Ammonia Tolerant Microorganisms from Anaerobic Waste Treatment Systems

Donna E. Fennell

The Rutgers EcoComplex
Rutgers Energy Institute
New Jersey Institute for Food, Nutrition and Health

FOOD WASTE-TO-LOW CARBON ENERGY CONFERENCE
APRIL 27-28, 2016
Acknowledgements

Serpil Guran and David Specca  
(EcoComplex)

Laurie Van Genderen  
(Burlington County, NJ)

Francis Bonaccorso  
(JMEUC)

Richard Crone  
(Pinehurst Acres Farm, PA)

Officers at Lamchabang Municipality,  
Chonburi, Thailand

Sunirat Rattana

Shaurya Prakash

Amanda Luther

Peter Strom

David Babson

Valdis Krumins
Anaerobic Digestion Metabolic Phases

Phase I: Hydrolysis

**Complex Organics**
Lipids, Polysaccharides, Proteins, Nucleic acids

- Cellulolytic and other hydrolytic bacteria
- Proteolytic bacteria

**Simple Organics**
Fatty acids, Monosaccharides, Amino acids & Peptides

**Phase II: Fermentation (Acidogenesis)**

- Short-Chain Fatty Acids
  - Propionate, Butyrate

- Obligate proton-reducing bacteria
- Acetotrophic methanogens (Archaea)
- Hydrogenotrophic methanogens (Archaea)

- Carbon Dioxide (CO₂)
- Acetate (CH₃COO⁻)

- Hydrogen (H₂)
- Syntrophic acetate oxidizing bacteria
- Acetogenic bacteria

**Phase III: Methanogenesis**

- Methane (CH₄)
- Carbon Dioxide (CO₂)

Modified from Klass 1984, Stams 1994, Rittmann and McCarty 2001
C:N Ratio, TAN and Ammonia Inhibition

- Ammonia/TAN inhibition of digesters common
- Prevent buildup of Ammonia/TAN
  - Blend feedstocks to increase C:N ratio
  - Adjust pH
  - Remove ammonia

\[
\text{NH}_3 + \text{H}^+ \leftrightarrow \text{NH}_4^+ \\
\text{Total Ammonia-Nitrogen (TAN)}
\]

\[
\text{NH}_3\text{-N} + \text{NH}_4^+\text{-N}
\]
Ammonia and C:N Ratio

- TAN concentration is a function of the available organic nitrogen in the feedstock.

- The C:N ratio is the ratio of carbon to nitrogen for a feedstock (g/g).

<table>
<thead>
<tr>
<th>C:N Ratio</th>
<th>Raw Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-10</td>
<td>Slaughterhouse waste</td>
</tr>
<tr>
<td>10-20</td>
<td>Cattle manure</td>
</tr>
<tr>
<td>20-30</td>
<td>Hay-general</td>
</tr>
<tr>
<td>30-40</td>
<td>Corn silage</td>
</tr>
<tr>
<td>40-50</td>
<td>Fruit wastes</td>
</tr>
<tr>
<td>50-60</td>
<td>Leaves</td>
</tr>
<tr>
<td>60-70</td>
<td>Corn stalks</td>
</tr>
<tr>
<td>70-80</td>
<td>Straw-oat</td>
</tr>
<tr>
<td>80-90</td>
<td>Straw-general</td>
</tr>
<tr>
<td>90-100</td>
<td>Paper pulp</td>
</tr>
<tr>
<td>100-200</td>
<td>Straw-wheat</td>
</tr>
<tr>
<td>200-300</td>
<td>Hard wood bark</td>
</tr>
<tr>
<td>300+</td>
<td>Soft woods</td>
</tr>
</tbody>
</table>

Ammonia & Anaerobic Digestion

- Ammonia is toxic to anaerobic microbes
- Ammonia causes eutrophication
- Ammonia removal is energy intensive (nitrification)

- Ammonia can be recovered for fertilizer
- Ammonia could be used directly as a fuel
- Could “bio”-ammonia could be reformed to hydrogen as a fuel?
Anaerobic digestion for methane generation and ammonia reforming for hydrogen production: A thermodynamic energy balance of a model system to demonstrate net energy feasibility

David M. Babson\textsuperscript{a}, Karen Bellman\textsuperscript{b}, Shaurya Prakash\textsuperscript{b,}\textsuperscript{*}, Donna E. Fennell\textsuperscript{a,}\textsuperscript{**}

\textsuperscript{a}Department of Environmental Sciences, Rutgers University, 14 College Farm Road, New Brunswick, NJ 08901, United States
\textsuperscript{b}Department of Mechanical and Aerospace Engineering, The Ohio State University, 201 W. 19th Ave., Columbus, OH 43210, United States
Anaerobic Digestion-Bioammonia to H₂

Biomass Feedstock → Anaerobic Digester

CH₄ + CO₂ → Ammonia Recovery System

TAN → NH₃

NH₃ → H₂ + N₂

Ammonia Reforming System

CH₄ + CO₂
• Methane production decreases as C:N ratio decreases – less carbon is available to be converted to methane.

• When normalized to potential methane for a specific C:N ratio, equivalent energy increases because in addition to CH₄, NH₃ is recovered as H₂.
Bioammonia Recovery

• Operating at high TAN with low C:N wastes advantageous for energy balance
Microbial Communities Shift at High TAN

- Syntrophic acetate oxidation (SAO) coupled to hydrogenotrophic methanogenesis are dominant pathways in digesters at high TAN concentrations (3-7 g NH\(_4^+\)-N/L)

\[
\begin{align*}
CH_3COO^- + 4H_2O & \rightarrow 2HCO_3^- + 4H_2 + H^+ & \Delta G^{0'} = +104.6 \text{ kJ} \cdot \text{mol}^{-1} \\
4H_2 + HCO_3^- + H^+ & \rightarrow CH_4 + 3H_2O & \Delta G^{0'} = -135.6 \text{ kJ} \cdot \text{mol}^{-1}
\end{align*}
\]

- *Tepidanaerobacter sp.* is syntrophic acetate oxidizing bacteria found in high TAN systems
- *Methanosarcina sp.* and other hydrogenotrophic methanogens become dominant during the SAO pathway (Schnurer et al. 1994; Schnurer and Nordberg 2008; Westerholm et al. 2011; Müller et al. 2015)

- Little is known about ammonia-tolerant communities in diverse anaerobic treatment systems
Study Hypotheses

1. Anaerobic digesters can be operated at high TAN once microbial communities have acclimated.

2. Ammonia tolerant microorganisms are present in high ammonia systems.

3. Organisms that produce ammonia have ammonia-specific stress responses.
Study I: Enrichment and identification of ammonia tolerant microorganisms from two landfill leachates

**Inoculum 1**
Bioreactor landfill leachate, Burlington County, NJ, USA

**Inoculum 2**
Conventional landfill leachate, Lamchabang Municipality, Chonburi, Thailand
Study II: Enrichment and identification of ammonia tolerant microorganisms from two anaerobic digesters

**Inoculum 1**
Swine waste digestate, Pinehurst Acres Farm, PA

**Inoculum 2**
Wastewater sludge digestate, JMEUC, NJ
Study I and II: Experimental Design

- Microcosm enrichments
- Semi-continuous operation at 35°C
- HRT = 140 days
- Glutamate (C₅H₉NO₄) as sole carbon and energy source
- TAN from background to 12.5 g/L

\[
\text{Glutamate}^- + 3\text{H}_2\text{O} \rightarrow 2\text{acetate}^- + \text{HCO}_3^- + \text{H}^+ + \text{NH}_4^+ + \text{H}_2
\]

\[
\text{Glutamate}^- + 4\text{H}_2\text{O} \rightarrow \text{propionate}^- + 2\text{HCO}_3^- + \text{NH}_4^+ + 2\text{H}_2
\]
Thailand enrichments resistant to higher TAN than NJ enrichments
Results – Landfill Bacterial DGGE

TAN Concentrations

<table>
<thead>
<tr>
<th>B</th>
<th>5</th>
<th>7.5</th>
<th>10</th>
<th>12.5</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>L</th>
<th>5</th>
<th>7.5</th>
<th>10</th>
<th>12.5</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Tepidanaerobacter sp.

Thailand

New Jersey

A- Active control, B- Background control, L- Leachate;
Thailand samples day 218, New Jersey samples day 292
Results – Landfill Archaeal DGGE

Methanoculleus sp.

Methanosarcina sp.

Thailand

A- Active control, B- Background control, L- Leachate;
Thailand samples day 218, New Jersey samples day 292
Results – Pyrosequencing (Order)

Bacterial Community

Day 218
- **Clostridium sp.**
- **Flavobacteriales**
- **Thermoanaerobacterales**
- **Clostridiales**

Day 292
- **Tepidanaerobacter sp.**
- **Flavobacteriales**
- **Bacteroidales**
- **Synergistales**

Archaeal Community

Day 218
- **Methanobacterales**
- **Bacteroidales**
- **Synergistales**
- **Candidatus_cloacamonas**

Day 292
- **Methanosarcinales**
- **Thermoplasmatales**
- **Methanobacterales**
- **Methanomicrobiales**

Other
Do Ammonia-Tolerant Microbes Come from High Ammonia Environments?

### Ammonia

<table>
<thead>
<tr>
<th>Source</th>
<th>Ammonia Concentration (g N/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thailand leachate</td>
<td>1.3</td>
</tr>
<tr>
<td>New Jersey leachate</td>
<td>0.8</td>
</tr>
<tr>
<td>Swine waste digestate</td>
<td>3.9</td>
</tr>
<tr>
<td>Wastewater sludge digestate</td>
<td>2.0</td>
</tr>
</tbody>
</table>

- **TAN**
- **Free Ammonia**

### Methane

<table>
<thead>
<tr>
<th>Source</th>
<th>Methane Production % of No TAN Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thailand leachate</td>
<td>97.7</td>
</tr>
<tr>
<td>New Jersey leachate</td>
<td>98.0</td>
</tr>
<tr>
<td>Swine waste digestate</td>
<td>87.8</td>
</tr>
<tr>
<td>Wastewater sludge digestate</td>
<td>103.2</td>
</tr>
</tbody>
</table>

- **5 g TAN/L**
- **12.5 g TAN/L**
Summary

- Methanogenesis at TAN up to 12.5 g/L with ammonia tolerant microbial communities
- High ammonia waste treatment systems may not contain ammonia-tolerant microorganisms
  - Swine waste digester versus Thailand landfill
- Facilities should consider whether the intrinsic native community is ammonia-sensitive or tolerant
  - e.g., existing wastewater sludge digester facilities want to add additional substrates to produce more energy
- Ammonia tolerant communities contained or developed the SAOB *Tepidanaerobacter sp.* and included *Methanosarcina sp.* as the primary methanogens (confirming previous research)
Study III: Ammonia stress response of *Peptostreptococcus russellii*

- isolated from a swine manure holding pit (Whitehead et. al., 2011)
- displays a high specific activity of ammonia production from protein, peptides, and amino acids
Ammonia Toxicity: Specific Toxicity Models

NH₃ effect  NH₄⁺ effect

Sprott and Patel, 1986
Experimental methods

- Induce ammonia stress in pure cultures of *P. russellii*, during fermentative growth on peptides by addition of ammonium chloride.
- Grow at elevated pH (8.5) so both TAN and FAN are at inhibitory levels.
- Induce salt stress (sodium) to help identify an ammonia specific response.

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>TAN, g L⁻¹ (FAN, mg L⁻¹)</th>
<th>Sodium (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstressed</td>
<td>1 (35)</td>
<td>0.07</td>
</tr>
<tr>
<td>Ammonia stressed</td>
<td>4 (1000)</td>
<td>0.07</td>
</tr>
<tr>
<td>Sodium stressed</td>
<td>1 (35)</td>
<td>0.5</td>
</tr>
</tbody>
</table>
Experimental Design

Unstressed cultures

Ammonia stressed cultures

Sodium stressed cultures

Isolate and enrich for mRNA from cells in exponential growth phase

Sequence transcripts

Compare gene expression under stress against unstressed through differential expression analysis
Peptostreptococcus russellii Growth

![Graph showing growth of Peptostreptococcus russellii under different conditions. The graph plots Log_{10} Absorbance (660nm) against Time (h). There are three conditions shown: Unstressed with a growth rate of 0.54 h^{-1}, TAN with a growth rate of 0.35 h^{-1}, and Sodium with a growth rate of 0.43 h^{-1}.]
Differential mRNA Expression Relative to Unstressed Control

Sodium stressed
- log10(False Discovery Rate) vs Log2(Fold Change)

- Red: Under
- Green: Over
- Black: Not different

Ammonia stressed
- Blue boxes indicate significant changes

Log2(Fold Change) for both conditions.
## Differential mRNA Expression Results

<table>
<thead>
<tr>
<th>Transcripts</th>
<th>Sodium stress</th>
<th>TAN stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>overexpressed</td>
<td>328</td>
<td>344</td>
</tr>
<tr>
<td>under expressed</td>
<td>380</td>
<td>335</td>
</tr>
<tr>
<td>shared overexpressed</td>
<td>66</td>
<td>66</td>
</tr>
<tr>
<td>shared under expressed</td>
<td>68</td>
<td>68</td>
</tr>
</tbody>
</table>
Differently Regulated Fermentation Pathways

HAD 488

HAD 1432

CDS= protein coding sequence = predicted gene
Differently Regulated Fermentation Pathways

**HAD 488 (down)**
- Glutamate/Glutamine
- (R)-2-hydroxyglutaryl-CoA
- Glutaryl-CoA
- acetate, butyrate, NH$_4^+$, CO$_2$, H$_2$

**HAD 1432 (up)**
- Alanine/Serine
- (R)-Lactyl-CoA
- Acryloyl-CoA
- acetate, propionate, NH$_4^+$, CO$_2$
Glycogen Storage Under TAN Stress

CDS = protein coding sequence = predicted gene
Glycogen Storage Under TAN Stress

- Glycogen acts as a carbon and energy storage macromolecule
- Glycogen is produced under conditions where both carbon and energy are abundant but growth is inhibited
- Glycogen is degraded to provide carbon and energy when carbon or energy are limiting
- Possible down-regulation of metabolic and ribosomal enzymes forced the cells into non-growth state where energy and carbon is stored for later use as glycogen.
Summary

- *P. russellii* growth is inhibited by both ionized and unionized ammonia (data not shown)
- TAN stress induced a unique transcriptional response compared to sodium stress in *P. russellii*
- TAN stress caused *P. russellii* to shift amino acid utilization pathways – could be related to accumulation of intracellular osmolytes
- TAN stress induced glycogen production systems in *P. russellii* – poor growth conditions
- Three potassium transport systems upregulated under ammonia stress, including two likely TrkAH (similar to findings in methanogens)
Overall Findings

- Likely shift to SAO pathway and presence of Methanosarcina (+Methanoculleus) indicated ammonia tolerance in a comparative study of different waste treatment systems.

- In situ system TAN concentration was not necessarily correlated with native ammonia-tolerant microbial communities—did not predict the extreme tolerance of the Thailand landfill.

- The proteolytic bacterium P. russellii was more ammonia tolerant than methanogenic communities—supports two-phase digestion configuration.

- P. russellii had several unique responses to ammonia stress—similar to methanogens and to response predicted by Tepidanaerobacter genome (Müller et al., 2015).
Acknowledgements

Serpil Guran and David Specca (EcoComplex)
Laurie Van Genderen (Burlington County, NJ)
Francis Bonaccorso (JMEUC)
Richard Crone (Pinehurst Acres Farm, PA)
Officers at Lamchabang Municipality, Chonburi, Thailand

Shaurya Prakash
Peter Strom
Valdis Krumins

Sunirat Rattana
Amanda Luther
David Babson
Serpil Guran and David Specca (EcoComplex)
Laurie Van Genderen (Burlington County, NJ)
Francis Bonaccorso (JMEUC)
Richard Crone (Pinehurst Acres Farm, PA)
Officers at Lamchabang Municipality, Chonburi, Thailand

Shaurya Prakash
Peter Strom
Valdis Krumins