Microbiology of Wastewater Treatment

Lecture 25

Other Sources:
http://www.splammo.net/JLbactsite.html
Black, Microbiology Principles and Exploration 7th Ed
Madigan, Brock Biology of Microorganisms, 11th Ed.

What is wastewater?
✓ Industrial sources:
  - Petrochemical, dairy, food, pharmaceutical, metallurgical, etc.
✓ Domestic sources:
  - Form households and non-industrial businesses
✓ Domestic sewage:
  - Sinks, toilets, and showers
  - US domestic sewage varies little from community to community across the country
  - Our waste is not unique. We even flush our toilets at the same time.

What’s in domestic wastewater?
✓ Also called sewage: it looks like spent dishwater
✓ The chemists only care about the organic content, specifically carbohydrates, fats, and proteins.
✓ Sewage is 99.9% water and 0.02-0.04% solids
✓ Example: Washington DC, 200 tons of solids per day is produced
  - 40-50% is proteins, 40-50% carbohydrates, 5-10% fats
✓ What would happen if this was released into the surrounding environment?

Lecture Topics
✓ Why treat wastewater
✓ The wastewater treatment plant
✓ Important microbial processes
✓ Microbial monitoring

http://nsm1.nsm.iup.edu/simmons
Environmental consequences of not treating sewage:

- People can get sick from pathogen contaminated water. Big problem in developing nations.
- Releasing wastewater directly into a stream leads to oxygen depletion.
  - This is caused by aerobic respiration linked to high organic carbon loads in the waste.
  - Low oxygen in water can cause fish kills.
- The degree of oxygen consumption in wastewater can be quantified.
  - This is called the Biological Oxygen Demand (BOD)

http://ian.umces.edu

How to measure BOD

- Five day bioassay for oxygen consumption, BOD5
- 300 ml bioassay in special bottles.
- Uses an oxygen meter to measure dissolved oxygen (DO) consumption in a five day period
- The BOD5 is calculated by:
  \[ \text{BOD}_5 \text{ (mg/L)} = \frac{D_1 - D_2}{P} \]
  - \( D_1 \): initial DO (mg/L) of the sample
  - \( D_2 \): sample DO (mg/L) after 5 days
  - \( P \): decimal volumetric fraction of sample used.

<table>
<thead>
<tr>
<th>Source</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Domestic sewage</td>
<td>150-200 mg/L</td>
</tr>
<tr>
<td>Milk processing/cannery waste</td>
<td>5000-6000</td>
</tr>
<tr>
<td>Pulping operations</td>
<td>10,000-15,000 mg/L</td>
</tr>
</tbody>
</table>

Why the different numbers?

The need for wastewater treatment plants

- Goal of wastewater treatment:
  - Protect health
  - Preserve natural resources
  - Prevent ecological damage

- How to accomplish these goals:
  - Use wastewater treatment plants (WWTP)
  - The WWTP removes energy-rich organic matter before discharge into the environment.
  - And uses technology to prevent/lower the occurrence of water borne diseases.
Sewage Treatment

**Primary treatment:**
- Non-biological treatment
- Removes solids
- Waste has high nutrient load (e.g., C, N, S, and P)

**Secondary treatment:**
- Decreases dissolved organic carbon (DOC)
- Uses biological treatment
- Aerobic and anaerobic secondary treatment

Microbiology of anoxic secondary treatment

- This is used for breaking down solid waste.
- Done in an anoxic sludge digester.
- Solids are complex polymers e.g., cellulose and fiber.
  - Microbes secret lipases, proteases, amylases, etc.
- Fermentation is the major metabolism in this treatment
- Lots of methane is produced by methanogenic archaea.
- The methane is collected and used to generate electricity.
Aerobic secondary waste treatment: trickle filter

- Trickling filter is a bed of crushed rocks—the 1st-treated sewage is trickled over it. Lots of surfaces for microorganisms to attach to.
- Complete mineralization of waste to CO$_2$, ammonia, nitrate, sulfate, and phosphate.
- Same process occurring in a fish aquarium.

Aerobic Activated sludge

- Air bubbled through waste water.
- Bacteria form large flocs.
  - Zoogloea ramigera is one of the key species that forms a slime and is the base of the floc.
- After the flocs form they are allowed to settle out.
- Filamentous bacteria can cause sludge bulking problems—sludge thickens.

Important microbes in the sewage treatment plant

- Nitrifying bacteria
  - Aerobes
    - Convert nitrogenous waste into nitrate.
- Denitrifying bacteria
  - Anaerobes
    - Convert nitrate to N$_2$.
- Methanogens
  - Generate methane from acetate.
  - Or use H$_2$ and CO$_2$ to make methane.
  - Mostly archaea.
Nitrifying bacteria

- Ammonia is converted into nitrate
- Ammonia has a high BOD because NH$_3$ oxidation requires oxygen.
- Two groups of microbes are involved:
  - Ammonia oxidizing bacteria (AOB)
  - Nitrite oxidizing bacteria (NOB)
- AOB oxidize NH$_3$ to NO$_2^-$ in two steps:
  - Ammonia monoxygenase (AMO)
  - Hydroxylamine oxidoreductase (HAO)
- NOB oxidize NO$_2^-$ to NO$_3^-$
  - Uses the Nor enzyme complex
- Both AOB and NOB respire oxygen

Nitrification Enzymes

- AMO = converts ammonia to hydroxylamine (toxic)
- HOA = converts toxic hydroxylamine to nitrite
- NOR = nitrite oxidoreductase

Ammonia Oxidizing Bacteria (AOB):
- (A, B) Nitrosomonas
- (C, D) Nitrosolobus

Nitrite Oxidizing Bacteria (NOB):
- (E, F) Nitrospira
- (G, H) Nitrococcus
Sewage treatment and environmental monitoring

- Monitoring effluents and the surrounding environment is important (Why?):
  - Asses the efficacy of the treatment process
  - C, N, P, metals, microbes, effluent toxicity
- It is often too difficult to directly monitor a specific pathogen or virus/phage (Why?).
- Instead, monitoring is usually done for indicator organisms.

What is an indicator organism?

- An organism that can be readily cultured that indicates the presence of a pathogenic microorganism or correlates to a health problem.
- Five criteria for an indicator organism:
  - Consistently present in feces and at higher concentrations than pathogens.
  - Should not multiply outside the human intestinal tract.
  - Should be as resistant or more resistant than the pathogen to environmental conditions and to disinfection.
  - Easy to assay (culture and quantify) and differentiate from other organisms.
  - Environmental concentrations should correlate with pathogens or measurable health hazards.

### Common indicator bacteria

- **Coliforms:**
  - Facultatively aerobic, gram-negative, nonspore-forming, rod-shaped bacteria; ferment lactose with gas formation at 35°C within 48 hrs.
  - Usually enteric bacterial group (E. coli, Klebsiella, Citrobacter, Enterobacter, Serratia, Yersinia)
  - Poor indicator: often found outside of the intestinal tract
- **Fecal coliforms**
  - Thermotolerant coliforms (44.5°C), 20% of total coliforms.

### Monitoring indicators is done using culturing techniques and selective media.

<table>
<thead>
<tr>
<th>Test medium type</th>
<th>Ideal incubation temperature (°C)</th>
<th>Typical colony color, size, and morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total coliforms (TPC)</td>
<td>35 or 45</td>
<td>Colonies are round, raised, and smooth; 1 to 4 mm in diameter, and not with a golden-green metallic sheen.</td>
</tr>
<tr>
<td>Total coliforms (NC)</td>
<td>35 or 45</td>
<td>Colonies are non-lactose-fermenting, round, and smooth; 1 to 4 mm in diameter, and not with a golden-green metallic sheen.</td>
</tr>
<tr>
<td>Escherichia coli (E. coli)</td>
<td>35 or 45</td>
<td>Colonies are non-lactose-fermenting, round, and smooth; 1 to 4 mm in diameter, and not with a golden-green metallic sheen.</td>
</tr>
<tr>
<td>Escherichia coli (H+I)</td>
<td>35 or 45</td>
<td>Colonies are non-lactose-fermenting, round, and smooth; 1 to 4 mm in diameter, and not with a golden-green metallic sheen.</td>
</tr>
<tr>
<td>Escherichia coli (MD)</td>
<td>35 or 45</td>
<td>Colonies are non-lactose-fermenting, round, and smooth; 1 to 4 mm in diameter, and not with a golden-green metallic sheen.</td>
</tr>
<tr>
<td>Fecal coliforms (FC)</td>
<td>35 or 45</td>
<td>Colonies are round, raised, and smooth; 1 to 4 mm in diameter, and not with a golden-green metallic sheen.</td>
</tr>
<tr>
<td>Fecal streptococci (FS)</td>
<td>35 or 45</td>
<td>Colonies are round, raised, and smooth; 1 to 4 mm in diameter, and not with a golden-green metallic sheen.</td>
</tr>
<tr>
<td>Staphylococcus (SAL)</td>
<td>35 or 45</td>
<td>Colonies are round, raised, and smooth; 1 to 4 mm in diameter, and not with a golden-green metallic sheen.</td>
</tr>
<tr>
<td>Salmonella (SAL)</td>
<td>35 or 45</td>
<td>Colonies are round, raised, and smooth; 1 to 4 mm in diameter, and not with a golden-green metallic sheen.</td>
</tr>
<tr>
<td>Clostridium (PCR)</td>
<td>35 or 45</td>
<td>Colonies are round, raised, and smooth; 1 to 4 mm in diameter, and not with a golden-green metallic sheen.</td>
</tr>
</tbody>
</table>
**Most probable number (MPN) analysis for quantifying coliforms**

- Quantifies total coliform bacteria in water samples by three sub-tests
  - Presumptive, Confirmed, and Completed
- The MPN is used to monitor waste effluents, drinking water systems, and recreational waters.
- High MPN results can lead to beach closures.
- In a drinking water system, a positive for coliform is a HUGE deal. This would set off a series of events to find the source of coliform contamination.
  - Would you want to be drinking water with coliforms in it?

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### MPN: Presumptive Test

- Determines presence of coliforms (gas producers).
- Serial dilution of replicate lactose broth tubes

**Growth:**
- Yes
- No

**Fermentation:**
- Yes
- Gas
- No

<table>
<thead>
<tr>
<th>Volume of Dilution Added</th>
<th>Culture Results</th>
<th>Number of Positive Tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 ml</td>
<td>+ + + + +</td>
<td>5</td>
</tr>
<tr>
<td>1 ml</td>
<td>+ + +</td>
<td>2</td>
</tr>
<tr>
<td>0.1 ml</td>
<td>+ + + + +</td>
<td>0</td>
</tr>
</tbody>
</table>

**Water sample inoculation volume**

- 10 ml/tube
- 1 ml/tube
- 1:10 Dilution: 1 ml/tube
- 1:100 Dilution: 1 ml/tube

**Replicate tubes**

- Dilution: $10^3$, $10^4$, $10^5$
- Gas-positive tubes: 5, 5, 3, 1
- MPN Index (bacterial cells/100 ml):
  - $10^3$: 5
  - $10^4$: 5
  - $10^5$: 3

**Lauryle tryptose (controls lactose broth)**

Analysis: Gas-positive tubes after 24 to 48 hours of incubation give an MPN index. In this example, there are 1,100 coliform bacteria per 100 ml of the water sample.

**MPN index reports data as per 100 ml**
MPN: Confirmed Test

- Part two of the MPN test
- Positive tubes in the presumptive test are inoculated into brilliant green lactose bile broth (BGLB)
  - These tubes will confirm acid and gas production (fermentation end products).
  - Bile will inhibit gram positive organisms

Example MPN problem:
Glucose fermenters in lake water:

<table>
<thead>
<tr>
<th>Dilution of lake water:</th>
<th>1st dilution (10^-1)</th>
<th>2nd dilution (10^-2)</th>
<th>3rd dilution (10^-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount inoculated into each of three tubes of glucose fermentation broth</td>
<td>1 ml</td>
<td>0.1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Set of tubes (designations used below)</td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td># of tubes showing growth</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td># of tubes showing acid production</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

- Choose numbers with three consecutive sets of "dilution to extinction" for sugar fermenting organisms (tubes: C, D, E)
- The MPN table for 3-1-0 is 0.43 organisms (next slide).
- 0.43 organisms were inoculated into the middle tube (D).
- Use the dilution to calculate the MPN per ml
- Tube D had 0.1 ml of a 10^-3 dilution of lake water.
- Now calculate the MPN in the original sample:
  0.43 organisms X 10^3 = 0.1 ml = 4.3 x 10^3 glucose fermenters per ml
  The MPN index would be 4.3 x 10^3 per ml = 100 x 43 organisms per 100 ml

MPN: Completed Test

- Positive tubes from the confirmed test are analyzed by streak plating on eosin methylene blue (EMB) plates.
  - Incubated at 35°C for 24-48 hr
  - Only coliforms will turn dark with a metallic green sheen
  - Gram + inhibited by eosin/MB
- Coliform colonies are gram stained and to verify that they are gram negative and non-spore forming (see definition of coliforms).
Molecular techniques for monitoring indicators

✓ Molecular methods can be very sensitive.
  - In theory, one gene copy can be detected via PCR.
✓ Molecular methods can be rapid: <1 day for results.
✓ Instead of growing organisms the goal is to track the genetic signature of pathogens in environmental samples.
✓ DNA is extracted from a sample and PCR is usually done to detect some genetic marker that is only present in a particular pathogen or virus.
✓ What gene target would you go after?

Example: detecting toxic E. coli by PCR

✓ Shiga toxigenic Escherichia coli (STEC) cause deadly gastrointestinal disease in humans: e.g. O157 types.
✓ Here are some detectable virulence factors:
  - The hlyA gene for Enterohemolysin E. coli (EHEC)
  - The eaeA gene for intimin, encodes attaching and effacing (A/E) protein
  - The stx1 and stx2 genes, Shiga type 1 and 2 toxins; type 1 is more deadly
✓ Use multiplex PCR, which allows detection of multiple genes in one PCR.

Lecture summary

✓ Because of their diverse capabilities to degrade organic material, microorganisms are exploited for the treatment of wastewater (sewage).
✓ Secondary wastewater treatment relies on the power of microorganisms to breakdown solid waste into smaller molecules that other microbes can convert into nutrients.
✓ Nutrients are further converted either by nitrifying bacteria into nitrate and then denitrifiers convert the nitrate to N₂ gas.
✓ Small organic acids are eventually converted into methane gas by methanogenic archaea.
✓ The effluent released into the environment should have a low BOD and low numbers of pathogens.
✓ Monitoring is done to quantify indicators of pathogens and BOD to make sure the environment is safe for humans and wildlife.