Supporting Information

Glycerol-driven Denitrification: Process Kinetics, Microbial Ecology, and Operational Controls

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\[ R_c: \quad (0.20)CO_2 + (0.05)HCO_3^- + (0.05)NH_4^+ + H^+ + e^- = (0.05)C_5H_7O_2N + (0.45)H_2O \]

\[ R_d: \quad (0.21)CO_2 + H^+ + e^- = (0.07)C_3H_8O_3 + (0.21)H_2O \]

\[ \Delta G^0_a = 38.88 \frac{kJ}{eeq} \]

\[ R_{a, NO_3^- \rightarrow N_2} : \quad (0.20)NO_3^- + (1.20)H^+ + e^- = (0.10)N_2 + (0.60)H_2O \]

\[ \Delta G^0_a = -72.20 \frac{kJ}{eeq} \]

\[ R_{a, NO_2^- \rightarrow NO_2} : \quad (0.50)NO_3^- + H^+ + e^- = (0.50)NO_2^- + (0.50)H_2O \]

\[ \Delta G^0_a = -41.65 \frac{kJ}{eeq} \]

\[ R_{a, NO_2^- \rightarrow N_2} : \quad (0.33)NO_2^- + (1.33)H^+ + e^- = (0.17)N_2 + (0.67)H_2O \]

\[ \Delta G^0_a = -92.56 \frac{kJ}{eeq} \]

\[ R_{in, acetylCoA} : \quad (0.22)CO_2 + H^+ + e^- = (0.11)C_2H_3O + (0.33)H_2O_{1-3} \]

\[ \Delta G^0_{in} = 30.90 \frac{kJ}{eeq} \]

\[ R = f_e(R_a - R_d) + f_s(R_c - R_d) = f_eR_a + f_sR_c - R_d \]

\[ 1 = f_e + f_s \]

\[ R_{NO_3^- \rightarrow N_2} : \quad f_e\left[(0.20)NO_3^- + (0.07)C_3H_8O_2 + (0.20)H^+\right] + f_s\left[(0.05)NH_4^+ + (0.05)HCO_3^- + (0.07)C_3H_8O_3\right] \]

\[ = f_e\left[(0.10)N_2 + (0.39)H_2O + (0.21)CO_2\right] + f_s\left[(0.05)C_5H_7O_2N + (0.24)H_2O + (0.01)CO_2\right] \]

\[ R_{NO_2^- \rightarrow NO_2} : \quad f_e\left[(0.50)NO_3^- + (0.07)C_3H_8O_3\right] + f_s\left[(0.05)NH_4^+ + (0.05)HCO_3^- + (0.07)C_3H_8O_3\right] \]

\[ = f_e\left[(0.50)NO_2^- + (0.29)H_2O + (0.21)CO_2\right] + f_s\left[(0.05)C_5H_7O_2N + (0.24)H_2O + (0.01)CO_2\right] \]

\[ R_{NO_2^- \rightarrow N_2} : \quad f_e\left[(0.33)NO_2^- + (0.07)C_3H_8O_3 + (0.33)H^+\right] + f_s\left[(0.05)NH_4^+ + (0.05)HCO_3^- + (0.07)C_3H_8O_3\right] \]

\[ = f_e\left[(0.17)N_2 + (0.45)H_2O + (0.21)CO_2\right] + f_s\left[(0.05)C_5H_7O_2N + (0.24)H_2O + (0.01)CO_2\right] \]
\[ R_{i, \text{glycerol-acetylCoA}}: (0.07)C_3H_8O_3 + (0.01)CO_2 = (0.11)\text{acetylCoA} + (0.12)H_2O \]


A combination of TEEM1 and the modifications incorporated into the TEEM2 thermodynamic models was employed to determine stoichiometric coefficients for glycerol-driven chemoorganoheterotrophic denitrification.

\[
\Delta G^0_{ic} = \Delta G^0_{in} - \Delta G^0_d = 30.90 \frac{kJ}{eeq} - 38.88 \frac{kJ}{eeq} = -7.98 \frac{kJ}{eeq}
\]

\[
\Delta G^0_{in} = 30.90 \frac{kJ}{eeq} \text{ to represent the energy required to convert the cell carbon source to an intermediate compound (acetyl-CoA) prior to full oxidation.}
\]

\[
\Delta G^0_d = 38.88 \frac{kJ}{eeq} \text{ for the heterotrophic reaction.}
\]

\[
\Delta G^0_{ic} = \Delta G^0_{ic} - RT \ln[10^{-7}] = -7.98 \frac{kJ}{eeq} - \left( 0.008314 \frac{kJ}{mol \cdot K} \right) (273.15 + 23K) \ln[10^{-7}] = -7.98 \frac{kJ}{eeq}
\]

Assume average atmospheric CO\(_2\) concentration, \(P_{CO_2} = 409ppm = 4.09 \cdot 10^{-4} \text{atm}\),

\[
[CO_{2(g,headspace)}] = [CO_{2(aq)}] = \frac{P_{N_2}}{K_H} = \frac{4.09 \cdot 10^{-4} \text{atm}}{29.41 \text{ atm}} = 1.39 \cdot 10^{-5} \text{M}
\]

therefore,

\[
[C_3H_8O_3] = \left( 300 \frac{mg\text{COD}}{L} \right) \left( \frac{1g\text{COD}}{1000mg\text{COD}} \right) \left( \frac{1mol\text{COD}}{32g\text{COD}} \right) \left( \frac{1molC_3H_8O_3}{3.5mol\text{COD}} \right) = 2.68 \cdot 10^{-3} \text{M per}
\]

Assume all glycerol is converted to acetyl-CoA, \([C_3H_8O_3] = [\text{acetylCoA}]\).
System was operated at room temperature (23°C) and buffered at pH = 7.5, or

\[ [H^+] = 10^{-7.5} M. \]

\[ \Delta G_{ic} = -7.98 \frac{kJ}{eeq} + \left( 0.008314 \frac{kJ}{mol \cdot K} \right) (273.15 + 23K) \ln \left( \frac{[2.68 \cdot 10^{-3} M]^{0.11} [1M]^{0.12}}{[1.39 \cdot 10^{-5} M]^{0.008} [2.68 \cdot 10^{-3} M]^{0.07}} \right) = -8.34 \frac{kJ}{eeq} \]

\[ \Delta G'_r = \Delta G'_a - \Delta G'_d = -72.20 \frac{kJ}{eeq} - 38.88 \frac{kJ}{eeq} = -111.08 \frac{kJ}{eeq} \]

\[ \Delta G^0_r = \Delta G^0_r - RTv_H + \ln [10^{-7}] = -111.08 \frac{kJ}{eeq} - \left( 0.008314 \frac{kJ}{mol \cdot K} \right) (273.15 + 23K) \ln \left( \frac{[6.10 \cdot 10^{-4} M]^{0.10} [1.39 \cdot 10^{-5} M]^{0.21} [1M]^{0.39} [1.39 \cdot 10^{-5} M]^{0.21} [1M]^{0.39} [10^{-7}] \right) \]

\[ \Delta G_r = \Delta G^0_r + RTlnQ = \Delta G^0_r + RTln \left( \frac{[N_2]^{0.10} [CO_2]^{0.21} [H_2O]^{0.39}}{[NO_3^-]^{0.20} [C_3H_8O_3]^{0.07} [H^+]^{0.20}} \right) \]

Assume completely anoxic reactor with headspace saturated with N\(_2\)(g), therefore,

\[ [N_2(g, headspace)] = [N_2(aq)] = \frac{P_{N_2}}{K_H} = \frac{1 \text{ atm}}{1639.34 \text{ atm/M}} = 6.10 \cdot 10^{-4} M \]

Initial \[ [NO_3^-] = \left( \frac{100 \text{ mgN}}{L} \right) \left( \frac{1 \text{ gN}}{1000 \text{ mgN}} \right) \left( \frac{1 \text{ molN}}{14 gN} \right) = 7.14 \cdot 10^{-3} M \] per cycle.

\[ \Delta G_r = -119.02 \frac{kJ}{eeq} + \left( 0.008314 \frac{kJ}{mol \cdot K} \right) (273.15 + 23K) \ln \left( \frac{[6.10 \cdot 10^{-4} M]^{0.10}}{[7.14 \cdot 10^{-3} M]^{0.20} \cdot 2} \right) \]

\[ = -114.67 \frac{kJ}{eeq} \]

\[ A = \frac{\Delta G_s}{\varepsilon \Delta G_r} \]

\[ A_e \Delta G_r + \Delta G_s = 0 \], at steady-state, assuming that the energy transfer efficiency from the oxidation of electron donor to capture by the electron carrier is equal to that of the electron carrier to electrons captured for cell synthesis.\(^1\)
\[ \Delta G_s = \frac{\Delta G_{fa} - \Delta G_d}{\varepsilon^m} + \frac{\Delta G_{in} - \Delta G_{fa}}{\varepsilon^n} + \frac{\Delta G_{pc}}{\varepsilon} \],  \\
where \( \Delta G_{pc} = 18.8 \frac{kJ}{eeq} \) with NH\(_4^+\) as the nitrogen source for cell synthesis and C\(_5\)H\(_7\)O\(_2\)N is assumed as the cell relative composition. Sufficient NH\(_4^+\) was included in the feed stock for theoretical growth requirements and significant NH\(_4^+\) was always remaining in the effluent indicating that additional nitrogen sources (NO\(_3^-\) or NO\(_2^-\)) were not used for synthesis purposes as they are less energy efficient for the cell.

Since glycerol and acetyl-CoA are not C1 compounds, \( \Delta G_{fa} = 0 \) and \( m = n \).

\( n = -1 \) as \( \Delta G_p < 0 \).

\( \varepsilon = 0.40 \) was assumed based upon experimental data\(^2\) and reported operational influent COD:NO\(_3^-\)-N ratios.\(^4-6\)

\[ A = \frac{\Delta G_s}{\varepsilon \Delta G_r} = \frac{\frac{\Delta G_{ic} + \Delta G_{pc}}{\varepsilon}}{\frac{1}{\varepsilon} \Delta G_r} = \left( \frac{-8.34}{0.40} \frac{kJ}{eeq} \right) + \left( \frac{18.8}{0.40} \frac{kJ}{eeq} \right) - \left( \frac{0.40}{0.40} \right) (114.67 \frac{kJ}{eeq}) = 0.952 \]

\( f_s = \frac{1}{1 + A} = \frac{0.512}{1 + 0.952} = 0.512 \)

\( f_e = 1 - f_s = 1 - 0.513 = 0.488 \)

\( R = f_e R_a + f_s R_c - R_d = (0.488) R_a + (0.512) R_c - R_d \)

\( R_{NO_3^- \rightarrow N_2} = NO_3^- + (0.73)C_3H_8O_3 + (0.26)NH_4^+ + (0.26)HCO_3^- + H^+ = (0.50)N_2 + (1.15)CO_2 + (0.26)C_3H_7O_2N + (3.17)H_2O \)

\[ COD = (0.73 \text{ mol } C_3H_8O_3) \left( \frac{3.5 \text{ mol } O_2}{1 \text{ mol } C_3H_8O_3} \right) \left( \frac{32 \text{ g } O_2}{1 \text{ mol } O_2} \right) = 82.1 \text{ g } O_2 = 82.1 \text{ g } COD \]

\[ NO_3^- - N = (1 \text{ mol } NO_3^-) \left( \frac{14 \text{ g } NO_3^- - N}{1 \text{ mol } NO_3^-} \right) = 14 \text{ g } NO_3^- - N \]
Using this same process, assumptions of other energy-transfer efficiencies yield the following results:

<table>
<thead>
<tr>
<th>ε</th>
<th>0.30</th>
<th>0.40</th>
<th>0.50</th>
<th>0.60</th>
<th>0.70</th>
<th>0.80</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.809</td>
<td>0.951</td>
<td>0.583</td>
<td>0.383</td>
<td>0.262</td>
<td>0.184</td>
</tr>
<tr>
<td>f_s</td>
<td>0.356</td>
<td>0.513</td>
<td>0.632</td>
<td>0.723</td>
<td>0.792</td>
<td>0.845</td>
</tr>
<tr>
<td>f_e</td>
<td>0.644</td>
<td>0.487</td>
<td>0.368</td>
<td>0.277</td>
<td>0.208</td>
<td>0.155</td>
</tr>
</tbody>
</table>

As can be seen, the assumption of an energy-transfer efficiency has a drastic effect and, therefore, must be confirmed.


The Dissipation Method for predicting bacterial yield was employed to confirm assumptions used in the thermodynamic Reaction Energetics Method determination of COD requirements to support glycerol-driven denitrification.

\[
\frac{D^0_s}{r_{Ax}} = 200 + 18 \cdot (6 - C)^{1.8} + e^{\left[(3.8 - \gamma_D)^0.16 \cdot (3.6 + 0.4C)\right]},
\]

which describes the heat (Gibbs free energy) dissipated during growth or production of 1 C-mole of biomass.

\[C = 3,\] which represents the number of carbon atoms in a mole of glycerol.

\[\gamma_D = 4.667,\] degree of reductance of the carbon in glycerol as the electron donor.\(^7\)
\[
\frac{D_s^0}{r_{Ax}} = 200 + 18 \cdot (6 - 3)^{1.8} + e^{\left[(3.8 - 4.667)^2\right]^{0.16} \cdot \left[3.6 + (0.4)(3)\right]} = 428.06 \frac{kJ}{c\ mol}
\]

\[
Y_{DX} = \frac{Y_D \Delta G_e^0 - \Delta G_e^0}{Y_X (\Delta G_e^0 - \Delta G_e^0) + \left(\frac{D_s^0}{r_{Ax}} \cdot \frac{1}{Y_X}\right) + \left(\Delta G_e^0 - \Delta G_e^0\right)}
\]

which represents the bacterial cell yield on the electron donor.

\[
\Delta G_e^0 = 38.88 \frac{kJ}{eeq}, \text{Gibbs standard free energy for glycerol as the electron donor.}\]

\[
\Delta G_e^0 = -72.20 \frac{kJ}{eeq}, \text{Gibbs standard free energy for NO}_3^- \text{as the electron acceptor.}\]

\[
\Delta G_e^0 = 38.80 \frac{kJ}{eeq}, \text{assuming } \Delta G_{fX}^0 = -67 \frac{kJ}{c\ mol.}
\]

\[
Y_X = 4.2, \text{degree of reductance of the carbon in biomass.}\]

\[
Y_{DX} = \left(\frac{4.667}{4.2}\right) \left[\frac{38.88 \frac{kJ}{eeq} - \left(-72.20 \frac{kJ}{eeq}\right)}{38.88 \frac{kJ}{eeq} - \left(-72.20 \frac{kJ}{eeq}\right) + \left(428.06 \frac{kJ}{c\ mol} \cdot \frac{1}{4.2}\right) + \left(38.88 \frac{kJ}{eeq} - 38.88 \frac{kJ}{eeq}\right)}\right] = 0.580 \frac{c\ mol_X}{c\ mol_D}
\]

\[
Y_{DX} = 0.522 \frac{eeq_X}{eeq_D}
\]

In terms of eq, \(Y_{DX} = f_s^0\), therefore, \(f_s^0 = 0.522 \frac{eeq_X}{eeq_D}\).

\[
f_e^0 = 1 - f_s^0 = 1 - 0.522 = 0.487
\]

Comparison of \(f_s^0\) calculated using the Dissipation Method with \(f_s\) calculated using the Reaction Energetics Method indicates that the energy-transfer efficiency, \(\epsilon\), inherent in the Dissipation Method calculations is \(\epsilon = 0.406\). This confirms the validity of the assumption of \(\epsilon = 0.40\) in the Reaction Energetics Method calculations.
While these calculations are at standard state, it has been shown that there is little difference between predictions at standard state and non-standard state in certain instances provided system pH is close to neutral, substrate concentrations are low, and $\Delta G'_{eD} - \Delta G'_{eA} > \frac{kJ}{eeq}$. Additionally, as they are simply being used to confirm assumptions made using the reaction energetics method, calculations were not made to convert to non-standard state conditions.

3. Initial reactor buffering methodology prior to pH optimization batch assays.

For pH optimization batch assays, the medium was initially buffered to approximately pH 9.0 but left unbuffered for the remainder of each experiment during which the pH ranged from 7.2 to 9.0.
Table S1. Components of SBR feed including trace nutrients. "Trace nutrients were dissolved in deionized water.

<table>
<thead>
<tr>
<th>SBR Feed</th>
<th>(mg per 100 L SBR feed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO$_3$-N</td>
<td>10,000.0</td>
</tr>
<tr>
<td>NH$_4^+$-N</td>
<td>2,500.0</td>
</tr>
<tr>
<td>MgSO$_4$·7H$_2$O</td>
<td>20,000.0</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>8,700.0</td>
</tr>
<tr>
<td>CaCl$_2$·2H$_2$O</td>
<td>2,000.0</td>
</tr>
<tr>
<td>NaOH</td>
<td>for pH adjustment</td>
</tr>
<tr>
<td>Trace Nutrients*</td>
<td>(mg per 100 L SBR feed)</td>
</tr>
<tr>
<td>EDTA·Na$_2$</td>
<td>2,010.1</td>
</tr>
<tr>
<td>FeSO$_4$·7H$_2$O</td>
<td>500.4</td>
</tr>
<tr>
<td>MnCl$_2$·4H$_2$O</td>
<td>172.2</td>
</tr>
<tr>
<td>ZnSO$_4$·7H$_2$O</td>
<td>43.1</td>
</tr>
<tr>
<td>CuSO$_4$·5H$_2$O</td>
<td>25.0</td>
</tr>
<tr>
<td>CoCl$_2$·6H$_2$O</td>
<td>23.8</td>
</tr>
<tr>
<td>Na$_2$MoO$_4$·2H$_2$O</td>
<td>10.0</td>
</tr>
<tr>
<td>NiSO$_4$·6H$_2$O</td>
<td>2.1</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>1.1</td>
</tr>
</tbody>
</table>
Figure S1. Two feeding strategies, semi-continuous (green arrows; 75-min NO$_3^-$ feed with concurrent 72-min glycerol feed, influent COD:NO$_3^-$-N=2.4:1) and pulse (red arrows; each pulse contained 4-min NO$_3^-$ feed with concurrent 1-min glycerol feed, influent COD:NO$_3^-$-N=2.4:1), were investigated to determine their impact on NO$_2^-$ accumulation.
**Table S2.** Effluent sCOD and biomass concentrations supported at each respective influent COD:NO\textsubscript{3}−N ratio.

<table>
<thead>
<tr>
<th>Inf. COD:NO\textsubscript{3}−N</th>
<th>Avg X\textsubscript{reactor} [mg/L COD]</th>
<th>Avg sCOD\textsubscript{eff} [mg/L COD]</th>
<th>sCOD\textsubscript{eff}/sCOD\textsubscript{inf} [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5:1 (n=6)</td>
<td>345.4±50.4</td>
<td>6.9±6.0</td>
<td>2.8</td>
</tr>
<tr>
<td>2.8:1 (n=11)</td>
<td>423.4±35.4</td>
<td>5.3±3.1</td>
<td>1.9</td>
</tr>
<tr>
<td>3.0:1 (n=10)</td>
<td>448.2±60.9</td>
<td>9.6±9.5</td>
<td>0.3</td>
</tr>
<tr>
<td>4.0:1 (n=7)</td>
<td>493.4±39.3</td>
<td>2.2±4.3</td>
<td>0.6</td>
</tr>
<tr>
<td>5.0:1 (n=6)</td>
<td>692.4±25.6</td>
<td>18.7±5.6</td>
<td>3.7</td>
</tr>
</tbody>
</table>
**Table S3.** Results of Holm-Sidak post hoc multiple comparison analysis to determine between which NARs a significant difference exists (statistical significance exists at $p<0.05$ and is demarcated using bold font).

<table>
<thead>
<tr>
<th>Inf. COD:NO₃-N</th>
<th>2.5 (x̄=0.65)</th>
<th>2.8 (x̄=0.69)</th>
<th>3.0 (x̄=0.62)</th>
<th>4.0 (x̄=0.57)</th>
<th>5.0 (x̄=0.11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>0.496</td>
<td>0.755</td>
<td>0.319</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>2.8</td>
<td></td>
<td>0.147</td>
<td><strong>0.006</strong></td>
<td><strong>0.000</strong></td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td></td>
<td></td>
<td><strong>0.000</strong></td>
<td><strong>0.000</strong></td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td></td>
<td></td>
<td></td>
<td><strong>0.000</strong></td>
<td></td>
</tr>
</tbody>
</table>
Table S4. Denitrification performance under continuous and pulse operational feeding strategies.

<table>
<thead>
<tr>
<th>Influent COD:NO\textsubscript{3}-N</th>
<th>SRT [d]</th>
<th>Operational Feeding Strategy</th>
<th>Avg NO\textsubscript{3}-eff \textsubscript{2,-} [mg/L NO\textsubscript{3}-N]</th>
<th>Avg NO\textsubscript{2}-eff \textsubscript{2,-} [mg/L NO\textsubscript{2}-N]</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4</td>
<td>3</td>
<td>Pulse NO\textsubscript{3} \textsubscript{1}- Pulse COD</td>
<td>11.3 ± 3.3</td>
<td>86.4 ± 7.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Continuous NO\textsubscript{3} \textsubscript{1}- Continuous COD</td>
<td>16.0 ± 5.5</td>
<td>70.1 ± 8.4</td>
</tr>
</tbody>
</table>

Contrary to the continuous operational feeding strategy, the pulse operational feeding strategy reduced nearly 90% of the influent NO\textsubscript{3}\textsuperscript{-} despite the limited reaction time for late occurring pulses of NO\textsubscript{3}\textsuperscript{-} and glycerol indicating that influent NO\textsubscript{3}\textsuperscript{-} underwent rapid reduction upon entering the system. This observation was consistent with other studies which reported that specific denitrification rates are higher for pulse-type feeding strategies as compared to continuous feeding strategies resulting in a faster reduction of influent NO\textsubscript{3}\textsuperscript{3-}.\textsuperscript{11,12} Martins et al.\textsuperscript{11} determined that maximum specific denitrification rates were considerably lower for SBR systems with long feeding periods that mimicked continuously-fed, completely mixed systems, than in plug flow-type systems. Similarly, Ryu et al.\textsuperscript{12} found that denitrification rates were fastest during slug feeding followed in order by intermittent and continuous feeding strategies during their evaluation of fermented food waste as an external carbon source for nutrient removal in an SBR.
**Figure S2.** Representative ex situ NO$_3^-$-N (▲, solid line) and NO$_2^-$-N (○, dotted line) profiles at influent COD:NO$_3^-$-N ratios (a) 2.5, (b) 3.0, (c) 5.0. Ex situ batch assays were performed using biomass acclimated at each influent COD:NO$_3^-$-N ratio in the parent reactor for at least four SRTs.
Table S5. Estimations of richness and diversity in the microbial communities at influent COD:NO$_3^-$-N ratios.

<table>
<thead>
<tr>
<th>Inf. COD:NO$_3^-$-N</th>
<th>Shannon Index</th>
<th>Chao-1 Estimator</th>
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Figure S3. Principal Coordinates Analysis (PCoA) of weighted Unifrac distances analyzes and compares the beta diversity of microbial communities selected for at influent COD:NO$_3^-$-N ratios.
References


