Ecological Perspective on New Connectivity between Nitrogen and Carbon Cycle

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Nitrogen contamination is one of the 14 grand challenges prioritized by the National Academy of Engineering.

The innovative and effective N transformations through prokaryotic mediated pathways have been well received.

Example: Anaerobic ammonia oxidation was included in the overall N cycle after its inception in mid 1990’s.

Denitrification is an important component of the overall nitrogen cycle.
- Heterotrophic process → organic carbon
- Autotrophic process → hydrogen and reduced sulfur compounds.
Introduction: Recent Developments In N Cycle: Methane Coupled Denitrification

Denitrification with anoxic methane oxidation (DAMO) is a very recent development.

\[
P: 3\text{CH}_4 + 8\text{NO}_2^- + 8\text{H}^+ \rightarrow 3\text{CO}_2 + 4\text{N}_2 + 10\text{H}_2\text{O}
\]

\[
Q: \text{CH}_4 + 4\text{NO}_3^- \rightarrow \text{CO}_2 + 4\text{NO}_2 + 2\text{H}_2\text{O}
\]

Adapted from Galloway et al., 2008
Introduction: Connectivity Between C & N Cycle: Methane oxidation coupled to NO₂

\[ P: 3\text{CH}_4 + 8\text{NO}_2^- + 8\text{H}^+ \rightarrow 3\text{CO}_2 + 4\text{N}_2 + 10\text{H}_2\text{O} \]
DAMO Process in Sediments

- Water Column
- Methanogen: $\text{CH}_4 + 4\text{NO}_3^- \rightarrow \text{CO}_2 + 4\text{NO}_2 + 2\text{H}_2\text{O}$
- Dissolved nutrients
- DAMO process
- Methane produced
- Dissolved Nutrients
- Sediments
- Bed Rocks

Chemical reactions:

$P: 3\text{CH}_4 + 8\text{NO}_2^- + 8\text{H}^+ \rightarrow 3\text{CO}_2 + 4\text{N}_2 + 10\text{H}_2\text{O}$

$Q: \text{CH}_4 + 4\text{NO}_3^- \rightarrow \text{CO}_2 + 4\text{NO}_2 + 2\text{H}_2\text{O}$
**Introduction: Denitrifying Anaerobic Methane Oxidizing (DAMO) Prokaryotes**

<table>
<thead>
<tr>
<th><strong>Bacteria</strong></th>
<th><strong>Archaea</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>(NO$_2^-$)</td>
<td>(NO$_3^-$)</td>
</tr>
<tr>
<td>- <em>Candidatus</em> 'Methylomirabilis oxyfera* only known bacteria to show DAMO activity.</td>
<td>- <em>Candidatus</em> 'Methanoperedens nitroreducens*</td>
</tr>
<tr>
<td>- NC 10 phylum</td>
<td>- ANME-2d lineage</td>
</tr>
<tr>
<td>- NC10 phylum bacteria are ubiquitous.</td>
<td>- Enriched from freshwater sediments and wastewater sludge.</td>
</tr>
<tr>
<td>- Slow growth (1-2 weeks)</td>
<td>- Genome size of 3.2 (Mb)</td>
</tr>
<tr>
<td>- Genome size of 2.75 (Mb)</td>
<td>- Size of 1-3µm</td>
</tr>
<tr>
<td>- Gram negative</td>
<td>- Mesophilic</td>
</tr>
<tr>
<td>- Size of 0.8-1.1µm</td>
<td></td>
</tr>
<tr>
<td>- Mesophilic</td>
<td></td>
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</tbody>
</table>

Wu et al., 2012

Haroon et al., 2013
Research Objectives: So what is next?

Objectives in this study:

– to confirm the presence of DAMO activity in ecosystem (Jordan river, UT)

– to enrich the prokaryotes involved in DAMO process from the sediment (Jordan river, UT).

– to compare microbial diversity in the enriched DAMO reactors and the Jordan river
Methods and Results: Rate of DAMO process In Jordan River

20 grams of sediments
(a) 0-5 cms
(b) 5-10 cms
(c) 10-20 cms

<table>
<thead>
<tr>
<th>CH4 (Headspace)</th>
<th>0-5cms Depth (µM/L.day)</th>
<th>5-10cms Depth (µM/L.day)</th>
<th>10-20cms Depth (µM/L.day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAMO-NO2</td>
<td>0.77</td>
<td>0.6</td>
<td>0.78</td>
</tr>
<tr>
<td>DAMO-NO3</td>
<td>0.07</td>
<td>0.06</td>
<td>0.037</td>
</tr>
</tbody>
</table>

20 grams of sediments
(a) 0-5 cms
(b) 5-10 cms
(c) 10-20 cms
Methods and Results: Reactor Configuration: DAMO reactor

Lab scale DAMO reactor
Semi-continuously fed sequencing batch reactor (FBR)
• 1.9L reactor volume
• 6 day cycle includes 400ml of feeding* (NO₂ & NO₃) (2.78ml/h)
• HRT of 29 days
• SRT of 100 days
• Operating at 35°C
• Purged with 95% CH₄ : 5% CO₂ (8ml/min)
• pH 7.5 ± 0.2
• DO maintained below detection level (Anoxic)

*Feed composition based on Ettwig et al., 2009
Methods and Results: Reactor Performance

(A) Reactor start-up

(B) Denitrification became negligible.

(C) Monitoring frequency changed from 7 days to daily.

5.5~6.0mg/L. day
Methods and Results: Methane Oxidation Rates

<table>
<thead>
<tr>
<th></th>
<th>CH4 (Headspace)</th>
<th>March (mM/day)</th>
<th>December (mM/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAMO-NO2</td>
<td>0.021</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>DAMO-NO3</td>
<td>0.005</td>
<td>0.007</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>N2 (Headspace)</th>
<th>March (mM/day)</th>
<th>August (mM/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denitrification-NO2</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>Denitrification-NO3</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
</tbody>
</table>

CH4 mass balance in serum bottle (duplicate)

Average 80-85% = (CH4 lost from reactor/ CH4 expected based on stoichiometry) * 100

Currently on going experiment for Stable isotope probing
Methods and Results: 16S rRNA gene (NC10) quantification

Quantification of 16SrRNA gene specific to NC10 phylum qP2 F/R primers (Amplify position 1169 to 1460bps)

Melting curve analysis (Tm): 84.1-84.2°C)

Significant increase in 16SrRNA gene (NC10 phylum) copy number

Graphical representation of Copy number in logarithmic scale (base 10)
Methods and Results: 16S rRNA gene (NC10 phylum clones)

Major 4 clusters of NC10 phylum

Neighbor-joining method. Boot strap support values (1,000 replicates)
Methods and Results: High Throughput Sequencing

Next generation sequencing: Ion torrent
- Riverine sediments: 1300S sediment samples
- DAMO reactor / SLC Reactor (2months)

Taxonomic Binning of reads: MEGAN analysis
Methods and Results: Evidence Showing Enrichment of *Methylomirabilis* Sp.

Reads from each metagenome were mapped on *Candidatus Methylomirabilis oxyfera* genome.

<table>
<thead>
<tr>
<th></th>
<th>% Reads mapped</th>
<th>Length of genome covered (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAMO reactor</td>
<td>5.29</td>
<td>2,581,385</td>
</tr>
<tr>
<td>Jordan River sediments</td>
<td>1.39</td>
<td>1,511,690</td>
</tr>
</tbody>
</table>

Reactor enrichment after 2 months shows enrichment of organisms with G+C % of average of 59.6.

*Ca’ Methylomirabilis oxyfera* G+C % 58.6
On-going: T-RFLP: *pmo*A gene NC10 specific

T-RF profile of
(a) Jordan River Samples (JRM)
(b) (b) Enrichment reactor (AMO).
(c) DAMO control*

Results
T-RF cuts of 90-100 bps observed in all samples.

More detailed analysis under processing for developing a robust method for T-RFLP of *pmo*A gene specific to NC 10 phylum

* DNA extracted from DAMO enriched reactors from Netherlands
Conclusions

• Presence of DAMO activity in Jordan River
• Enrichment of bacteria capable of anoxic methane oxidation from sediments of riverine system was achieved.

Based on

– Cloning and sequencing of 16SrRNA gene (NC10 phylum)
– Quantification of 16S rRNA gene (NC10 phylum)
– Metagenomics: Enrichment of organism with G+C% average 59.6%
Thank you

Questions

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On Going Research

Ecosystem study:
(a) Methane Oxidation Rates
(b) Diversity of DAMO prokaryotes

Lab-scale enrichment
(a) Methane Oxidation Rates.
(b) Quantification of NC 10 Phylum.
(c) Metagenomics: Whole Community Analysis.
(d) Gene expression based study.

Enrichment

Wastewater study:
Can we use DAMO to achieve better nitrogen removal?

Geochemistry

pH, Temp and DO