BIOREMEDIATION APPROACHES AND TOOLS FOR BENZENE REMEDIATION UNDER ANAEROBIC CONDITIONS

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• Introduction –
• BTEX degradation and bioremediation
• Aerobic vs anaerobic
• Benzene degrading culture DGG-1
• Biomarkers
• Biotreatability studies
• Conclusions and Future work
BTEX Compounds

- Petroleum hydrocarbons of primary concern in groundwater are benzene, toluene, ethylbenzene and xylenes (BTEX)
- BTEX comprises ~18% of gasoline
- Benzene in particular is problematic potent carcinogen/ very mobile and most difficult to degrade under anaerobic conditions
<table>
<thead>
<tr>
<th>Category</th>
<th>Technology</th>
<th>Example Target Contaminants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic</td>
<td>Oxygen Addition, Nutrient Addition</td>
<td>Petroleum Hydrocarbons, Pesticides</td>
</tr>
<tr>
<td></td>
<td>Bioaugmentation</td>
<td>Petroleum Hydrocarbons, Pesticides</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>Electron Donor Addition</td>
<td>Chlorinated Solvents, Perchlorate, Oxidized Metals, Explosives, Nitrate</td>
</tr>
<tr>
<td></td>
<td>Bioaugmentation (KB-1® / KB-1® Plus/DGG-1)</td>
<td>PCE, TCE, DCE, VC and 1,2-DCA \ Chlorinated ethanes and methanes such as 1,1,1-TCA, carbon tetrachloride and chloroform; CFC-113 Benzene</td>
</tr>
<tr>
<td></td>
<td>Electron Acceptor Addition</td>
<td>Petroleum Hydrocarbons</td>
</tr>
<tr>
<td>Cometabolic</td>
<td>Gas infusion, Bioaugmentation</td>
<td>1,4-Dioxane, NDMA, Chloroform, TCE, DCE, VC, MTBE, Creosote, &gt;300 different compounds</td>
</tr>
<tr>
<td>Abiotic</td>
<td>Natural Attenuation, Reduced Metals</td>
<td>Chlorinated solvents, Oxidized metals,</td>
</tr>
</tbody>
</table>
Bioremediation

- **Biostimulation**: addition of amendments to increase biodegradation e.g., electron donors, electron acceptors, nutrients, etc.

- **Bioaugmentation**: addition of beneficial microorganisms to improve biodegradation
**Anaerobic vs. Aerobic Respiration**

**Aerobic respiration**
metabolic reactions and processes that take place in the cells of organisms that use oxygen as the terminal electron acceptor

**Anaerobic respiration**
metabolic reactions and processes that take place in the cells of organisms that use electron acceptors other than oxygen (e.g., sulfate, BTEX)
Overview of Microbial Metabolism

**Electron Donor or Substrate (Reduced)**
- Sugars, Proteins, Fats
- BTEX; H₂; Fe(II);

**Electron Donor or Substrate (Oxidized)**
- CO₂, H₂O, Fe(III)

**Electron Acceptor (Oxidized)**
- Oxygen,
- Nitrate (NO₃), Sulfate (SO₄)
- Fe(III), CO₂, chlorinated solvents

**Electron Acceptor (Reduced)**
- Water
- N₂, H₂S
- Fe(II), CH₄,
BTEX Bioremediation

- Aerobic bioremediation approaches rely on delivery of oxygen.

- Intrinsic microbial populations often capable of performing aerobic biodegradation.

- When contamination is deep or under naturally induced reducing conditions aerobic bioremediation can be difficult to establish and maintain.
Aerobic processes effective but when contamination is deep or under established reducing conditions, aerobic bioremediation can be difficult to establish and maintain.
Anaerobic BTEX Bioremediation

- Biodegradation of BTEX occurs under anaerobic conditions
  - Methanogenic
  - Nitrate reducing
  - Sulfate reducing
- Microbial populations – may be present at low concentration but growth is slow
- TEX degraders more ubiquitous than benzene degraders can becomes a bottleneck = need for bioaugmentation
- Benzene is biggest challenge due to its unsubstituted ring structure = need for bioaugmentation
Anaerobic Benzene Degradation

- Benzene to fatty acids, alcohols via primary fermenters (e.g., ORM2)
- Syntrophy

- Fatty acids, alcohols to acetate via secondary fermenters
- Acetate oxidized by acetate-oxidizing organisms
- Acetoclastic methanogens produce 
  - CH₄, CO₂
- Hydrogenotrophic methanogens

- Acetate combined with 
  - CO₂ + H₂S/N₂

- NO₃⁻-reducing bioprocesses
- SO₄²⁻-reducing bioprocesses
- Methanogenesis

- Denitrifying bacteria
- Sulfate-reducing bacteria

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Anaerobic Benzene Degradation

Benzene → Fatty acids, alcohols
- primary fermenters
- secondary fermenters

Fatty acids, alcohols → acetogens → Acetate → acetate-oxidizing organisms → CO₂
- denitrifying bacteria
- sulfate-reducing bacteria

Acetate → acetoclastic methanogens → CH₄, CO₂

H₂, formate → acetogens → Acetate
- hydrogenotrophic methanogens

NO₃⁻-reducing bioprocesses
SO₄²⁻-reducing bioprocesses
methanogenesis
Anaerobic Benzene Culture – DGG-1

Currently Scaling up to Field Scale volumes

Anaerobic benzene seed culture (above) benzene fermenter is ORM2 (right)
Photos Courtesy of University of Toronto

Edwards and Grbic-Galic, 1992
Introducing ORM2

- Benzene specialist derived from an oil refinery site in 2003
- Deltaproteobacterium not yet isolated in pure culture
- Slow growing ~ 30 day doubling time
- Has enzymes that ferment benzene
- Observed to degrade 40 mg/L
- Can ORM2 be used to bioaugment benzene sites?
DGG-1 Anaerobic Benzene Bioaugmentation Culture

Benzene concentration (μmol/L) vs. Time (day)

- Negative control
- Full strength culture
- Benzene amendment (~30 mg/L)
Identification of key microbes in degradation pathways

- Allows identification by qPCR analysis
  - Anaerobic Benzene – ORM-2
  - Sulfate degrading bacteria - SRB
Gene-Trac® Tests:

- **ORM2** – benzene degrader (sulfate reducing/methanogenic conditions)
- **SRB** - sulfate reducing bacteria
- **Peptococcaceae** - benzene degrader (under nitrate reducing conditions)
- **abcA** - (Peptococcaceae functional gene benzene carboxylase)
Biomarker Testing

- Are the required microorganisms indigenous to the site?
- Is bioaugmentation required?
- Impact of site amendments?
- Growth and spread of organisms in enhanced bioremediation?
Anaerobic Biotreatability Studies

Anaerobic conditions maintained during set up incubation and sampling in glove bags filled with N₂/CO₂/H₂ gas mixture

Degradation of BTEX monitored by GC under various conditions
Batch Treatability Study Design Features

- **Sterile Control**: Autoclaved and poisoned to inhibit microbes and measure possible abiotic losses.
- **Active Control**: Unamended.
- **Biostimulation**: Addition of organic electron donors.
- **Bioaugmentation+ Biostimulation**: Addition of known degrading populations e.g., KB-1.
- **Gas Addition**: H₂/O₂ addition etc. To measure impact of gas infusion/cometabolic processes e.g., propane addition.

Treatability studies are custom designed for each site.
Ontario Site Treatability Study

### Unbioaugmented

- **Benzene (mg/L):**
  - Days: 12, 77, 109
  - Graph showing Benzene levels decreasing over time.

- **Gene copy number /mL:**
  - 1E+4, 1E+5, 1E+6, 1E+7, 1E+8
  - Days: 12, 77, 109
  - Graph showing gene copy numbers for Total bacteria.

  - ORM2 ND

### Bioaugmented

- **Benzene (mg/L):**
  - Days: 12, 77, 109
  - Graph showing Benzene levels decreasing over time.

- **Gene copy number /mL:**
  - 1E+4, 1E+5, 1E+6, 1E+7, 1E+8
  - Days: 12, 77, 109
  - Graph showing gene copy numbers for ORM2 Benzene degrader.

- **ORM2 ND**
BTEX Degrade under Sulfate Reducing Conditions – Ontario Site Microcosms

DGG-1

Concentration (mmol/bottle)

Days

Benzene
Toluene
Ethyl Benzene
o-Xylene
p,m-Xylene

Bioaugmented with DGG-1

~260 mg/L

~100 mg/L
We are developing Cultures Can Degrade Multiple Substrates Simultaneously
SiREM collaborator on 3 year grant with University of Toronto (Elizabeth Edwards) and Federated Co-operatives Limited

**Project Goals:**
- Bioaugmentation culture scale-up
- Treatability Testing
- Develop molecular genetic tests to track key organisms
- Data for regulatory approvals (safety/performance)
- Field pilot testing (Co-op site)

Do you have a benzene site? Please let us know!
Conclusions con’t and Future Work

• Scale up to 100L of anaerobic benzene culture for field scale applications - in progress
• Lab treatability studies underway for 4 sites - indicate the DGG-1 culture is effective under simulated site conditions
• Molecular tools to quantify key microorganisms/functional genes have been test under development
• Cultures for the TEX compounds being tested can be combined
• Field testing is planned for 2018
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