day 33, after which SBR operation remained stable and the cycle profiles were repetitive (profiles for day 2 to day 75 are shown in appendix Figures 28 and 29). Once again, Figure 9 for reactor B displays an apparent advantage of aerobic methanol addition over Figure 10 for reactor A.
Figure 9: Nitrate profile for acclimation of reactor A to methanol

Figure 10: Nitrate profile for acclimation of reactor B to methanol
3.1.2 Specific Denitrification Rates

Clearly, as shown in all the nitrate figures, the slope of the NO$_3$-N profiles for reactor B is greater than that of reactor A. To better compare these reactors specific denitrification rates (SDNRs) were calculated. An SDNR is calculated by measuring the change in NO$_3$-N overtime in comparison to each reactors individual MLVSS concentration.

$$\text{SDNR} = \frac{\text{mg NO}_3\text{-N}}{\text{g MLVSS} \times \text{Hour}}$$

The SDNR values between SBR A and SBR B are quite similar, as shown in Table 4. This supports the theory that the aerobically grown methylotrophs have similar anoxic denitrification potential as strict anoxically grown methanol utilizing bacteria. If the methylotrophic bacteria grown aerobically were not facultative or had slower denitrifying kinetics, it would not change the MLVSS concentration in the reactor, but one would assume the SDNR would be lower due to the non-denitrifying biomass. A lower SDNR in reactor B was not the case as shown in Table 4, but was exactly the opposite on most occasions.

Note: MLVSS term in the SDNR calculation does not account for the possible microbial population differences between reactors A and B. All biomass is lumped into a sum MLVSS concentration.
Table 4: Specific denitrification rate (SDNR) and nitrate slopes overtime for reactors A and B

<table>
<thead>
<tr>
<th>Sampling Days</th>
<th>SDNR (mgNO3-N/gVSS/hr)</th>
<th>Slope (mgNO3-N/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SBR A</td>
<td>SBR B</td>
</tr>
<tr>
<td>2</td>
<td>0.7</td>
<td>0.9</td>
</tr>
<tr>
<td>5</td>
<td>3.0</td>
<td>2.7</td>
</tr>
<tr>
<td>7</td>
<td>3.1</td>
<td>6.7</td>
</tr>
<tr>
<td>13</td>
<td>8.8</td>
<td>15.2</td>
</tr>
<tr>
<td>15</td>
<td>10.8</td>
<td>12.2</td>
</tr>
<tr>
<td>18</td>
<td>9.7</td>
<td>9.1</td>
</tr>
<tr>
<td>21</td>
<td>8.8</td>
<td>9.3</td>
</tr>
<tr>
<td>33</td>
<td>20</td>
<td>17</td>
</tr>
<tr>
<td>36</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td>39</td>
<td>25</td>
<td>21</td>
</tr>
<tr>
<td>42</td>
<td>20</td>
<td>21</td>
</tr>
<tr>
<td>47</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>53</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>63</td>
<td>18</td>
<td>22</td>
</tr>
<tr>
<td>75</td>
<td>20</td>
<td>24</td>
</tr>
<tr>
<td><strong>Mean =</strong></td>
<td><strong>21.1</strong></td>
<td><strong>21.5</strong></td>
</tr>
<tr>
<td><strong>St. Dev. =</strong></td>
<td><strong>2.6</strong></td>
<td><strong>2.6</strong></td>
</tr>
</tbody>
</table>

- Mean and Standard Deviation were taken from day 33 to day 75

3.1.3 Anoxic Methanol Utilization (COD Profiles)

As discussed in section 4.1, reactor B exhibited a greater slope than reactor A for the NO3-N profiles, and the same case is shown for the COD profiles. Below in Figure 11, is a typical COD profile for both reactors. The aerobic methanol addition of reactor B is evident and the uptake rate is rapid.
Figures 12 and 13 are shown below to better observe the acclimation of the SBR to the external carbon source, methanol. The COD cycle profiles are show from day 2 to day 33, after which SBR operation remained stable and the cycle profiles were repetitive.

The SBRs were originally designed assuming an approximate COD (as methanol) to N (as NO₃-N) ratio of 4.7. The actual COD/N for both reactors turned out to be a lower value, which left the reactors nitrate limited.

Note: Only two samples were taken (beginning and end of aerobic) for the aerobic sampling of COD during a cycle analysis. Therefore, the aerobic slope shown is not an actual slope, but the total change in COD within the time period.
**Figure 12:** COD profiles of reactor A from day 2 to day 33

**Figure 13:** COD profiles of reactor B from day 2 to day 33
3.1.4 Anoxic COD/NO$_3$-N Ratio

When comparing SBRs A and B, it is interesting to consider the COD to NO$_3$-N ratio. This ratio varies in the reported literature between 2.5 and 4 g COD/g NO$_3$-N (section 1.2.6). As shown in Table 5, reactor B has a higher COD/NO$_3$-N ratio than reactor A (both based on the COD added anoxically only), but compared to literature values, they are relatively low. The reason for reactor B having a greater COD/N ratio than reactor A is unclear, and this will be discussed in further detail in section 4.1.1.

Table 5: SBRs A and B ratio of methanol added per nitrate as nitrogen removed (COD/N and MeOH/N ratios)

<table>
<thead>
<tr>
<th>Sampling Days</th>
<th>SBR A gCOD/gNO$_3$-N</th>
<th>SBR B gCOD/gNO$_3$-N</th>
<th>SBR A gMeOH/gNO$_3$-N</th>
<th>SBR B gMeOH/gNO$_3$-N</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2.86</td>
<td>4.37</td>
<td>1.91</td>
<td>2.91</td>
</tr>
<tr>
<td>7</td>
<td>5.39</td>
<td>4.72</td>
<td>3.59</td>
<td>3.15</td>
</tr>
<tr>
<td>13</td>
<td>3.54</td>
<td>3.60</td>
<td>2.36</td>
<td>2.40</td>
</tr>
<tr>
<td>15</td>
<td>4.18</td>
<td>4.02</td>
<td>2.79</td>
<td>2.68</td>
</tr>
<tr>
<td>18</td>
<td>4.72</td>
<td>4.51</td>
<td>3.15</td>
<td>3.01</td>
</tr>
<tr>
<td>21</td>
<td>5.07</td>
<td>4.53</td>
<td>3.38</td>
<td>3.02</td>
</tr>
<tr>
<td>33</td>
<td>4.02</td>
<td>4.26</td>
<td>2.68</td>
<td>2.84</td>
</tr>
<tr>
<td>36</td>
<td>3.84</td>
<td>4.13</td>
<td>2.56</td>
<td>2.75</td>
</tr>
<tr>
<td>39</td>
<td>3.26</td>
<td>4.26</td>
<td>2.17</td>
<td>2.84</td>
</tr>
<tr>
<td>42</td>
<td>3.38</td>
<td>4.33</td>
<td>2.25</td>
<td>2.89</td>
</tr>
<tr>
<td>47</td>
<td>3.43</td>
<td>4.37</td>
<td>2.29</td>
<td>2.91</td>
</tr>
<tr>
<td>53</td>
<td>3.54</td>
<td>4.37</td>
<td>2.36</td>
<td>2.91</td>
</tr>
<tr>
<td>63</td>
<td>3.54</td>
<td>4.36</td>
<td>2.36</td>
<td>2.91</td>
</tr>
<tr>
<td>75</td>
<td>3.67</td>
<td>4.35</td>
<td>2.45</td>
<td>2.90</td>
</tr>
<tr>
<td>Mean =</td>
<td>3.59</td>
<td>4.30</td>
<td>2.39</td>
<td>2.87</td>
</tr>
<tr>
<td>St. Dev. =</td>
<td>0.25</td>
<td>0.08</td>
<td>0.17</td>
<td>0.06</td>
</tr>
</tbody>
</table>

- Mean and Standard Deviation were taken from day 33 to day 75

3.1.5 Aerobic Methanol Utilization

Figure 14 shows the aerobic profile of methanol uptake compared to the dissolved oxygen profile. The air flow in the reactors was maintained at a rate sufficient to prevent
the DO from dropping below 1.0 mg/L during methanol loading. Once the COD was consumed, the DO would rapidly increase and be above 7 mg/L by the end of the aerobic cycle. This was why it was critical to allow a long enough time for stripping the DO out with nitrogen gas.

![Reactor B, Methanol Aerobic Profile](image)

**Figure 14:** COD vs. dissolved oxygen profile for aerobic period of reactor B

Figure 15 displays a typical dissolved oxygen profile of SBR B throughout the duration of the cycle. The only difference between SBR B and SBR A for the DO profile is that A does not exert aerobic oxygen demand due to methanol.
When comparing growth rates for a facultative microorganism, the aerobic growth rates are usually greater than anoxic growth rates. To investigate, two tests on reactor B were conducted by running both an aerobic and anoxic test at the same time. Mixed liquor was taken from SBR B with the experiments starting shortly thereafter. This method of testing was done to guarantee the MLVSS concentration was the same in both of the ex-situ batch reactors, so that the slope of methanol utilization could be compared between the two. As expected, the slope for the aerobic methanol was greater than the slope of anoxic methanol uptake as shown in Table 6. This would suggest that the methylotrophs might have a greater $\mu_{\text{max}}$ aerobically than anoxically. If this were truly the case, then it would help to explain advantage of reactor B over reactor A, and the short duration of acclimation time that it took reactor B to fully denitrify.
Table 6: SBR B, aerobic versus anoxic methanol uptake.
Note: Day 18 testing was performed in-situ for both aerobic and anoxic during a normal SBR cycle. Day 47 testing was performed ex-situ for aerobic to allow a longer duration and in-situ SBR for anoxic.

<table>
<thead>
<tr>
<th>Sampling days</th>
<th>SBR B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slope (mgCOD/hr)</td>
</tr>
<tr>
<td></td>
<td>Aerobic</td>
</tr>
<tr>
<td>18</td>
<td>78.8</td>
</tr>
<tr>
<td>47</td>
<td>252.4</td>
</tr>
</tbody>
</table>

3.1.6 Nitrite Concentration versus Acclimation

It is usually assumed when denitrification occurs that the nitrate is converted to nitrogen gas. Nitrite is an intermediate that is not usually factored in nitrogen removal, and accumulation in a denitrification process is abnormal. Throughout the operation of these SBRs nitrite was most always present and sometimes at very large concentrations.

In general, SBR B exhibited little to no nitrite accumulation, but SBR A had nitrite concentrations that would sometimes approach 10 mg NO₂-N/L at the end of the cycle. The reason for the high concentrations of nitrite in A is unclear, but this is further discussed in section 4.2.2. Below in Figures 16 and 17 are shown the nitrite profiles overtime for both the SBRs.
Reactor A, NO2-N overtime

Figure 16: Nitrite profile for reactor A overtime

Reactor B, NO2-N overtime

Figure 17: Nitrite profile for reactor B overtime
### 3.1.7 SBR Operational Design Parameters

Other analyses were critical to monitor conditions in the SBRs, such as MLSS/MLVSS and effluent TSS concentrations. The MLSS/MLVSS concentration for the entire operation of the SBRs is shown in Figure 18. Reactor A and B both received 90 mg COD/L anoxically, and reactor B receives half-again as much aerobically. As expected, reactor B MLSS stabilized at a greater value than reactor A. These MLVSS concentrations shown below were used to calculate the SNDR of the separate SBR.

![Figure 18: MLSS concentration for reactors A and B over the duration of the SBR operation](image)

As the SBRs stabilized over time, the ratio of VSS/TSS also increased, as shown below in Figure 19. Initially the ratio of VSS/TSS for the Piscataway WWTP was 0.69. This low initial VSS/TSS ratio was not surprising when considering all the different constituents in influent wastewater. With nothing but soluble synthetic wastewater being
fed to the SBR and after 50 days of operation the ratio stabilized at just above 0.90 g VSS/ g TSS.

![Graph showing the ratio of VSS to TSS for reactors A and B during SBR operation](image)

**Figure 19:** Ratio of VSS to TSS for reactors A and B during SBR operation

Another factor influenced by the duration of the SBR operation was the effluent TSS concentration. The longer the SBRs were operated the more the settling improved, as shown below in Figure 20. The TSS effluent concentration was measured over time to accurately predict reactor SRT. The SRT, throughout the SBR operation, is shown below in Table 7 for reactors A and B. As previously mentioned in section 2.2.6 the target SRT was 12 days, which the SBRs closely followed. It was more important that there was relatively no difference in SRT between SBRs A and B to ensure one of the reactors did not have an advantage over the other.
Figure 20: TSS effluent concentration for reactors A and B overtime

Table 7: Actual overall SRT for the reactors with wastage and TSS effluent

<table>
<thead>
<tr>
<th>Sampling Days</th>
<th>SBR A</th>
<th>SBR B</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>12.0</td>
<td>12.2</td>
</tr>
<tr>
<td>5</td>
<td>12.7</td>
<td>12.9</td>
</tr>
<tr>
<td>7</td>
<td>12.4</td>
<td>12.8</td>
</tr>
<tr>
<td>13</td>
<td>11.9</td>
<td>12.6</td>
</tr>
<tr>
<td>15</td>
<td>11.6</td>
<td>11.5</td>
</tr>
<tr>
<td>18</td>
<td>11.5</td>
<td>12.0</td>
</tr>
<tr>
<td>21</td>
<td>11.4</td>
<td>12.6</td>
</tr>
<tr>
<td>33</td>
<td>10.4</td>
<td>11.8</td>
</tr>
<tr>
<td>36</td>
<td>10.6</td>
<td>12.1</td>
</tr>
<tr>
<td>39</td>
<td>12.4</td>
<td>12.7</td>
</tr>
<tr>
<td>42</td>
<td>12.5</td>
<td>12.5</td>
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<tr>
<td>47</td>
<td>11.8</td>
<td>12.2</td>
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<tr>
<td>53</td>
<td>12.0</td>
<td>12.4</td>
</tr>
<tr>
<td>63</td>
<td>13.1</td>
<td>13.1</td>
</tr>
<tr>
<td>75</td>
<td>13.1</td>
<td>13.2</td>
</tr>
</tbody>
</table>

Mean = 12.0
St. Dev. = 0.8

- Mean and Standard Deviation were taken from day 2 to day 75
3.2 High Food to Microorganism (High F/M) Experiments

3.2.1 Strictly Anoxic High F/M

After stabilization of the SBRs (according to SDNR data), numerous High F/M measurements were conducted comparing SBRs A and B. These high F/M batch experiments, as described in section 2.3, were setup using the effluent and adding a small amount of mixed-liquor from the parent reactor. The COD to N ratio in the experiments was approximately 600 to 100, with a typical MLSS concentration of less than 50 mg/L.

A representative high F/M experiment for samples from reactors A and B is shown in Figure 21. Maximum specific growth rate calculated from all of the high F/M tests is summarized in Table 8. The differences in $\mu_{\text{max}}$ values between the two reactors are not significant when comparing the results (within the error of the method), thus suggesting reactors A and B had very similar anoxic growth rates. If these results were truly representative of the SBRs, it would appear the aerobic methanol addition that occurred in SBR B had no adverse impact on anoxic growth rates in the high F/M tests.
Figure 21: Typical high F/M experiment for reactors A and B

Table 8: Reactors A and B high F/M maximum specific growth rate

<table>
<thead>
<tr>
<th>Test</th>
<th>µmax (d^{-1})</th>
<th>µmax (d^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.63</td>
<td>0.46</td>
</tr>
<tr>
<td>2</td>
<td>0.40</td>
<td>0.38</td>
</tr>
<tr>
<td>3</td>
<td>0.48</td>
<td>0.58</td>
</tr>
<tr>
<td>4</td>
<td>0.49</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Mean = 0.50 0.50
St. Dev. = 0.10 0.10

For each high F/M experiment, the COD was also measured for every nitrate sample taken. Below in Figures 22 and 23 are the COD/N ratios for reactors A and B. The COD/N for these experiments was estimated at 4.7. The experimental values from both reactors closely match typical literature values. The COD/N ratio slope was only shown for one set of high F/M tests, since all other tests closely resembled these numbers.
ranging from 4.4 to 5.7 g/g. Below in Table 9 is the summary of COD/N ratios for the high F/M experiments.

Reactor A (mgMeOH COD/mgNO3-N)

\[ y = 4.6275x + 144.44 \]

\[ R^2 = 0.9952 \]

**Figure 22:** Typical COD/N ratio shown for reactor A high F/M experiment

Reactor B (mgMeOH COD/mgNO3-N)

\[ y = 4.7425x + 99.28 \]

\[ R^2 = 0.9793 \]

**Figure 23:** Typical COD/N ratio shown for reactor B high F/M experiment
### Table 9: Reactors A and B COD/N and MeOH/N ratios for high F/M experiments

<table>
<thead>
<tr>
<th>Test</th>
<th>gCOD/ gNO3-N ratio</th>
<th>gMeOH/gNO3-N</th>
<th>Reactor A</th>
<th>Reactor B</th>
<th>Reactor A</th>
<th>Reactor B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.39</td>
<td>4.73</td>
<td>2.93</td>
<td>3.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4.63</td>
<td>5.09</td>
<td>3.09</td>
<td>3.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5.68</td>
<td>4.61</td>
<td>3.79</td>
<td>3.07</td>
<td></td>
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<tr>
<td>4</td>
<td></td>
<td>4.74</td>
<td></td>
<td></td>
<td>3.16</td>
<td></td>
</tr>
<tr>
<td>Mean =</td>
<td>4.90</td>
<td>4.79</td>
<td>3.27</td>
<td>3.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>St. Dev. =</td>
<td>0.69</td>
<td>0.21</td>
<td>0.46</td>
<td>0.14</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The nitrite concentration was measured for every sample in the high F/M experiments. Nitrite was monitored to see if complete denitrification occurred. However, often extreme nitrite accumulation occurred in the experiments. At the start of the experiments the reactor NO₂-N concentrations were consistently less than 1.0 mg/L. Depending on the experiment, the nitrite concentrations varied from 1 to 26 plus mg/L by the end of the experiment (anything over 26 mg NO₂-N/L is out of the dilution range for measurement by the HACH method). It appears that nitrate reduction was affected at NO₂-N concentrations approaching 15 mg/L. This decrease in the denitrification rate is most likely due to the high concentration of the nitrite ions, which are known to inhibit bacterial growth (Almeida et al, 1995b).

When looking at the test results and nitrite accumulation, it is difficult to determine what might have caused such high and sporadic nitrite values. Shown below in Table 10 is the ending nitrite concentration recorded for high F/M experiments. These results and possible reasons for nitrite accumulation are later discussed more in depth in section 4.2.2.
Table 10: Reactors A and B end of experiment nitrite accumulation

<table>
<thead>
<tr>
<th>Test</th>
<th>Reactor A</th>
<th>Reactor B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>18</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>&gt;26</td>
</tr>
<tr>
<td>4</td>
<td>&gt;26</td>
<td>23</td>
</tr>
</tbody>
</table>

3.2.2 Aerobic/Anoxic High F/M

Aerobic/Anoxic high F/M experiments were conducted to further test the hypothesis that aerobically grown methylotrophs can denitrify with similar kinetics. These tests were done by using both SBR sludges and Blue Plains AWTP nitrification/denitrification sludge. The experiments were ran in much the same manner as the strictly anoxic high F/M experiments, with the only difference being each sludge was tested with and without a preliminary aerobic methanol period prior to the standard anoxic contact time. This preliminary aerobic period tested the hypothesis by allowing methylotrophic biomass to grown aerobically and to then switch the reactors conditions anoxic. The two NO\textsubscript{3}-N profiles between the strictly anoxic reactor and the aerobic/anoxic reactor could then be compared to see if there was an advantage, disadvantage, or no effect of a pre-aerobic period. The anoxic nitrate slope of the aerobic/anoxic reactor should be steeper than that of the strictly anoxic reactor if there were an advantage to adding methanol aerobically.
Figure 24: Reactor A High F/M Experiment, anoxic vs. aerobic

The data for both the experiments is shown below in Figures 24 and 25. These aerobic/anoxic experiments suggest that methylotrophs grown under aerobic conditions in the High F/M reactor could immediately denitrify, and it would appear they also have a similar anoxic $\mu_{\text{max}}$. 
High F/M Experiment, Blue Plains

Notes:
BP 1 - strictly anoxic
BP 2 - Only anoxic period shown, aerobic 36 hours prior to anoxic

Figure 25: Blue Plains High F/M Experiment, anoxic vs. aerobic/anoxic
CHAPTER 4: DISCUSSION

4.1 Gujer and Irene’s Theory

4.1.1 Aerobically Grown vs. Anoxically Grown Denitrifiers

Gujer and Irene (1999) proposed that there was a significant difference in the kinetics of methylotrophic microorganisms grown aerobically versus anoxically. He represented this difference by the X1 and X2 microorganisms. In the SBR setup for this experiment reactor B was designed to favor the X1 biomass, while reactor A was intended to enrich X2 biomass.

In SBR A after day 5, there was no significant methanol residual remaining in the reactor at the end of the anoxic period. So, if the SBRs were representative of what Gujer and Irene described as X1 and X2 biomass then SBR A would be dominated with an X2 microorganism. The X1 microorganism would also be prevalent in SBR B since there is aerobic methanol addition. In order for this theory to be correct in our situation it would be necessary for SBR A to have a greater SDNR than SBR B. As shown by the SDNRs in Table 4, once stabilized SBR A never had a greater SDNR than SBR B.

The only part of Gujer and Irene’s theory that might suggest an X1 and X2 methylotrophic biomass was the difference in COD/N ratios observed in the SBRs. This difference in COD/N values between the SBRs for denitrification might propose a difference in yields needed for microorganism growth. These yields can be estimated from the COD/N ratio, as shown in Section 1.2.6. Gujer and Irene cited that the X1 biomass (SBR B) would have a greater yield (COD/N ratio) than the X2 biomass (SBR A). According to the paper, X2 would have a 3.4 gMeOH / gN ratio (5.1 COD/N) and X1 would be 4.5 gMeOH/ gN (6.75 COD/N). As shown in Section 1.2.1, the theoretical
value when not including biological growth for denitrification with methanol is 1.90 grams MeOH is required per gram of NO$_3$-N removed (2.86 gCOD/ gNO$_3$-N). When looking at the SBR values, SBR A (X2) had an average MeOH/N value of 2.4, and SBR B had an average MeOH/N value of 2.9 (estimated yields based on the COD/N ratio for SBR A and B are 0.21 and 0.34, respectively). The values for both of the SBRs operated in this study were significantly lower than Gujer and Irene’s (1999) predicted values.

Foglar et al (2004) reported that dosing methanol in excess of 3.0 MeOH/N was inefficient and would result in higher yields. This is one possibility when comparing the COD/N ratio on reactors A and B, considering SBR B receives half again as much methanol as SBR A.

### 4.1.2 Further Investigation of Gujer and Irene’s Theory

It is important look at the aspects that could be further investigated or details of the research that could have been done differently if given more time. First, if these SBRs were to be operated again it would be beneficial to run SBR A strictly anoxic to ensure that it is operated in conditions that would grow X2 microorganisms only. These strictly anoxic SDNRs then could be compared to the current SDNRs of reactor A. Another way to operate reactor B to investigate the theory of the X1 organism would be to keep it strictly aerobic with methanol addition for a couple weeks and then cycle aerobic/anoxic to see if the same SDNRs occurred. This strictly aerobic SBR could also be used to see if it would be faster to acclimate a sludge to methanol by adding it aerobically.
4.2 SBR Results

Upon determining the scope of this research, it was decided the best method to investigate our hypothesis was by SBRs that were started with an unbiased sludge towards methanol. Although, batch experiments on Blue Plains sludge would have been a possibility instead of SBRs, the SBRs allowed a consistent operation with the synthetic feed. The SBRs also allow the visualization of the acclimation period required by methanol in each SBR.

4.2.1 Nitrite Accumulation

The reason for the unusually high nitrite in SBR A and in the high F/M experiments is unknown. One of the reasons why this could have occurred is because SBR B always completely denitrified and SBR A never really achieved complete denitrification. It could be seen that reactor B would accumulate a small amount of nitrite during an anoxic cycle, but as soon as SBR completely denitrified the nitrite would disappear. Betlach and Tiedje (1981) suggested that some microorganisms that are capable of both nitrate and nitrite reduction still accumulate nitrite since nitrate reduction occurs faster than the nitrite reduction rate.

A second reason could be the MLSS concentration in the reactors. There is some correlation to support a theory that the lower MLSS concentration then the worse problem there will be with nitrite accumulation since SBR B had a greater MLSS concentration than that of SBR A. The high F/M experiments experienced much of the same problem the lower the MLSS concentrations became. The question that this raises is the nitrite problem occurring simply because the MLSS is low or is it due to the loss of specific nitrite reducing bacteria? It can be seen that SBR A did not have any significant
nitrite accumulation until after two full weeks of operation when looking at the nitrite profiles overtime. The conditions in SBR A could have possibly selected for partial denitrifiers that are only capable of nitrate reduction (Henze, 1992). Lemmer et al (1997) also suggested that the majority of facultative heterotrophic bacteria are only capable of nitrate reduction, which could also lead to nitrite accumulation.

Another interesting point in nitrite accumulation in SBR A is it increases up to day about Day 63. From Day 63 till Day 75 (last cycle analysis taken) nitrite concentration steadily decreases. The reason for this decrease in nitrite concentration is unsure, but the only adjustment that was made during this period of time was the feed source was switched from sodium nitrate to potassium nitrate. In order to better understand this problem, it would be helpful to analyze the gas phase intermediates in the denitrification process.

4.3 High F/M Results

4.3.1 Development of the Anoxic High F/M Test

There have been multiple changes in the anoxic High Food-to-Microorganism methods since first published by Dold et al (2005). Many of developments have been made through trial and error. One of the bigger challenges when performing these tests is to ensure the conditions in the reactor remain anoxic. Initially, these experiments were conducted with the reactor contents exposed to atmosphere. They were then upgraded to a foam cover with nitrogen gas bubbling, and finally to a sealed reactor with nitrogen gas. One would assume, with zero dissolved oxygen in the reactors, that the tests would be simple and straight forward, but its hard to tell how much these conditions really simulate what occurs at a full-scale WWTP.
Another point to consider is how the growth rate is calculated from these tests. When assuming the curvature of nitrate disappearance is directly correlated to the growth of microorganisms leaves room for discussion. The nitrate slope from the majority of the high F/Ms of SBR sludge were smooth and consistent, but with a few of the tests this was not the case (see section below in 4.3.4). Below are just some of the problems and questions that were encountered when running these kinds of experiments.

4.3.2 Impact of MLVSS on the High F/M Tests

The amount of mixed-liquor to add in a high F/M test was critical in producing usable results. If too much mixed liquor were added then the experiment would denitrify too fast and not allow enough time for growth of new microorganisms. Therefore, with minimum growth and short duration too little curvature on the NO$_3$-N profile would not allow accurate prediction of $\mu_{\text{max}}$. If not enough mixed liquor was added to the experiment then nitrate disappearance may not occur at all, and the duration of the test would exceed the ideal time frame of 4 to 5 days.

Past experiments performed for anoxic high F/M and the WERF manual of Methods for wastewater characterization in activated sludge modeling were referenced when trying to calculate how much mixed-liquor to add. With the WERF manual on nitrifying high F/M experiments, it cites a VSS of around 35mg/L as a good starting point for a nitrifying high F/M. When looking at past denitrifying high F/M experiments at Blue Plains, the VSS concentrations varied between 80 and 120 mg/L. For the first 3 tests that were run on the denitrifying SBRs a target of 35 mg VSS/L was used. This estimate came from knowing that the active fraction in the SBRs was greater than that at Blue Plains. This amount of mixed-liquor added turned out to be too much, with the
experiments denitrifying to quickly. For these initial experiments, the amount of NO$_3$-N disappearance the first day was between 30 and 40 mg/L. To design the next set of experiments the SDNRs from the parent SBR were used to back calculate on how much sludge to add. The back calculated value targeted a conversion of 5 mg NO$_3$-N /L/day. This target number of 5 mg/L/day gave an estimate of 10 mg VSS/L initially needed in the high F/M experiments. Using this method produced predictable and usable results.

4.3.3 Strict Anoxic High F/M Tests

The anoxic high F/M experiments produced values of $\mu_{\text{max}}$ that were consistent between the two reactors. The growth rates were estimated by a least squares minimization analysis method as described by Dold et al (2005). The observed variation of the growth rates for these experiments at 20°C was between 0.4 to 0.6 d$^{-1}$, as shown in Table 8. When comparing these results to previous papers it raises the question on where might the differences be coming from. The anoxic growth rates for methanol at 13°C reported by Mokhayeri et al (2006) were approximately 0.5 d$^{-1}$. Tchobanoglous et al (2003) reported values of 0.52 and 1.86 d$^{-1}$ for temperatures of 10°C and 20°C, respectively. Gujer and Irene (1999) predicted results from modeling the growth rates of the methylotrophs at 20°C were 1.3 for strict anoxically grown and 0.81 for the aerobically grown denitrifiers.

What caused the different values in experiments and what could be done to impact these observed values is still in question. It is unclear whether all the stated values could be coming from different microorganism or whether these differences are coming from testing methods. These questions can not be answered from the limited
research that was done for this project, but in the future more research will be conducted on this same topic by researchers such as those at Blue Plains AWTP

4.3.4 Aerobic/Anoxic High F/M Experiments

Four of these aerobic/anoxic high F/M tests were conducted, with only two of the experiments working as predicted, with aerobic growth occurring and then denitrifying. The two successful tests were run on SBR A sludge and on Blue Plains AWTP sludge. Two tests were tried on SBR B sludge, but both of these tests were stopped with inconclusive results. Below in Figure 26 is the reactor B aerobic and anoxic profile of NO$_3$-N for both of the failed experiments. The nitrate profile was supposed to drop off sharply during the anoxic period with the supposed growth of facultative methylotrophs grown aerobically. Instead of distinct change occurring once anoxic, the reactor showed poor denitrification that was sporadic and slower than that of the strictly anoxic experiment. These inconclusive results are interesting considering that SBR B was use to receiving methanol aerobically, but did not produce the expected results on the aerobic/anoxic high F/M.
Figure 26: Failed aerobic/anoxic high F/M experiments for reactor B. Note: These two experiments were conducted approximately a week apart, with both ending in similar non-conclusive results.

When this test was run on Blue Plains sludge the results were not as expected either. There was an extremely long lag between the aerobic period and before the reactor denitrified. The conditions were anoxic (the DO in the reactor was measured at zero an hour after the air was shut off) but yet the microorganisms were not denitrifying. Even after the reactor began to denitrify it came up with a different than expected slope. The slope would start off low and then become real steep towards the end of the experiment. When trying to use Excel Solver to fit the slope for a growth rate, it was unable to fit the last half of the experiment due to the change in slope. This slope would imply two different growth rates from looking at the NO$_3$-N disappearance. It is unsure of where this difference in slopes is coming, but it is unlikely that it is actually from growth of microorganisms during the anoxic period. It is possible that this surge in nitrate disappearance towards the end of the experiment could be coming from the
biomass grown aerobically that may have taken a longer time to adjust to the anoxic conditions. It may simply be the time needed for the microorganism to adjust from oxygen to using nitrate as the electron acceptor (although lag we are observing is in days). This curve is shown below in Figure 27.

![Figure 27](image)

**Figure 27:** Blue Plains aerobic/anoxic high F/M illustrating the difference in curvature

### 4.4 Molecular Biology Consideration

With this research there are multiple factors that were assumed due to the lack of information and to simplify discussion. The biomass in the reactors is discussed as a general denitrifying biomass instead of specific populations of microorganisms that may be performing different functions. This assumption and the resulting tests, such as the SDNR and High F/M, make no differentiation in the growth rates between different kinds of denitrifiers. It is a possibility that the microorganisms responsible for denitrification in SBRs A and B are exactly the same microbes. It is also possible that the maximum
growth rate measured in the high F/M test is from the same microorganisms responsible for denitrification in the SBRs. So, when discussing research results it is important to realize that there may actually be a difference in microorganisms grown aerobically, anoxically, and under high F/M conditions, but due to the limit scope of this project the identification of these microorganisms has yet been performed. Hopefully, in the near future, the molecular results from Columbia University will help clear up some of these questions and help to further define who is responsible for denitrification.

4.5 Conclusions and Impact on Future WWTP Design

After reviewing this study, it is possible offer insight to many of the WWTPs that are currently using methanol to drive post denitrification. This study suggests a feasible solution to many of these plants to increase denitrification capability with as little disruption as possible to the current treatment process in anticipation of stricter limits on total nitrogen. Evidence from this research would suggest that the aerobically grown facultative methylotrophic microorganisms have the potential to denitrify with kinetics similar to those grown anoxically. These same microorganisms, also, seem to have a greater aerobic $\mu_{\text{max}}$ than that of the anoxically grown, as suggested from the aerobic-methanol batch tests.

When using methanol as an external carbon source for denitrification this research could have impact on future design of WWTPs. Against tradition thought, methanol left over after the anoxic zone may not be truly “wasted”. This would allow WWTPs with post denitrification, such as Blue Plains, to feed methanol in the aerobic/nitrification step in the same sludge system prior denitrification. The bleeding of methanol into an aerobic reactor in a nutrient removal process could help to boost denitrifying capability when the
biomass is under stressed conditions and the SRT may be close to washout out. Also, by feeding methanol in the aerobic zone it may allow WWTPs that are restricted by space to be able to maintain a denitrifying biomass during stressed conditions with smaller anoxic tanks. When first adding methanol to drive denitrification this aerobic feeding of methanol could help to decrease the acclimation time require by WWTPs to fully denitrify.

The negative aspect of aerobic dosing of methanol to aid denitrifying biomass is the addition cost that is associated with the methanol (this is assuming that there is no adverse kinetic differences between aerobic and anoxically grown denitrifiers, as suggested by Gujer and Irene, 1999). This addition methanol cost can be justified for short term, critical situations to prevent washout of selected microorganisms.
REFERENCES


**APPENDIX**

**SBR A**

![Graph showing anoxic nitrate profiles for reactor A from day 2 to day 72.](image)

**Figure 28:** Anoxic nitrate profiles for reactor A from day 2 to day 72

**SBR B**

![Graph showing anoxic nitrate profiles for reactor B from day 2 to day 72.](image)

**Figure 29:** Anoxic nitrate profiles for reactor B from day 2 to day 72