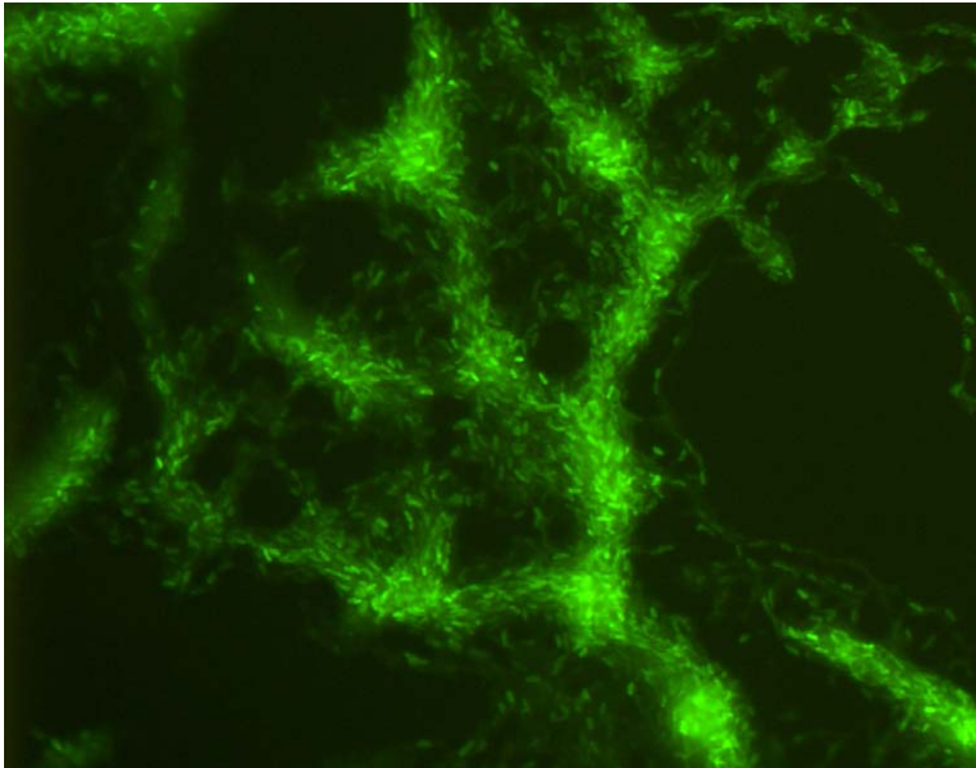
Welcome to
Environmental Microbiology

BIO 369L



Fall 2007

Dr. Egbert Schwartz

GTA
Camille E. Naaktgeboren

Table of Contents

	Page
Syllabus	i-iii
Laboratory Rules	iv
Laboratory Protocol and Safety	v-vi
Waste Disposal	vii
Laboratory Materials	viii
Environmental Microbiology Laboratory Overview	1
Week 1 – Overview, Goals for Class, Lab Safety and Protocol, Tour Rio de Flag Reclamation Facility	2
Week 2 – Microscopy, Microbial Counts: Heterotrophs and Coliforms	3-7
Week 3 – Enrichments: Toluene Degraders and Denitrifiers, MPN	8-12
Week 4 – Toluene Enrichment and Isolation Continued	13
Week 5 – Enzyme Activity Probes, Visualization of Viruses, Toluene Enrichment and Isolation Continued	14-16
Week 6 – Toluene Isolate: DNA Extraction and Gel, Microscopy, Enzyme Activity Probes, Discuss PCR Protocol	17-22
Week 7 – PCR Reaction for 16S rDNA, PCR Amplicon Gel	23
Week 8 – Cloning PCR Products, Ampicillin Experiment	24-27
Week 9 – Cloning Analysis	28-30
Weeks 10 & 11 – Bioremediation Experiment	31
Week 12 – Tour EnGGen Facility, Analyze DNA Sequences	32-36
Appendix – Media Recipes	37-41
Bibliography	42

Environmental Microbiology Laboratory
BIO 369L
Fall 2006

Syllabus

Instructors

Dr. Egbert Schwartz
Office: 250 Biology Building
Phone: 523-6168
Egbert.Schwartz@nau.edu

GTA

Camille Naaktgeboren
Camille.Naaktgeboren@nau.edu

Goals for Laboratory

The goal of this laboratory is to provide students with knowledge and techniques used in environmental microbiology.

We designed this laboratory experience to complement the lecture component of the course, but with special emphasis on water quality as a platform for exploring a wide variety of cutting edge techniques used in environmental microbiology. We will collect non-treated and treated samples from the Rio de Flag Reclamation Facility; these samples will provide the material with which we will assess microbiological water quality parameters, enrichment principles, functional activity measurements, and molecular analysis.

Assessment

Grading will be based on active participation in laboratory exercises and discussions (25%), a final exam (50%), and a final project (25%).

Professional Conduct

THIS IS TO BE CONSIDERED A PROFESSIONAL LABORATORY AT ALL TIMES. In the laboratory, you may work with opportunistic pathogens and you need to handle all cultures with extreme caution to avoid exposing yourself and others to potentially disease-causing organisms. Laboratory Rules, Protocol, and Safety are on pages iv-vi. These rules must be followed at all times in the lab. However, emphasis is placed on the following:

1. No eating or drinking in the lab!
2. Wash your hands at the beginning and end of each lab.

3. Wipe lab bench with 3% Osyl at the beginning and end of each lab.
4. A lab coat or an old button-front shirt is required at all times. This is not so much to protect your clothing from stains as it is to facilitate the easy removal and sterilization that would be necessary should any culture be spilled on you, as well as to prevent removal of organisms outside the lab.
5. Extreme caution should be used at all times when you are handling microorganisms. ALWAYS USE STERILE TECHNIQUE.
6. No mouth-pipetting!

NOTE: Continual careless behavior or any behavior deemed dangerous or inappropriate by the lab instructor will result in **expulsion from both the lab and the lecture**.

Academic Dishonesty

Cheating and/or plagiarism will result in appropriate disciplinary action such as a grade of "F" on that assignment or for the entire course.

Tentative Laboratory Schedule

(subject to modification as required)

Week	Date	Topic / Activity
	August 27	No laboratory sessions
	September 3	Labor Day Holiday – No laboratory sessions
1	September 10	Overview, Goals for Class, Lab Safety and Protocol Tour Rio de Flag Reclamation Facility
2	September 17	Microscopy Microbial Counts
3	September 24	Enrichments: Toluene Degraders and Denitrifiers Most Probable Number
4	October 1	Toluene Enrichment and Isolation Continued
5	October 8	Enzyme Activity Probes, Visualization of Viruses, Toluene Enrichment and Isolation Continued
6	October 15	Toluene Isolate: DNA Extraction and Gel, Microscopy, Enzyme Activity Probes, Discuss PCR Protocol

7	October 22	PCR Reaction for 16S rDNA PCR Amplicon Gel
8	October 29	Cloning PCR Products Ampicillin Experiment
9	November 5	Cloning Analysis
10	November 12	Bioremediation Experiment
11	November 19	Bioremediation Experiment Continued
12	November 26	Tour EnGGen Facility Analyze DNA Sequences
13	December 3	FINAL EXAM! Reading Week – Last Laboratory Session
	December 10	Finals Week – No Laboratory Sessions

Laboratory Rules

In this laboratory, students will be working with bacteria that can be pathogenic for humans and animals. The safety of each student is dependent upon the strict observance of laboratory rules. Exhibition of carelessness by a student that may endanger the well-being of others will result in the dismissal of that student from the class. The following rules MUST be observed:

1. **Always** wear a lab coat when working in the laboratory.
2. Eating, drinking, or smoking is not permitted in this laboratory.
3. Familiarize yourself completely with the details of each experiment before coming to the laboratory.
4. The microscope made available to you is a precise and delicate instrument that should be handled with care. If your microscope is not in good working condition, consult with your lab instructor.
5. LABEL EVERYTHING that needs to be labeled LEGIBLY with name, **date**, section, and experiment.
6. Flame-sterilize inoculating loops and needles BEFORE and AFTER using. Assume these instruments are contaminated until sterilized in the flame.
7. If your hands become contaminated with infectious material, rinse well with disinfectant solution followed by a thorough scrubbing with soap and water.
8. An occasional accident is inevitable. If the floor or table top is contaminated by infectious material, NOTIFY THE INSTRUCTOR.
9. In case of injury, notify the instructor **immediately.**
10. Not all bacteria on a fixed slide may be dead. Discard all used microscope slides in trays to be autoclaved. Do not try to clean and re-use them.
11. Place all contaminated materials to be sterilized in appropriately designated containers. Contaminated pipettes should be put into Nalgene buckets.
12. Put paper and other non-contaminated waste material into the wastebasket. Do not discard paper, cotton, or cover slips into the sinks.
13. Do not lay culture tubes on the bench top or lean them against a fixture. Place them securely in the racks provided for that purpose.
14. High-speed photography shows that applying bacterial suspensions onto slides with a loop, mixing bacteria with water on a slide preparatory to fixing, and staining all produce aerosols containing live bacteria.
15. Cultures must not be removed from the laboratory under any circumstances.
16. Coats and other materials (other than lab books) are not to be placed on the lab benches. They must be hung on hooks or placed on the shelf provided.
17. Please notify your lab instructor of an unavoidable absence BEFORE that lab.
18. YOU MUST WASH YOUR HANDS EACH TIME YOU ENTER OR LEAVE THE LABORATORY.

NOTE: Because of the nature of diagnostic microbiology, you will be expected to work outside of your lab class time. A culture may require 18, 24, 72, etc. hours of growth, which may necessitate examination of bacterial cultures at times other than regularly scheduled lab periods.

Laboratory Protocol & Safety

1. ***Following directions and preparation:*** It is critical for your safety and for the smooth operation of the laboratory that you follow all instructions issued by your lab instructor. Do not begin work until instructed to do so. Failure to follow directions will result in dismissal from the class (both lab and lecture). If this occurs after the deadline for withdrawal, you will receive a grade of “F” for the class. It is essential that the student read the laboratory exercise before attending the class. Since most laboratory sessions will require the full three hours to complete, it is important to arrive on time and prepared to work. Each student will keep notes from lectures given by your laboratory instructor and a written record of all data from your experiments. Blank sheets for notes and data are provided at the end of each exercise in your notebook.

2. ***Lab Coat:*** All students are required to wear a lab coat while in the lab, which should have at least ¾ sleeves. It does not have to be a formal, white lab garment. An old, lightweight, long-sleeved shirt will be adequate; but the garment must not be integral to your street clothes. Most of the stains that are used cannot be removed by laundering, and there may be situations that require the lab coat to be removed and autoclaved. Therefore, the lab coat needs to be a garment that you can put on and take off as you enter and leave the lab. Also, sandals or open-toed shoes will not be allowed in lab.

3. ***Disinfecting Lab Bench Tops:*** At the beginning and end of each lab period, the lab bench will be disinfected with a solution of 3% Osyl. This will be done by squirting a small amount of Osyl (red bottle in student bins) onto the tabletop and using a **wet** sponge to wipe the surface. The surface should then be allowed to air-dry. Sponges are located at each sink in the lab. Do not try to spread Osyl across the bench top with a dry sponge.

4. ***Hand Washing:*** At the beginning and end of each lab period, the student will wash his or her hands with an antimicrobial soap (Vironox-9).

5. ***Eating and Drinking:*** There will be no eating, drinking, gum-chewing, smoking, or any other hand-to-mouth activities (e.g. – applying lip balm, chewing on pens or pencils, etc.) while in the lab.

6. ***Long Hair:*** Students with long hair will be required to restrain it at the back of the head or in a fashion that will prevent it from coming into contact with the flame of a Bunsen burner.

7. ***Mouth-pipetting:*** Absolutely no mouth-pipetting of any liquid will be allowed. Use a pipette aid.

8. ***Labels:*** Do not label the caps (screw-cap or Morton cap) of culture tubes as they are difficult to clean and caps can easily be switched on tubes, which can lead confusion

9. ***Decontamination and waste disposal:*** Deposit used or contaminated petri plates and other contaminated plastic materials (except plastic pipettes) into the bins that hold autoclave bags. Do not place waste that is not meant to be autoclaved (e.g. – paper towels used to dry your hands) into these bins. Never throw anything contaminated into the garbage cans. Culture tubes and other contaminated materials must be autoclaved before they can be disposed of or recycled. Remove any tape from contaminated culture tubes before placing them in the racks designated for this purpose. Do not place contaminated culture tubes into the glass recycling bins. Discard glass and plastic pipettes into the Nalgene buckets that are located on the bench tops. Never put pipettes into the plate disposal bags as they can puncture the bags, which then leak during autoclaving. Decontaminate a spilled culture by placing paper towels over the spilled material and pouring Osyl onto the paper towels. Allow them to stand at least 15 minutes before cleaning up.

10. ***Broken Glass:*** Deposit broken glass (e.g. – broken slides, cover slips, glass pipettes, etc.) into the containers marked for broken glass. Never dispose of broken glass in the garbage cans or autoclave bags as this can present a significant hazard to the custodial staff.

11. ***Accidents:*** Report any accident to your laboratory instructor immediately. Make sure that you are familiar with the location of the emergency shower, eye-wash stations, fire extinguishers, chemical spill kits, and first aid kits.

Waste Disposal

Item	Disposal
Petri plates	Biohazard bag for petri plates at front of lab (leave lid on plate)
Plastic pipettes, pipette tips	Nalgene buckets on bench (tips down)
Plastic centrifuge tubes, Steriloops	Nalgene buckets on bench
Microscope slides, coverslips	Stainless steel trays on bench
Pasteur pipettes (glass)	Stainless steel trays on bench
Uncontaminated broken glass	Cardboard glass disposal box
Test tubes	Racks on trash cart at back of lab
Reusable flasks, bottles, etc.	Trash cart at back of lab
Ethidium bromide (EtBr) waste including gels and gloves	Biohazard bag for EtBr waste

Laboratory Materials

1. Each student will share a lab drawer with other students in other sections and classes who sit at the same assigned seat. Each lab drawer contains:

    -One inoculating loop
    -One inoculating needle
    -One grease pencil
    -One flint striker for lighting Bunsen burners
    -One pair of forceps
    -One packet of blotting paper
    -One packet of lens paper
    -One box of cover slips
    -One pair of safety glasses
    -One blue pipette pump (for 1ml and 2ml pipettes)
    -One green pipette pump (for 5ml and 10ml pipettes)
    -One laminated sheet with diagrams for staining procedures
    -One 6” ruler

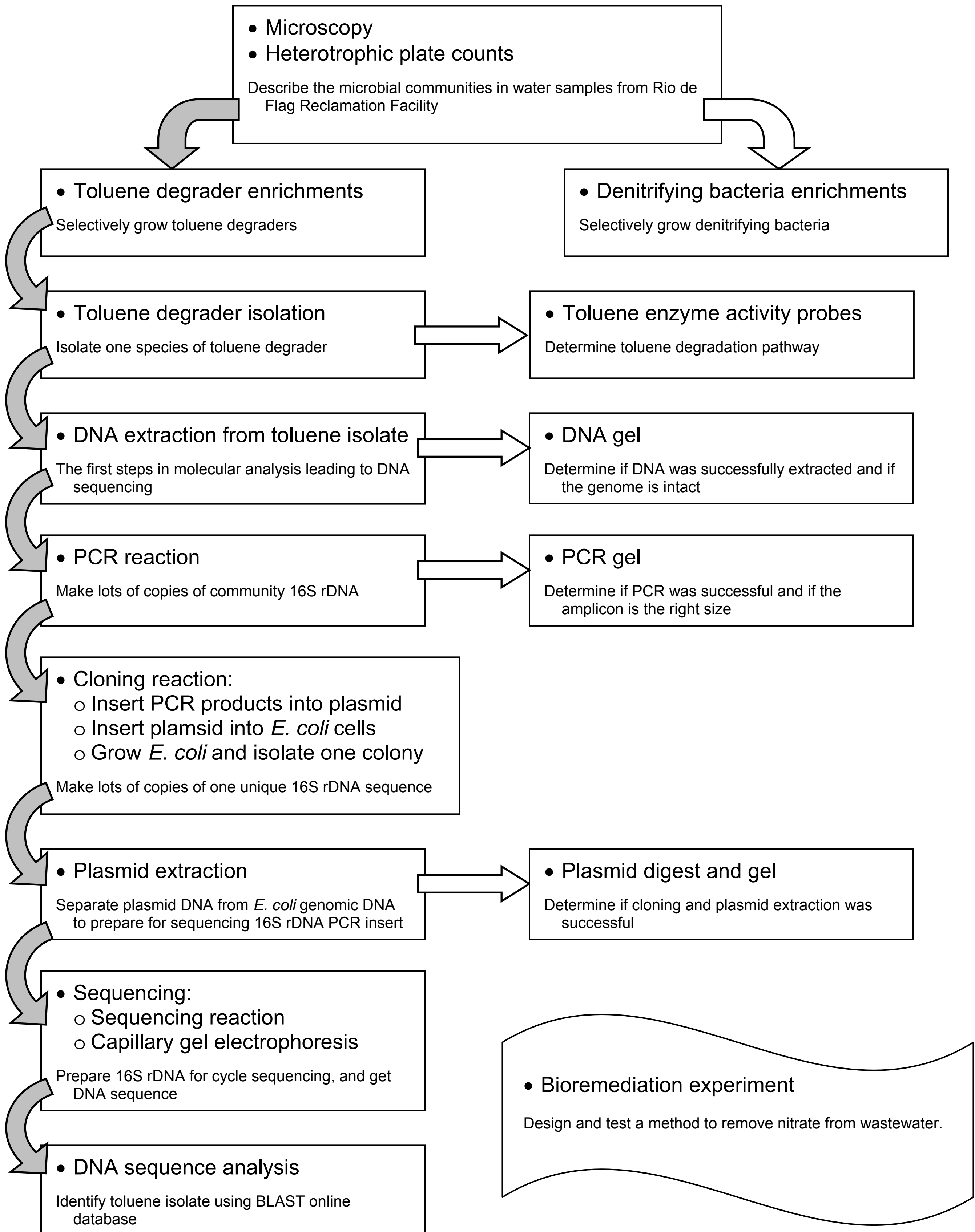
2. On top of each lab bench are:

    -Red squeeze bottles containing 3% Osyl (can be refilled)
    -Squeeze bottles containing 70% ethanol (refillable)
    -Squeeze bottles containing 95% ethanol
    -Squeeze bottles containing distilled water (refillable)
    -Sponges
    -Long, covered stainless steel or plastic trays for depositing contaminated slides and Pasteur pipettes
    -Nalgene buckets for disposal of plastic pipettes
    -Bunsen burners
    -Plastic bins containing stains, immersion oil, and lens cleaner
    -Vortex mixers

3. Each student will supply:
    -One Sharpie marking pen (fine-tip works best)
    -One lab coat

4. Gloves will also be provided at the front of the room (TA bench).

Environmental Microbiology Laboratory Overview



Week 1
Overview, Goals for Class, Lab Safety and Protocol
Tour Rio de Flag Reclamation Facility

Purpose

a) Understand goals and expectations for the lab.

b) Understand the main wastewater treatment steps at Rio de Flag Reclamation Facility.

Background

Main treatment steps at Rio de Flag Reclamation Facility:

1) Primary screen – removes larger solids (larger solids are disposed in the landfill)
2) Primary clarifier – removes smaller solids (smaller solids go to Wildcat Facility for anaerobic treatment)
3) Aeration – removes biological oxygen demand and pathogens – this is the big party
4) Secondary clarifier – removes floc
5) Anaerobic treatment – reduces nitrate concentration
6) Sand and carbon filter – removes fine particles
7) UV irradiation – kills bacteria and viruses
8) Chlorination – kills bacteria and viruses

Note:
Wetlands receive non-chlorinated water.
Reclaimed water for irrigation is chlorinated.

Week 2
Microscopy
Microbial Counts

Purpose

Describe microbial communities at 3 steps along the wastewater treatment process:
Influent – incoming sewage after removal of larger solids
Mixed liquor – the big party leaving the aeration chamber
Effluent – after UV sterilization

Background

Drinking water standards vs. reclaimed water standards
Arizona complies with the maximum contaminant levels established by the EPA.
(Arizona Department of Environmental Quality 2005)

Drinking water:
Total heterotrophs < 500 cfu/mL
Fecal coliforms = 0
Enteric viruses = 99.99% removal/inactivation
(Environmental Protection Agency 2005)

Reclaimed water:
Class A = okay for human contact
Classes B and C = no human contact
(Environmental Protection Agency 2005)

Microscope Care and Use

Reminders:

a) Raise the condenser to focus light on your sample.
b) When using phase contrast, check that the setting on the condenser dial matches the objective lens. Otherwise, 0 = bright field and DK = dark field.
c) First, find the field of focus with the 10x objective. (Do not lower the stage.) Then swing the 40x objective into place and focus with the fine adjustment knob only. Repeat for 100x.
d) Use immersion oil only on the 100x objective.
e) Clean eyepieces and objectives with lens tissue only.

Microscope cleanup:
a) Eyepieces cleaned and pushed together
b) Objectives cleaned (particularly 100x) and in proper position
c) Stage cleaned and lowered
d) Condenser cleaned and lowered
e) Stain splatters cleaned
f) Rheostat set at 1 and power switch off
g) Power cord wrapped around hanger
h) Dust cover on
i) Microscope placed on correct shelf (matching lab bench number) in correct position

Staining Techniques

(See staining techniques sheet in lab drawers, too.)

Wet Mount
1) Place 1 or 2 drops of sample on a microscope slide.
2) Place a coverslip over the sample.
3) View with phase contrast or dark field.

Simple Stain
1) Prepare a dry mount:
    1a) Microbes from a liquid culture: spread on slide –OR–
    1b) Microbes from a plate: mix cells in a drop of distilled water, spread on slide
    1c) Air dry
2) Gently heat fix slide in flame.
3) Flood with Methylene Blue for 1 minute. Rinse.
4) Blot dry and view with bright field.

Gram Stain
1) Prepare a dry mount.
2) Gently heat fix slide in flame.
3) Flood with Crystal Violet for 1 minute. Rinse.
4) Flood with Gram's Iodine for 1 minute. Rinse.
5) Decolorize with ethanol – 3 seconds with 95%, longer with 70%. Rinse.
6) Flood with Safranin for 2 minutes. Rinse.
7) Blot dry and view with bright field.

    Gram-positive = purple or blue
    Gram-negative = pink or red

Total Heterotroph Counts

Heterotrophs will grow on nutrient agar (NA) plates. Prepare serial dilutions of mixed liquor and effluent water samples to be sure that some of the plates have countable colonies (30-300 colonies).

Make spread plates in the following dilutions.

Mixed liquor – 10^{-3}, 10^{-6}, 10^{-9}
Effluent – 10^{-1}

Influent plates will be prepared for comparison. Influent plate dilutions: 10^{-9}, 10^{-12}, 10^{-15}.

<u>Serial dilutions</u>

1) Label test tubes: 10^{-1}, 10^{-2}, 10^{-3}, etc.
2) Add 4.5 mL saline (0.75% NaCl) to test tubes.
3) Transfer 0.5 mL of the water sample into the 10^{-1} tube. Mix.
4) Transfer 0.5 mL from the 10^{-1} tube into the 10^{-2} tube. Mix.
5) Transfer 0.5 mL from the 10^{-2} tube into the 10^{-3} tube, etc.

<u>Spread plates</u>

1) Label NA plates: name or initials, date, water sample, plate dilution.
2) Add 0.1 mL of the water sample or appropriate dilution to each plate. (This is a 1:10 dilution step, therefore plating 0.1 mL of the 10^{-2} dilution results in a 10^{-3} plate.)
3) Dip hockey stick in ethanol and flame sterilize. Cool hockey stick.
4) Spread sample on the plate.
5) Incubate at room temperature.

Count colonies next week.

Results

<u>a) Microscopy</u>

Do you expect to see different microbial communities in the influent, mixed liquor, and effluent water samples?

Describe the microbial community in the influent.

Describe the microbial community in the mixed liquor.

Describe the microbial community in the effluent.

b) Total Heterotrophs

Which water sample do you expect to have the greatest number of heterotrophs?

Do you expect to have any heterotrophs in the effluent sample?

Record class data:
- How many heterotrophs are in the influent sample?
- How many heterotrophs are in the mixed liquor sample?
- How many heterotrophs are in the effluent sample?

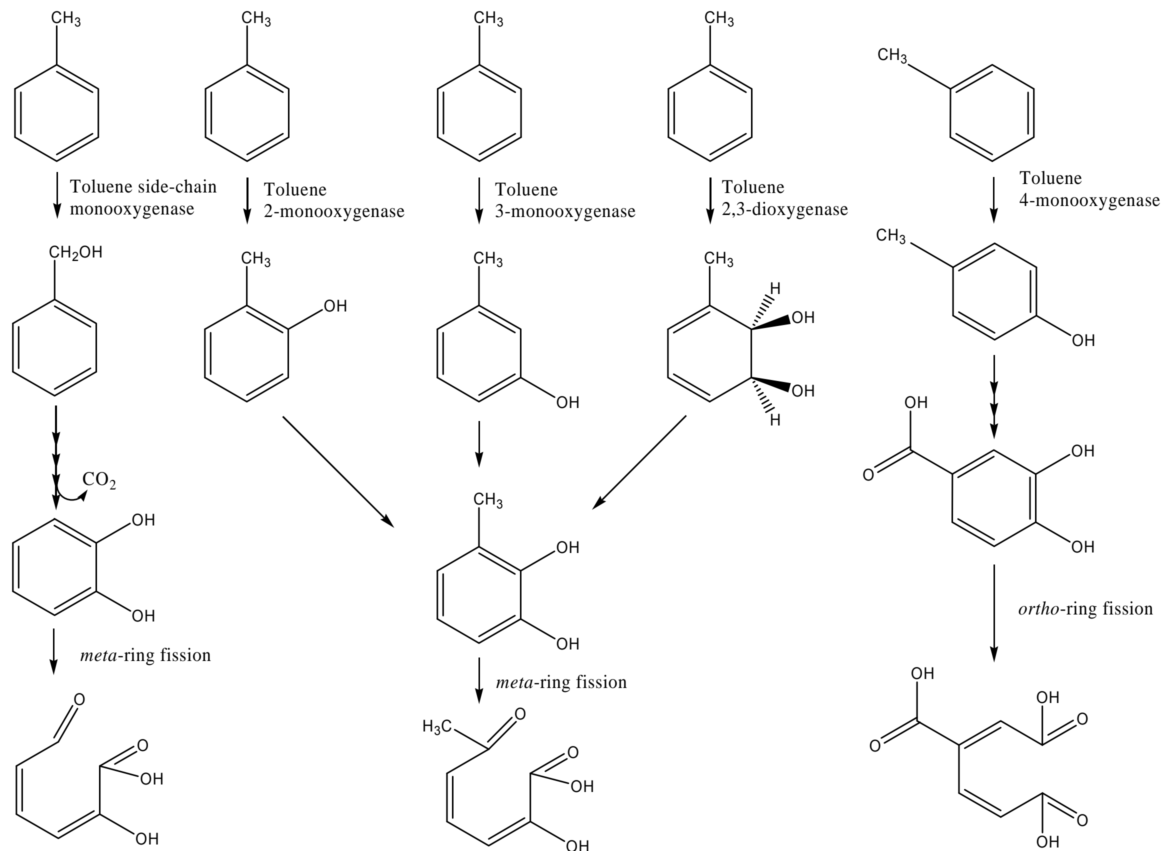
Week 3
Enrichments: Toluene Degraders and Denitrifiers
Most Probable Number (MPN)

Purpose

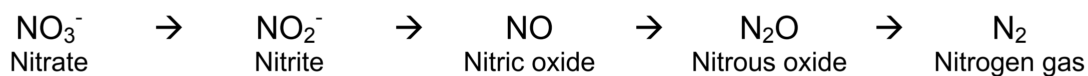
a) Selectively grow microbes that degrade toluene.
b) Selectively grow denitrifying bacteria.
c) Estimate the number of toluene degraders and denitrifiers in Rid de Flag water samples based upon MPN.

Background

Toluene degradation pathways



Denitrification



Enrichment for Toluene Degrading Bacteria

Toluene degrading bacteria can use toluene as a sole carbon and energy source.

1) Inoculate a Wheaton bottle of toluene minimal medium with 1mL of mixed liquor.
2) Inoculate another bottle with effluent.
3) In the fume hood, add 40μL of toluene into the test tube in each bottle. Tighten caps.
4) Incubate at room temperature with shaking.

During the week, observe toluene enrichments for growth. Transfer 1mL of turbid toluene enrichment into fresh toluene minimal medium and add toluene to the test tube. Transfer at least twice before the next lab.

Toluene enrichments will be prepared with influent for comparison.

Enrichment for Denitrifying Bacteria

Denitrifying bacteria can use nitrate (or other oxidized forms of nitrogen) as a terminal electron acceptor when oxygen is not available.

1) Inoculate a tube of denitrifying minimal medium with several drops of mixed liquor until the tube is filled to the top.
2) Inoculate another tube with effluent.
3) Tighten caps.
4) Incubate at room temperature for a week.

5) Next week, observe denitrifying enrichments for growth and add nitrite reagents (steps 6 and 7).

6) Add nitrite reagents to determine if NO_3^- was reduced to NO_2^-.
    a) Transfer 0.5 mL of the enrichment culture to a clean test tube.
    b) Add an equal number of drops of nitrite reagents A and B (about 5 drops).
    c) Incubate at room temperature for 15 minutes.
    d) Color change to red indicates reduction of NO_3^- to NO_2^-.
        RED = POSITIVE. DO NOT ADD ZINC.
    e) If no color change after 15 minutes, continue on to step 7.

7) Add zinc powder to determine if NO_3^- was reduced to nitrogen gases.
    a) Add a few grains of zinc.
    b) Incubate at room temperature for 15 minutes.
    c) No color change indicates reduction of NO_3^- to nitrogen gases.
    d) Color change to red indicates no reduction of NO_3^-.
        RED = NEGATIVE
    Note: Zinc is a heavy metal and must be disposed appropriately.

Denitrifying enrichments will be prepared with influent for comparison.

Most Probable Number (MPN)

MPN is determined based upon the presence or absence of microbes in increasingly diluted concentrations. The most dilute concentration of cells able to grow is assumed to be 10 cells (Madigan et al. 2003). MPN may be based upon visible growth (such as turbid liquid cultures) or other measurable characteristics of growth (such as the reduction of nitrate to nitrite).

<u>MPN – Toluene Degrading Bacteria</u>

1) Prepare serial dilutions of water samples: 10^{-2} through 10^{-6}.
2) Inoculate 5 Wheaton bottles of toluene minimal medium with 100 μL of the 10^{-2} dilution (final dilution = 10^{-3}).
3) Repeat for each of the remaining dilutions (final dilutions = 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}).
4) Add 40μL of toluene into the test tube in each bottle.
5) Incubate at room temperature with shaking for 2 weeks.

6) Observe each bottle for growth (turbidity), and score each bottle as positive or negative for growth.

7) Calculate the most probable number of toluene degraders in the original sample. (See sample MPN results on page 12.)
    a) Choose P_1 = the greatest number of positive tubes in the greatest dilution.
    b) P_2 and P_3 = the number of positive tubes for the next two greater dilutions.
    c) Use the MPN table (see page 11) to find the most probable number.
    d) Multiply by the dilution factor for P_2 to determine the number in the original sample.

<u>MPN – Denitrifying Bacteria</u>

1) Prepare serial dilutions of water samples: 10^{-2} through 10^{-6}.
2) Inoculate 5 tubes of denitrifying minimal media with 100 μL of each dilution (final dilutions = 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}).
3) Incubate at room temperature for 2 weeks.

4) Test for the presence of NO_3^-.
    a) Add nitrite reagents A and B.
        RED = POSITIVE, no color change = negative.
    b) If no color change after 15 minutes, add zinc powder.
        RED = NEGATIVE, no color change = positive.
    c) Score each tube as positive or negative for nitrate reduction.

6) Calculate the most probable number of denitrifiers in the original sample.
MPN Table

P_1 Value	P_2 Value	P_3 Value					
		0	1	2	3	4	5
0	0	-	0.018	0.036	0.054	0.072	0.090
0	1	0.018	0.036	0.055	0.073	0.091	0.110
0	2	0.037	0.055	0.074	0.092	0.110	0.130
0	3	0.056	0.074	0.093	0.110	0.130	0.150
0	4	0.075	0.094	0.110	0.130	0.150	0.170
0	5	0.094	0.110	0.130	0.150	0.170	0.190
1	0	0.020	0.040	0.060	0.080	0.100	0.120
1	1	0.040	0.061	0.081	0.100	0.120	0.140
1	2	0.061	0.082	0.100	0.120	0.150	0.170
1	3	0.083	0.100	0.130	0.150	0.170	0.190
1	4	0.110	0.130	0.150	0.170	0.190	0.220
1	5	0.130	0.160	0.170	0.190	0.220	0.240
2	0	0.045	0.068	0.091	0.120	0.140	0.160
2	1	0.068	0.092	0.120	0.140	0.170	0.190
2	2	0.093	0.120	0.140	0.170	0.190	0.220
2	3	0.120	0.140	0.170	0.200	0.220	0.250
2	4	0.150	0.170	0.200	0.230	0.250	0.280
2	5	0.170	0.200	0.230	0.260	0.290	0.320
3	0	0.078	0.110	0.130	0.160	0.200	0.230
3	1	0.110	0.140	0.170	0.200	0.230	0.270
3	2	0.140	0.170	0.200	0.240	0.270	0.310
3	3	0.170	0.210	0.240	0.280	0.310	0.350
3	4	0.210	0.240	0.280	0.320	0.360	0.400
3	5	0.250	0.290	0.320	0.370	0.410	0.450
4	0	0.130	0.170	0.210	0.250	0.300	0.360
4	1	0.170	0.210	0.260	0.310	0.360	0.420
4	2	0.220	0.260	0.320	0.380	0.440	0.500
4	3	0.270	0.330	0.390	0.450	0.520	0.590
4	4	0.340	0.400	0.470	0.540	0.620	0.690
4	5	0.410	0.480	0.560	0.640	0.720	0.810
5	0	0.230	0.310	0.430	0.580	0.760	0.950
5	1	0.330	0.460	0.640	0.840	1.100	1.300
5	2	0.490	0.700	0.950	1.200	1.500	1.800
5	3	0.790	1.100	1.400	1.800	2.100	2.500
5	4	1.300	1.700	2.200	2.800	3.500	4.300
5	5	2.400	3.500	5.400	9.200	16.000	-

Sample MPN Results

Dilution	Results	P_1, P_2, P_3	MPN
10^{-3}	5 pos/0 neg		
10^{-4}	5 pos/0 neg	$P_1 = 5$	
10^{-5}	3 pos/2 neg	$P_2 = 3$	1.4×10^5 microbes per mL
10^{-6}	2 pos/3 neg	$P_3 = 2$	
10^{-7}	0 pos/5 neg		

Results

a) Toluene Enrichment

Do you expect toluene degraders to be present in Rio de Flag water samples? (Think about growth requirements.)

b) Denitrifying Bacteria Enrichment

Do you expect denitrifying bacteria to be present in Rio de Flag water samples? (Again, think about growth requirements.)

c) MPN Counts

Record data:

How many toluene degraders are present in Rio de Flag water samples?

How many denitrifying bacteria are present in Rio de Flag water samples?

How accurate do you think these estimates are? Can you think of other ways to quantify toluene degraders and denitrifying bacteria?

Week 4
Toluene Enrichment and Isolation Continued

Purpose
Isolate one species of toluene degrading bacteria.

Isolation of a Toluene Degrader
Prepare spread plates of toluene enrichment cultures with final plate dilutions of 10^{-2}, 10^{-4}, and 10^{-6}. (Refer to page 5 for serial dilution procedure and spread plate procedure.)

Results
a) Isolation

Record results.

<u>Week 5</u>
Enzyme Activity Probes
Visualization of Viruses
Toluene Enrichment and Isolation Continued

Purpose

a) Understand how enzyme activity probes work and what these probes test.

b) Understand how viral concentrations change through the wastewater treatment process.

c) Isolate one species of toluene degrading bacteria. Grow a plate culture of the isolate to test enzyme activity probes, and grow a liquid culture of the isolate for DNA extractions.

Enzyme Activity Probes

Observe prepared plates of toluene degraders. Compare enzyme activity probe results with the table below.

Pathway / Probe	**PA**	**3HPA**	**cinn**	**3ET**
Toluene 2-monooxygenase	Yellow	Yellow	None	None
Toluene 3-monooxygenase	Yellow	Yellow	None	None
Toluene 4-monooxygenase	None	None	None	None
Toluene 2,3-dioxygenase	Yellow	Yellow	Orange	None
Toluene side-chain-monooxygenase	None	None	None	Yellow

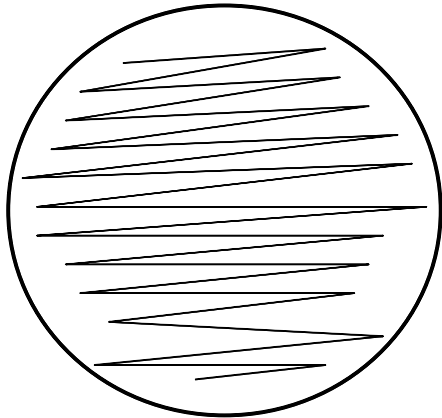
<u>Toluene enzyme activity probes:</u>
PA = phenylacetylene
3HPA = 3-hydroxyphenylacetylene
cinn = *trans*-cinnamonitrile
3ET = 3-ethynyltoluene

Visualization of Viruses

View prepared slides (YOPRO stain to visualize viruses) of influent, mixed liquor, effluent, tap water, and bottled water via epifluorescent microscopy.

Toluene Isolate Cultures

Chose one colony from your spread plates to inoculate both a plate and liquid media. Streak across the entire surface of the plate:



Add 40µL of toluene into the test tube in each bottle, and incubate at room temperature with shaking. Incubate plates at room temperature in a desiccator with an open tube containing 100 µL of toluene.

Results

<u>a) Enzyme Activity Probes</u>

What kind of information do you get from enzyme activity probes?

How is this information different from the information you get from molecular analysis (such as PCR amplification of toluene degradation genes)?

b) Visualization of Viruses

Do you expect viruses to be eliminated by the time reclaimed water leaves the treatment plant? (Think about both enteric viruses and bacteriophage.)

c) Toluene Isolate Cultures

Why will you use one colony to inoculate both a Wheaton bottle and a plate? Why not use two colonies?

Week 6
Toluene Isolate:
DNA Extraction and Gel, Microscopy, Enzyme Activity Probes
Discuss Polymerase Chain Reaction (PCR) Protocol

Purpose

a) Extract DNA from toluene isolate culture. Visualize extracted DNA via gel electrophoresis.
b) View toluene isolate via microscopy.
c) Use enzyme activity probes to determine toluene degradation pathway of toluene isolate.
d) Preview PCR protocol.

Protocol for DNA Extraction from Isolate (adapted from MoBio Laboratories, Inc., UltraClean Microbial DNA Isolation Kit, Catalog #12224-50)

Be sure to wear gloves during this procedure. If you are not comfortable using the micropipettes, please ask for help!

1. Add 1.5 ml of your culture to a microcentrifuge tube labeled “1” and spin in the centrifuge at full speed for 5 minutes.

2. Dump the supernatant into a waste beaker, spin the tube again for 30 seconds, and use the 200 μl pipetter to remove any supernatant left in the tube.
The growth media is removed from the cells. Media can contain high concentrations of salt, which can interfere with the DNA extraction procedure.

3. Resuspend the cells in 150 μl of microbead solution from the tube labeled “MB”. Flick the tube with your finger to get the cells back in solution. Transfer the suspension to the microbead tube labeled “B”.
The microbead tube contains glass beads, which help break up the cells.

4. Add 25 μl of MD1 solution and mix by turning the tube upside down several times.
Solution MD1 contains SDS, a detergent that dissolves cell membranes.

5. Incubate the tube at 65˚C for 10 minutes.
The detergent is more efficient at hotter temperatures, lysing a greater fraction of bacterial cells.

6. Vortex the tube for 2 minutes.
Cells that were not lysed by the detergent are mechanically disrupted by the glass beads.

7. Centrifuge tubes at full speed for one minute.
Cell debris and glass beads, which are large and heavy, form a pellet on the bottom of the tube. DNA stays in solution, also called the supernatant.

8. Transfer 150 µl of supernatant to a new tube labeled “2”.

9. Add 50 µl of solution MD2, vortex for 5 seconds, and incubate on ice for 5 minutes.
MD2 contains a protein precipitation reagent, which helps remove proteins from the DNA in solution.

10. Centrifuge the tubes for one minute at full speed.
Proteins form a pellet on the bottom of the tube, and the DNA is still in solution.

11. Avoiding the pellet transfer 150 µl to a new tube labeled “3”.

12. Add 450 µl of solution MD3 and vortex for 5 seconds.
MD3 is a salt solution that promotes DNA to bind to silica. The silica is present in the spin filter of tube 4.

13. Load the contents of tube 3 onto the spin filter of tube 4, centrifuge at full speed for 30 seconds. Discard the flow through liquid into the waste beaker.
The DNA is now bound to the spin filter and the salt is in the flow through solution.

14. Add 150 µl of solution MD4 to the spin filter and spin in the centrifuge at full speed for 30 seconds. Discard the flow through liquid into the waste beaker.
MD4 is an ethanol based wash solution to further clean the DNA. DNA is not soluble in ethanol so ethanol will not wash the DNA out of the spin filter.

15. Centrifuge the empty spin filter again at full speed for 1 minute.
Centrifuge the spin filter again to be sure that all of the ethanol is removed from the spin filter. Ethanol can interfere with subsequent PCR reactions.

16. Place the spin filter into a new tube labeled “5”.

17. Add 50 µl of solution MD5 to the center of the membrane in the spin-filter.
MD5 is a sterile buffer. DNA is soluble in the buffer. Because all the salts are washed out, the DNA can disassociate from the silica and dissolve in the buffer.

18. Centrifuge at full speed for 30 seconds.
The buffer and the DNA are released from the spin filter and are at the bottom of the tube.

19. Take the spin filter out of the tube and discard.
The spin filter does not contain any DNA and can be thrown away.

20. The DNA is in the tube and ready for PCR reactions.

Protocol for DNA Gel

1) Prepare 1% agarose, 1xTBE gel. (See recipe below.)
2) Align gel properly – negatively charged DNA moves toward the positive charge (black to red).
3) Load 2 µL loading dye and 10 µL extracted DNA into a well.
4) Load one well in each gel with 2 µL Lambda Hind III marker.
5) Run gel at 100 V for 20 min.
6) Stain and destain gel.
7) Visualize DNA.

NOTE: Discard gloves after contact with ethidium bromide stain (into ethidium bromide contaminated waste).

1% Agarose, 1xTBE Gel
27 mL distilled H_2O
3 mL 10xTBE buffer
0.3 g agarose

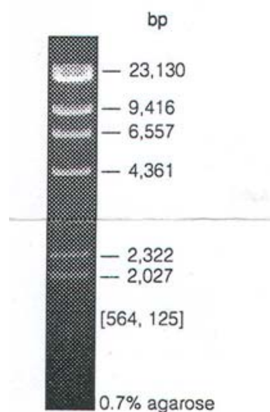
1) Combine ingredients in flask, melt in microwave until clear, cool slightly.
2) Set-up equipment including comb, slowly pour gel.
3) Allow gel to set (about 30 min), gently remove comb.

1xTBE Running Buffer
270 mL distilled H_2O
30 mL 10xTBE buffer

Ethidium Bromide (EtBr) Stain
100 µL EtBr
100 mL distilled H_2O

Destain
distilled H_2O

Lambda *Hind* III Marker



Microscopy
Prepare and view wet mounts of toluene isolate.
Prepare and view Gram stain of toluene isolate.

Enzyme Activity Probes

1) Divide toluene isolate plate into quadrants.
2) Add 100 µL of each toluene enzyme activity probe to one quadrant.
3) Incubate plate in the fume hood with lid off for about 15 minutes.
4) Determine toluene degradation pathway based upon probe results.

Protocol for PCR Reaction of Bacterial 16S rDNA Gene from Isolate

You will use conserved bacterial primers to amplify part of the 16S rDNA gene from your bacterial isolate. Set up the reaction as follows. (Be sure to understand the purpose of each ingredient.)

Add the following ingredients to a thin-walled PCR tube.

1. 5 µl of 10xPCR buffer
2. 10 µl of 1 pmoles/µl primer 27F
3. 10 µl of 1 pmoles/µl primer 1492R
4. 10 µl of 1 mM dNTPs (a mixture of nucleotides: dATP, dTTP, dGTP, dCTP)
5. 10 µl of 5 mM $MgCl_2$
6. 3 µl of 1 units/µl Taq DNA polymerase
7. 2 µl of genomic DNA extracted from your isolate

The total volume of your PCR reaction will be 50 µl.

After you add all the ingredients of your PCR reaction, leave the tube on ice until the whole class is ready to load the samples into the PCR machine.

PCR Reaction – Thermal Cycler Program

30 Cycles:			
Denaturation	94˚C	30 sec	*melt DNA template*
Annealing	53˚C	30 sec	*anneal primers to template*
Elongation	72˚C	1 min	*extend primers to make PCR product*
Final elongation	72˚C	5 min	*be sure that PCR products are complete*

PCR Gel

After the PCR reaction is complete, analyze PCR products on a 1% agarose, 1xTBE gel.

Results

a) DNA Extraction

If your extraction was successful, you should see one band.

What size band do you expect to see?

What may have happened if you see a smear instead of a band?

Record your results.

b) Microscopy

If you have a pure culture of your isolate, you should see one cell shape. If your culture is not pure, you should see many cell shapes.

Does your isolate appear to be pure?

What is the cell morphology of your isolate?

Is your isolate motile?

Is your isolate Gram positive or Gram negative?

c) Enzyme Activity Probes

Positive probe responses (color change from clear to yellow or orange) indicate that your isolate has active toluene degradation enzymes.

What were the probe results for your isolate?

Which toluene degradation pathway does your isolate have?

Week 7
PCR Reaction for 16S rDNA
PCR Amplicon Gel

Purpose

Amplify 16S rDNA from extracted DNA, and view PCR products via gel electrophoresis.

PCR Reaction for 16S rDNA

See protocol in Week 6.

PCR Amplicon Gel

Analyze PCR products on a 1% agarose, 1xTBE gel.

Results

a) PCR Reaction

If your PCR reaction was successful, you should see one band.

What size band do you expect to see?

What may have happened if you see a band of a different size or if you see multiple bands?

Record your results.

Week 8
Cloning PCR Products
Ampicillin Experiment

Purpose

a) Use cloning to make lots of copies of one unique 16S rDNA gene sequence.

b) Design an experiment to determine which microbes can grow in the presence of ampicillin.

Background

Cloning steps

1) Insert PCR products into vector (plasmid).
2) Insert plasmids into competent *Escherichia coli* cells.
3) Grow *E. coli* on LBA plates (Luria-Bertani plates with ampicillin).
4) Isolate one *E. coli* colony.

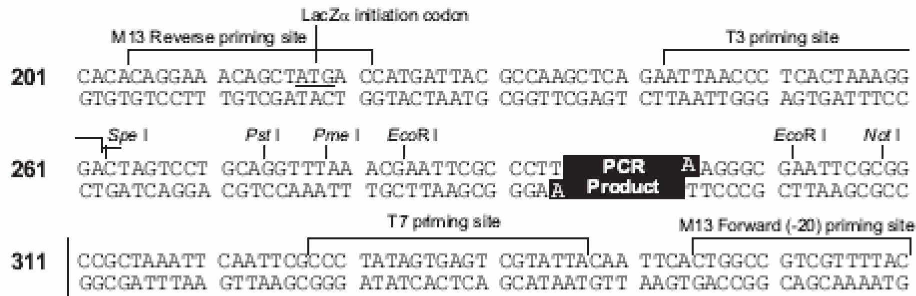
pCR4-TOPO Plasmid

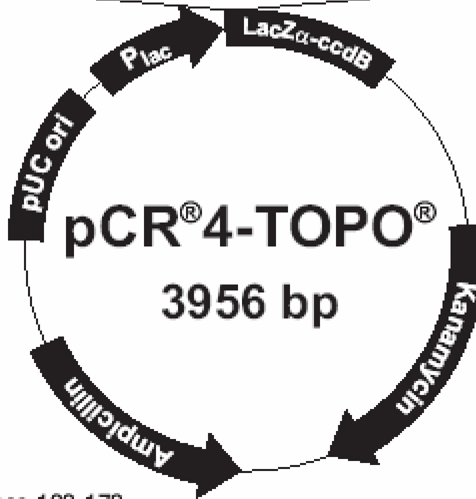
The plasmid used in the cloning procedure has both an ampicillin resistance gene and a suicide gene to allow easy identification of *E. coli* that have a plasmid with a PCR insert:

a) *E. coli* do not grow in the presence of ampicillin, unless they have ampicillin resistance genes. Only *E. coli* that took up plasmids can grow in the presence of ampicillin.

b) The suicide gene spans the opening in the vector. If a plasmid closes on itself without a PCR insert, the suicide gene is functional and the *E. coli* cell that took up this plasmid dies. When a plasmid has a PCR insert, the suicide gene is interrupted and the *E. coli* cell that took up this plasmid lives.

<u>Map of pCR4-TOPO Plasmid</u> (from Invitrogen Corporation, TOPO TA Cloning Kit for Sequencing, Catalog # K4575-J10)





Comments for pCR®4-TOPO®
3956 nucleotides

lac promoter region: bases 2-216
  CAP binding site: bases 95-132
  RNA polymerase binding site: bases 133-178
  Lac repressor binding site: bases 179-199
Start of transcription: base 179
M13 Reverse priming site: bases 205-221
LacZα-*ccdB* gene fusion: bases 217-810
  LacZα portion of fusion: bases 217-497
  *ccd*B portion of fusion: bases 508-810
T3 priming site: bases 243-262
TOPO® Cloning site: bases 294-295
T7 priming site: bases 328-347
M13 Forward (-20) priming site: bases 355-370
Kanamycin promoter: bases 1021-1070
Kanamycin resistance gene: bases 1159-1953
Ampicillin (*bla*) resistance gene: bases 2203-3063 (c)
Ampicillin (*bla*) promoter: bases 3064-3160 (c)
pUC origin: bases 3161-3834
(c) = complementary strand

Protocol for Cloning PCR Products (adapted from Invitrogen Corporation, TOPO TA Cloning Kit for Sequencing, Catalog # K4575-J10)

<u>Insert the PCR product into the plasmid</u>

1. Add the following ingredients to a microcentrifuge tube.

   2µl of your PCR product (*this is the insert*)
   2µl of pCR4 TOPO vector (*this is the plasmid*)
   2µl of salt solution

2. Incubate cloning reaction at room temperature for 5 minutes, then put the tube on ice.

<u>Put the plasmid with your insert into *E. coli* bacteria</u>

3. Add 2 µl of your cloning reaction to a tube of competent cells.
Competent E. coli *cells have been treated so that they will readily take up DNA from the environment.*

4. Mix gently by tapping the tube.
5. Incubate the reaction on ice for 10 minutes.
6. Heat shock the cells by heating your tube to **exactly** 42˚C for **exactly** 30 seconds.
7. Immediately transfer the tube to ice.

8. Add 250µl of SOC medium to your tube containing *E. coli* cells and cloning reaction.
The E. coli *cells are first allowed to grow in medium without antibiotics. This allows them to grow and make antibiotic resistance proteins (enzymes that can break down the antibiotic).*

9. Incubate the tube in a shaking incubator at 37˚C for one hour.

<u>Grow *E. coli* on LBA plates</u>

10. Plate 10µl and 100µl of your media on a LBA plate.
This plate contains antibiotics - only bacteria that have taken up a plasmid can grow. In addition, the colonies that grow must have an insert in their plasmid because the plasmid contains a suicide gene that must be disrupted by an insert for the bacteria to grow.

11. Incubate plates overnight at 37˚C.

<u>Isolate one *E. coli* colony</u>

12. Pick one isolated colony into LBA broth.

With the assumption assume that one colony comes from one bacterium, the broth culture will have lots of copies of one unique PCR insert. (One PCR insert into one plasmid, one plasmid into one E. coli*…)*

13. Incubate overnight with shaking at 37˚C.

Ampicillin Experiment

Design an experiment to determine which microbes can grow in the presence of ampicillin.

Results

a) Cloning

If *E. coli* colonies grow on your LBA plates, what does this tell you?

b) Ampicillin Experiment

How does ampicillin inhibit microbial growth?

Does ampicillin inhibit growth of all bacteria? Or does ampicillin target a particular type of bacteria (such as Gram positives or Gram negatives)?

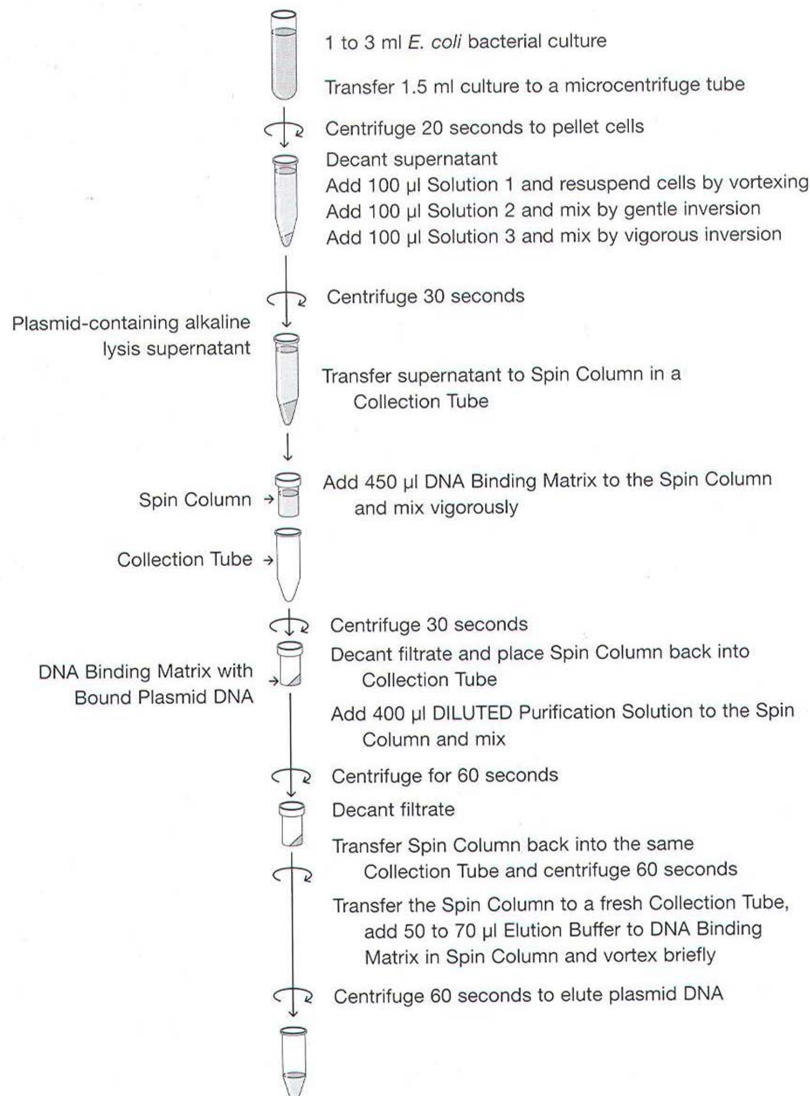
Does ampicillin inhibit growth of fungi or other eukaryotic microbes?

Week 9
Cloning Analysis

Purpose

a) Extract plasmids from *E. coli* cells to prepare for sequencing the PCR insert.
b) Determine if cloning and plasmid extraction was successful by digesting plasmids and running a gel.

Overview of Plasmid Extraction Protocol (from Eppendorf, Perfectprep Plasmid Mini kit, catalog # 0032 005.454)



Protocol for Plasmid Extraction (adapted from Eppendorf, Perfectprep Plasmid Mini kit, catalog # 0032 005.454)

1. Add 1.5 ml of your *E. coli* culture to a microcentrifuge tube, and centrifuge at full speed for 20 seconds.

2. Discard the supernatant. Do not disturb the pellet.

3. Resuspend the cells in 100 µl of Solution 1. Vortex vigorously.

4. Add 100 µl of Solution 2 and mix well by gently turning the tube upside down several times. Do not vortex.
   (E. coli *cells are lysed in this step. The lysate should be relatively clear with no visible clumps of cells.*)

5. Add 100 µl of Solution 3 and mix well by vigorously turning the tube upside down several times. Do not vortex.
   (*The lysate is neutralized in this step.*)

6. Centrifuge at full speed for 30 seconds and transfer the supernatant to a Spin Column in a Collection Tube.
   (*Plasmid DNA remains in solution while cell debris, proteins, and chromosomal DNA form the pellet.*)

7. Vigorously and thoroughly mix the DNA Binding Matrix suspension before pipetting.

8. Add 450 µl of the DNA Binding Matrix to the Spin Column and mix by vigorously turning the tube upside down several times.

9. Centrifuge the Spin Column/Collection Tube assembly at full speed for 30 seconds. Discard the flow through, and place the Spin Column back into the Collection Tube.

10. Add 400 µl of the DILUTED Purification Solution to the Spin Column and shake a few times. Centrifuge at full speed for 60 seconds.

11. Discard the flow through, and place the Spin Column back in the Collection Tube. Centrifuge at full speed for 60 seconds.

12. Transfer the Spin Column to a fresh Collection Tube and add 50 to 70 µl of Elution Buffer to the DNA Binding Matrix. Vortex briefly.

13. Centrifuge at full speed for 60 seconds.

14. Discard the Spin Column and cap the Collection Tube.
    (Purified plasmid DNA is now in the tube and ready for DNA sequencing reaction.)

<u>Note:</u> Keep your purified plasmid DNA for sequencing!

Restriction Digest

The restriction site for EcoRI endonuclease is:

There are two EcoRI restriction sites in the cloning vector (see the plasmid map on page 25). After using EcoRI to cut the extracted plasmids, we expect to see two bands of DNA in the gel. One band is the sequencing vector, the other band is the cloned PCR product.

Protocol for Restriction Digest

Add the following ingredients to a microcentrifuge tube.

3 μl of plasmid extract
2 μl of EcoRI
2 μl of restriction buffer
2 μl of BSA solution
11 μl of sterile water

Incubate the tube at 37˚C for 30 minutes.

Analyze 10 μl of the restriction digest on a 1% agarose, 1xTBE gel.

Note:
Discard your restriction digest at the end of lab, but remember to keep your purified plasmid DNA.

Results
a) Cloning

If your cloning procedure and plasmid extraction were successful, you should have two bands in your restriction digest gel.

Can you predict the sizes of these bands?

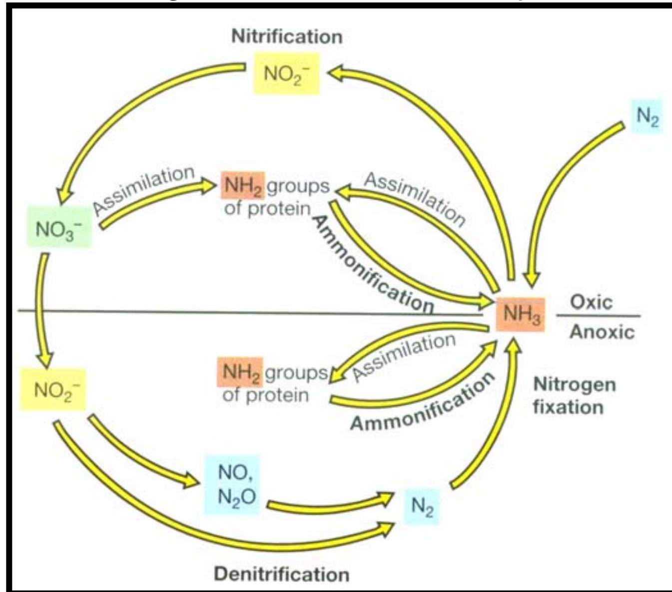
How would you interpret the results if there were three bands in the gel?

Record your results.

Weeks 10 and 11
Bioremediation Experiment

Purpose

You will be given a waste water sample that contains high concentrations of nitrate.



Nitrate is the most common groundwater pollutant and may cause illnesses such as blue baby syndrome. Therefore nitrate needs to be removed during the water treatment process. Your task is to design and test two separate methods, one involving denitrification and a second that uses nitrogen assimilation, to remove nitrate from wastewater with microorganisms. Once you get the contaminated sample you will have to determine the nitrate concentration. You will have two weeks to implement your remediation strategies after which you will measure the nitrate concentrations again to determine if your approach was successful.

You will have to submit a brief report (3 pages or less) of your experiments that describes 1. the approaches you designed 2. a materials and methods section 3. results of your nitrate measurements and microbial growth and 4 a brief conclusion in which you describe if the methods worked and which you method you think would work faster.

Week 12
Tour Environmental Genetics and Genomics (EnGGen) Facility
DNA Sequence Analysis

Purpose

a) Understand sequencing reactions and methods of sequencing DNA.

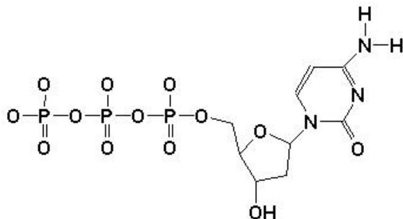
b) Compare 16s rDNA sequences to published sequences using BLAST online database. Identify the closest match and the closest cultured match.

Background

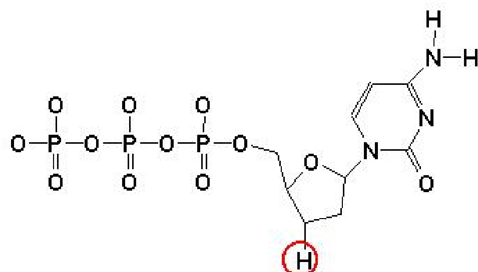
DNA Sequencing Reactions

Similar to PCR reactions, DNA sequencing reactions use a DNA polymerase to build a new strand of DNA. Unlike PCR reactions, DNA sequencing reactions use four special nucleotides in addition to the deoxynucleotides (dNTP) used in PCR reactions. The special nucleotides are dideoxynucleotides (ddNTP) and lack the 3' hydroxyl group that is necessary to add another nucleotide in 5' to 3' DNA synthesis. Therefore, when a ddNTP is incorporated in a growing DNA strand, DNA polymerization is terminated. Each type of ddNTP (A, T, C, G) is labeled with a different fluorescent molecule.

Deoxycytosine (dCTP) – normal nucleotide – most of the cytosine in a DNA sequencing reaction.



Dideoxycytosine (ddCTP) – terminal nucleotide with fluorescent label – some of the cytosine in a DNA sequencing reaction.



+ Fluorescent label

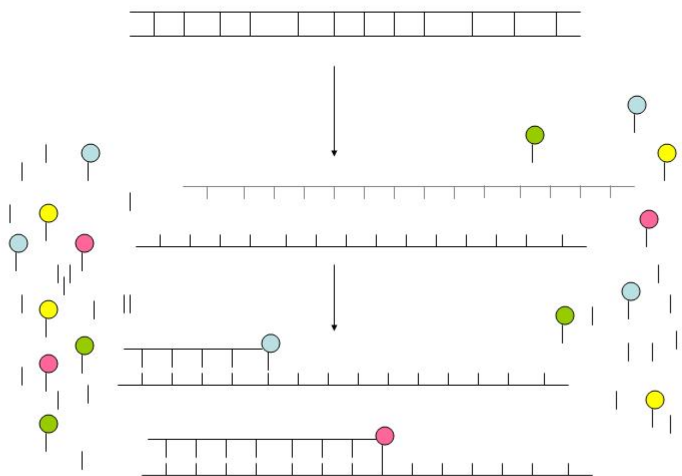
(different fluorescent labels for ddATP, ddTTP, ddCTP, and ddGTP)

DNA sequencing reaction ingredients:

- Purified plasmid DNA
- One primer
- DNA polymerase
- dNTPs (normal nucleotides)
- 2'-3' ddNTPs (terminal nucleotides with fluorescent label)

In cycle sequencing, fluorescently labeled ddNTPs occupy terminal positions in each new copy of the DNA template. Since ddATP, ddTTP, ddCTP, and ddGTP each have different fluorescent labels, it is possible to determine which nucleotide is at the end of a terminated strand. In cycle sequencing reactions, fragments of all sizes are generated and the terminal ddNTP of each fragment is determined. Thus a 500 basepair DNA sequence will have 500 different size fragments, and each fragment will have a single terminal ddNTP.

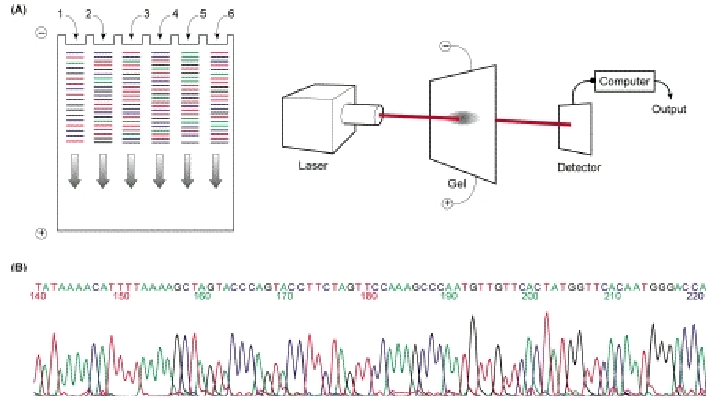
The following diagram shows how DNA sequencing products are generated (University of Wisconsin 2005). Only 2 sequencing products are shown, though in reality all fragment sizes would be generated in the sequencing reaction.



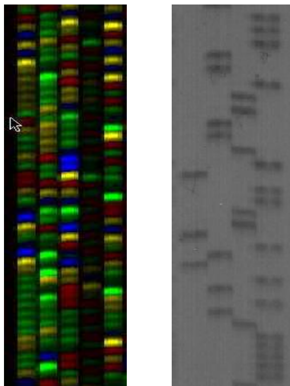
For gel sequencing, the sequencing product is loaded onto a gel and electrophoresed. Fragments that differ in size by one nucleotide can be separated in these gels. The smallest fragments migrate fastest. A laser beam at the end of the gel detects each different fluorescent label. Based on the order of the four different fluorescent signals, an electropherogram is generated, which gives the DNA sequence. (See gel sequencing diagram on page 34.)

Gel sequencing is being replaced by capillary gel electrophoresis, which is more automated and repeatable, but works on similar theory. The different size fragments migrate at different rates through the capillary system, and the different fluorescent markers are detected by a sensor after they move through the capillary system.

Gel sequencing diagram with electropherogram (University of Wisconsin 2005).



DNA sequencing gels (University of Wisconsin 2005).



The first gel is an example of cycle sequencing in which each ddNTP has a different fluorescent label. An electropherogram is generated to determine the DNA sequence.

The second gel is an earlier method of DNA sequencing in which four separate DNA reactions are required: one reaction has labeled ddATP, one has labeled ddTTP, one has labeled ddCTP, and one has labeled ddGTP. The DNA sequence is determined by analyzing all four sequencing reactions on a gel.

Note:
Your purified plasmid DNA will be prepared for cycle sequencing using Applied Biosystems Big Dye cycle sequencing kit for sequencing reaction and cleanup, and will be sequenced via capillary gel electrophoresis at NAU’s EnGGen facility.

DNA Sequence Analysis

1. Identify plasmid sequence.

2. Identify 27F or 1492R primer sequence:
   27F: AGA GTT TGA TCC TGG CTC AG
   1492R: TAC GGY TAC CTT GTT ACG ACT T

3. Avoid bad sequence – avoid N's.
4. Submit sequence to BLAST online database: http://www.ncbi.nlm.nih.gov/BLAST/
5. Hit format button to format request.

6. Identify and describe the closest match – include E value and % identity.

7. Identify and describe the closest match with a cultured organism (genus and species) – include E value and % identity.

8. Use taxonomy tool to further describe your microbe.

9. Find a paper in the library about your microbe.

<u>Sample sequence:</u>

NNAGTNNGNTGCAGGTTTAAACGAATTCGCCCTTAGAGTTTGATCCTGGCTCAGTC
GAACGAGAAAGTGGAGCAATCCATGAGTAAAGTGGCGACCGCGGTGAGTAACACG
TGACTAACCTACCTCCAAGTGGGGAATAACTCCGGGAAACCGGGGCTAATACCGC
ATAACATCTTCGGGTCGAAAGAACTGAAGATCAAAGCAGCAATGCGCTGGGAGAG
GGGGTCGCGGCTGATTAGCTGGTTGGCGGGGTAACGGCCCACCAAGGCGAAGAT
CGGTATCCGGCCTGAGAGGGCGCACGGACACACTGGAACTGAAACACGGTCCAG
ACTCCTACGGGAGGCAGCAGTGGGGAATTTTGCGCAATGGGGGAAACCCTGACG
CAGCAACGCCGCGTGGAGGATGAAGTCCCTTGGGACGTAAACNTNNNCACAANAA
CCAAAGACGATAANTGACCGGTACCTGGGTGGNANNGAANGCACCCGGCCTAAC
CTCTNNTGNCCAGCANGCCCGCGGGTAAATACAAGAGGGGGTGCAAGG

Results

<u>a) DNA Sequencing</u>

Why do DNA sequencing reactions use only one primer, while PCR reactions use two primers?

b) DNA Sequence Analysis

What is the closest match to your DNA sequence? Include E value and % identity.

What is the closest cultured match to your DNA sequence? Include E value and % identity.

Are you confident that your isolate is this species?

What additional information did you find to support that your isolate is this species?

Appendix
Media Recipes

Nutrient Agar Plates

1 L	dH_2O
23 g	Nutrient Agar Powder

Stir with heat to dissolve.
Autoclave.
Cool. Pour plates.

Note:
23g Nutrient Agar Powder = 3g beef extract, 5g peptone, and 15g agar.

1% Rosalic Acid

100 mL	dH_2O
0.8 g	NaOH
1 g	Rosalic Acid

Stir to dissolve.
Filter sterilize.

Minimal Medium (Liquid) for Toluene Degrading Bacteria

1.0 g	$(NH_4)_2 SO_4$
950 mL	dH_2O

Stir to dissolve.
Autoclave.
After cooling, add filter sterilized:

40 mL	1M Phosphate Buffer
10 mL	Modified Hutner’s Concentrated Mineral Base Solution

1M Phosphate Buffer

104.3 g	Na_2HPO_4 (anhydrous)
42.9 g	KH_2PO_4 (anhydrous)
1050 ml	dH_2O

Stir to dissolve (1-2 hr)
Adjust pH to 7.3 with NaOH or HCl
Autoclave

Modified Hutner's Concentrated Mineral Base Solution

20 g	Nitrilotriacetic acid (NTA – free acid, not salt)
600 mL	dH_2O

Dissolve fully. Neutralize with KOH (about 14.6 g)
Add remaining ingredients one at a time, dissolving fully before adding the next.

28.9 g	$MgSO_4$ anhydrous (or 59.3 g $MgSO_4{\bullet}7H_2O$)
6.67 g	$CaCl_2{\bullet}2H_2O$
0.0185 g	$(NH_4)_6Mo_7O_{24}{\bullet}4H_2O$
0.198 g	$FeSO_4{\bullet}7H_2O$
100 mL	Metals 44 solution

Adjust pH to 6.8 with KOH or HCl (should need about 100 mL of 1M KOH). When adjusting pH, a precipitate often forms but eventually redissolves. When pH is near 6.8 the color of the solution changes from a deep yellow to straw.

Bring up to a final volume of 1 L.
Stores for 1 year at 4˚C.

Metals 44

2.5 g	EDTA (free acid, not sodium salt)
10.95 g	$ZnSO_4{\bullet}7H_2O$
5.0 g	$FeSO_4{\bullet}7H_2O$
1.54 g	$MnSO_4{\bullet}H_2O$
0.392 g	$CuSO_4{\bullet}5H_2O$ (or 0.251 g anhydrous $CuSO_4$)
0.250 g	$Co(NO_3)_2{\bullet}6H_2O$
0.177 g	$Na_2B_4O_7{\bullet}10H_2O$
800 mL	dH_2O

Stir to dissolve (may need to warm and add a few drops of 10N NaOH).

To retard precipitation, add a few drops of concentrated H_2SO_4 beyond necessary to neutralize any added NaOH.

Bring up to a final volume of 1 L.
Clear, lime green solution stores indefinitely at 4˚C.

Minimal Medium (Plates) for Toluene Degrading Bacteria

1.0 g	$(NH_4)_2\ SO_4$
950 mL	dH_2O
17 g	Noble Agar

Stir to dissolve.
Autoclave.
After cooling, add filter sterilized:

40 mL	1M Phosphate Buffer
10 mL	Modified Hutner's Concentrated Mineral Base Solution

Pour plates.

Minimal Medium for Denitrifying Bacteria

980.0 mL	Minimal Medium Solution A
10.0 mL	Minimal Medium Solution B
10.0 mL	Minimal Medium Solution C

Mix thoroughly.

Minimal Medium Solution A

5.0 g	KNO_3
4.0 g	Carbon source – acetate ($NaC_2H_3O_2$)
1.0 g	$(NH_4)_2SO_4$
0.87 g	K_2HPO_4•$3H_2O$ (or 0.66 g anhydrous K_2HPO_4)
0.54 g	KH_2PO_4
980.0 mL	dH_2O

Stir to dissolve.
Bring up to a final volume of 1 L.
Autoclave 15 min 15 psi pressure 121˚C.
Cool to 25˚C.

Minimal Medium Solution B

2.0 g	$MgSO_4 \cdot 7H_2O$
100.0 mL	dH_2O

Stir to dissolve.
Bring up to a final volume of 100 mL.
Autoclave 15 min 15 psi pressure 121˚C.
Cool to 25˚C.

Minimal Medium Solution C

0.2 g	$CaCl_2 \cdot 2H_2O$
0.1 g	$FeSO_4 \cdot 7H_2O$
0.05 g	$MnSO_4 \cdot H_2O$
0.01 g	$CuSO_4 \cdot 5H_2O$
0.01 g	$Na_2MoO_4 \cdot 2H_2O$
100.0 mL	0.1N HCl

Stir to dissolve.
Bring up to a final volume of 100 mL.
Autoclave 15 min 15 psi pressure 121˚C.
Cool to 25˚C.

Enzyme Activity Probes

5mM probe in 40mM phosphate buffer

LB (Luria-Bertani) Plates

1 L	dH_2O
25 g	LB Broth Powder
15 g	Noble Agar

Stir with heat to dissolve.
Autoclave.
Cool. Pour plates.

Note:
25g LB Broth Powder = 10g tryptone, 5g yeast extract, 10g sodium chloride.

LBA (Luria-Bertani with Ampicillin) Plates

1 L	dH_2O
25 g	LB Broth Powder
15 g	Noble Agar

Stir with heat to dissolve.
Autoclave.
Cool. Add filter sterilized:

5 mL Ampicillin stock solution (50ug/mL final concentration)

Mix well.
Pour plates.

LBA Broth

1 L	dH_2O
25 g	LB Broth Powder

Stir with heat to dissolve.
Autoclave.
Cool. Add filter sterilized:

5 mL Ampicillin stock solution (50ug/mL final concentration)

Mix well.

Bibliography

Arizona Department of Environmental Quality. 2005. Homepage www.azdeq.gov. (www.azdeq.gov/environ/water/dw/health.html)

Environmental Protection Agency. 2005. Homepage: www.azdeq.gov. (www.azdeq.gov/environ/water/dw/health.html)

Madigan MT, Martinko JM, Parker J. 2003. Brock Biology of Microorganisms, 10th edition. Upper Saddle River, New Jersey. Prentice Hall.

University of Wisconsin. 2005. Homepage www.uwlax.edu. (www.bioweb.uwlax.edu/GenWeb/Molecular/Theory/DNA_sequencing/dna_sequencing.htm)