Enumeration and Identification of Enteric and Pseudomonad Proteobacteria from Agriculture-Impacted Rivers, and an Exploration of Fecal Viruses and Protozoa

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Abstract

In this exercise, students analyze water samples from two local rivers, emphasizing MacConkey-selected Gram Negative Proteobacteria. They use lactose and oxidase testing to sort observed colonies into Enterics (both lactose + and lactose -) and Pseudomonads. While Enterics are typically associated with transient fecal contamination, Pseudomonads represent natural soil/water dwellers that play important roles in decomposition and bioremediation. Each student sub-cultures and identifies river-derived and provided Enteric controls using a standard array of biochemical tests. Each student also analyzes waterborne and fecal-associated protozoa and viruses, using provided slides and on-line Centers for Disease Control (CDC) resources.

FIGURE ONE - Miscellaneous Images

Willamette River in Independence, Oregon

MacConkey Plate, Willamette River

Oxidase Disk Testing: Escherichia (left), Pseudomonas (right)

Bioremediation/Indole Assay

Activity

INTRODUCTION

Learning Objectives.

Upon completion of this activity, students will be able to (1) understand direct counting methods, having applied these techniques to a local river habitat; (2) recognize and sort Enteric and Pseudomonad colonies, including understanding implications of their relative numbers; (3) identify provided Enteric controls and river-derived isolates using standard biochemical tests and keys, demonstrating the power and limitations of culture-dependent approaches when dealing with unknown populations; (4) view and research viruses and protozoa associated with feces and waterborne illness.

Background.

This laboratory exercise has been carried out at Western Oregon University. A portion of it was included as one component of our poster entitled "A General Microbiology Lab Curriculum Featuring Culture-Dependent and -Independent Approaches and Computer-Based Project Presentations" at the 2004 American Society for Microbiology General Meeting. This exercise applies many fundamental microbiology principles and techniques (e.g. direct count enumeration, selective and differential media, identification using phenotypic tests and keys) to river habitats that are impacted by agriculture via fecal contamination and/or fertilizer run-off. After collecting and plating water samples from two local rivers, students sort and enumerate MacConkey-selected colonies into Enterics (both lactose + and lactose -) and Pseudomonads. Each student then uses a standard battery of Enteric-based phenotypic tests to identify two river-derived lactose + Enterics and two assigned Enteric controls. The pre-lab lecture for this unit emphasizes a detailed comparison of Enteric vs. Pseudomonad Proteobacteria in terms of phenotypic traits and roles in medicine, ecology, and bioremediation. Since 2002, all class coverage of Enteric pathogens has been moved to lab, freeing up more lecture time later for other disease topics. Additional microscopy and on-line assignments were developed in 2003 to include protozoal and viral agents associated with fecal and waterborne transmission.

This curriculum was first implemented in 1999 for the laboratory component of General Microbiology (Biology 331), a course that all Biology Majors are required to take. The typical annual impact is 32 students per year. Students complete this laboratory exercise during weeks four and five of this ten-week course, concurrent with the beginning of lectures about indigenous flora and bacterial pathogens. By this point in the lab, they have also mastered several microbiological skills, including aseptic technique, dilution series, direct counting, Gram Staining, and various environmental enrichment techniques. The course requires Brock Biology of Microorganisms (4), an invaluable resource for bacterial diversity, water quality issues, and bioremediation. The same instructor (Boomer) who has developed and delivered lectures also runs the laboratory (which meets Tuesdays and Thursdays for 2 hours); first-person portions of this report refer to Boomer. Students receive all instruction materials at the beginning of the term.

PROCEDURE

Materials.

This exercise should be run with students working individually, although team set-up for initial collection, plating, and counting is appropriate. All prepared slides and cultures below are available through commercial venders. Although control cultures are available through the American Type Culture Collection (ATCC), the cost of these materials is highly prohibitive in comparison with what biological supply companies like Wards ($30-200 vs. $7-15 per culture); thus, catalog numbers for media below refer to this source. I rely on a lab preparator who makes all media over the course of 6-8 hours (spread over 2 days) using pre-mixed base reagents from Fisher Scientific; catalog numbers for media below refer to this source, with specific sub-brands are indicated (e.g. Difco, Remel). Media recipes are also available in most standard microbiology lab manuals (2). All media can be made 4-6 weeks in advance and should be wrapped in plastic and refrigerated until use. The media-independent lab set-up time for this lab is 3-5 hours per session over 2-3 days. This approximation assumes instructors have all control cultures actively growing; initial set-up/maintenance of controls will add about 1 week of set-up, albeit only 2-3 hours total during that time. Each student needs:

Session One

5 ml river water (collected within 12 hours of lab, ideally assessed for temperature and pH)
5 MacConkey Agar Plates (Difco MacConkey Agar Base, DF0818-17-3)
5 sterile disposable pipettes
Dally rod and alcohol/flame for sterilization

Internet access, although assignments can be completed as homework elsewhere
Slides: Entamoeba histolytica, Cryptosporidium parvum, Giardia lamblia, Balantidium coli*
Microscopes and oil immersion supplies

**Slides should be the trophozoite form; one slide for every four students is adequate.*

Session Two
3 MacConkey Agar Plates (plus 1-2 extras for accidents and failures)
5-8 oxidase test disks provided in empty petri dishes with forceps*
Oxidase controls on Nutrient Agar (I use Escherichia coli and Pseudomonas putida)
10-20 sterile toothpicks

**Review Safety and Handling section for important additional information.*

Sessions Three and Four
4 Citrate Agar Slants (Difco Simmons Citrate Agar Base, DF0950-17-1)
4 Glucose Ferment Tubes (Remel Phenol Red Broth Base, R062202, plus 1% glucose)
4 SIM (Sulfur/Indole/Motility) tubes (Remel SIM Media, R454412)
Any 2 Enteric controls/unknowns, nearly all of which are biosafety level 2 (BSL2) pathogens*

**Recommended culture information based on provided identification key (Appendix One)*

Culture	Wards Catalog #	Kind of Culture
Escherichia coli	85 W 0400	Live Tube
Citrobacter freundii**	85 W 1848	Freeze-Dried Pellet
Salmonella typhimurium**	85 W 1956	Freeze-Dried Pellet
Klebsiella pneumoniae**	85 W 1880	Freeze-Dried Pellet
Proteus mirabilis**	85 W 1895	Freeze-Dried Pellet
Shigella flexneri**	85 W 1932	Freeze-Dried Pellet
Pseudomonas putida	85 W 0710	Live Tube

***Known pathogens, handle as BSL2 agents. Additional information about BSL2 agents and facility requirements is described in the Safety and Handling Issues section and Appendix One.*

Student Version.

Lab Procedures - **APPENDIX ONE**

Introduction to Pseudomonads and Enteric Proteobacteria
Many Proteobacteria can be found in local river habitats, two subgroups of which provide the focus of this unit: Enterics and Pseudomonads. Enterics are oxidase (-) rods that metabolize simple sugars. Coliforms, sometimes called fecal indicators, typically refer to lactose (+) Enterics. Although Coliforms include important GI bacteria such as Escherichia (both pathogenic strains and normal flora), there are disease-relevant lactose (-) Enterics (e.g. Salmonella). Pseudomonas are oxidase (+) rods that utilize both simple sugars as well as a polymeric carbon sources. Some Pseudomonads are distinguished by their ability to oxidize hydrocarbons and xenobiotics (e.g. PAH and PCB) and thus may be selected in industrially-polluted habitats. While Enterics often represent transient river contaminants, Pseudomonads thrive naturally in soil, mud, and water - with some causing inhaled or contact diseases. In this lab unit, you will compare local rivers using MacConkey agar, a selective and differential medium used to assess water quality. In addition to counting and identifying river lactose (+) isolates, you will also sort and count lactose (-) isolates using the oxidase test.

Overview - Individual Waterborne Protozoa and Virus Assignments
Many protozoa and viruses can also be found naturally occurring or as contaminants in rivers. Given the complexities of isolating and describing these agents, each person will explore several important fecal-associated and/or waterborne protozoa (Entamoeba histolytica, Cryptosporidium parvum, Giardia lamblia) and viruses (Poliovirus, Norwalk Agent/Norovirus, Hepatitis A) using prepared slides, your textbook, and the CDC Website. During the final hour of this lab unit, each student will set up 1 assigned protozoa slide, center a representative agent in focus, and show your instructor. You should have all previous questions done so you will be able to perform this task.

ACTIVITIES - SESSION ONE

Collection and Plating - Team and Individual
At least 2 students from different teams will accompany me to Independence Park at 7:30 a.m.
On Site: Collect 10 ml water, performing pH and temperature assessments on-site as directed
In Lab: Gently swirl or invert river water before removing every sample to avoid settling
Each person will spread 0.2 ml onto each of 5 MacConkey plates; incubate 37°C

ACTIVITIES - SESSION TWO

Individual Sub-Culture and Assessment
Lac+ Colonies - COLIFORMS
Select 3 different lactose (+) colonies; perfectly streak onto MacConkey plates; incubate 37°C
Check plate within 24 hours; Good - store in refrigerator; Bad - repeat until perfect by NEXT LAB!

Good = a properly streaked plate that contains uniform colony types and single, pure colonies – the latter of which are needed and will be used for upcoming identification testing.

Lac- Colonies Oxidase Sorting – PSEUDOMONADS OR ENTERICS?
Handle oxidase disks with forceps in empty petri dishes to avoid contact with skin
SORT 10 lac negative colonies into oxidase + or - categories; Why? Significance?

Press colonies into oxidase test disc using toothpick; perform 1 at a time to avoid confusion

ACTIVITIES - SESSION THREE/FOUR

Individual Identification Projects
Each person will set up and use the following tests to make a best-guess identification of 2 river-derived lactose + EntericsAND fully identify 2 assigned Enteric controls from the ID table. The latter set will be graded all or nothing. When recording data, DESCRIBE what you see (colors, cloudiness, etc.), NOT just +/-.

Citrate: Heavily inoculate 1 isolate per slant tube. After 2 days of growth, blue = (+); green = (-). Some organisms do not grow on this media so make sure to distinguish between growth/green and no growth (which is also green).

Glucose: Inoculate 1 isolate per broth tube. After 2 days of growth, yellow = glucose to acid; gas = bubble in Durham tube.

Sulfur-Indole: Inoculate 1 isolate per golden tube by loop-plunging into semi-sold agar. After 2 days of growth, black = sulfur (+); golden = sulfur (-) For indole production, add 10-15 drops Kovacs and wait 2 minutes. If Kovacs turns red, indole (+); if not, indole (-).

ID Chart For Enterics - From Text p. 353 and Bergey's Manual
Var = varies by strain, NG = no or limited growth usually obtained using this media

	Sulfur	Indole	Lactose	Glucose-Acid/Gas	Citrate
Escherichia coli	-	+	+	+/+	-
Citrobacter freundii	+	-	+	+/+	+
Citrobacter intermedius	-	+	+	+/+	+
Salmonella typhimurium	+	-	-	+/+	+
Klebsiella pneumoniae	-	-	+	+/+	+
Proteus mirabilis	+	+	-	+/+	NG
Shigella flexneri	-	var	-	+/-	-

SAFETY AND HANDLING SECTION

Some controls listed above are classified as mild to moderate pathogens. Until this lab, you have only worked with mild pathogens that are classified as biosafety level 1 (BSL1) agents, appropriate for basic and introductory teaching labs; during these lab activities, you may encounter BSL2 agents, appropriate for advanced teaching labs but requiring more precautions and experience. To ensure your safety and familiarize you with federally-defined lab safety standards, you are being provided with relevant excerpts from the CDC/NIH "Biosafety in Microbiological and Biomedical Laboratories" handbook, the entire contents (including definitions of higher BSL facility definitions and a list of pathogens for each category) of which can be found on-line at:

http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

Excerpts from the Biosafety in Microbiological and Biomedical Laboratories Handbook:

Biosafety Level 1 practices, safety equipment, and facility design and construction are appropriate for undergraduate and secondary educational training and teaching laboratories, and for other laboratories in which work is done with defined and characterized strains of viable microorganisms not known to consistently cause disease in healthy adult humans. *Bacillus subtilis, Naegleria gruberi,* infectious canine hepatitis virus, and exempt organisms under the NIH Recombinant DNA Guidelines are representative of microorganisms meeting these criteria. Many agents not ordinarily associated with disease processes in humans are, however, opportunistic pathogens and may cause infection in the young, the aged, and immunodeficient or immunosuppressed individuals. Vaccine strains that have undergone multiple *in vivo* passages should not be considered avirulent simply because they are vaccine strains. Biosafety Level 1 represents a basic level of containment that relies on standard microbiological practices with no special primary or secondary barriers recommended, other than a sink for handwashing.

Biosafety Level 2 practices, equipment, and facility design and construction are applicable to clinical, diagnostic, teaching, and other laboratories in which work is done with the broad spectrum of indigenous moderate-risk agents that are present in the community and associated with human disease of varying severity. With good microbiological techniques, these agents can be used safely in activities conducted on the open bench, provided the potential for producing splashes or aerosols is low. Hepatitis B virus, HIV, the salmonellae, and *Toxoplasma* spp. are representative of microorganisms assigned to this containment level. Biosafety Level 2 is appropriate when work is done with any human-derived blood, body fluids, tissues, or primary human cell lines where the presence of an infectious agent may be unknown. (Laboratory personnel working with human-derived materials should refer to the OSHA *Bloodborne Pathogen Standard* for specific required precautions.) Primary hazards to personnel working with these agents relate to accidental percutaneous or mucous membrane exposures, or ingestion of infectious materials. Extreme caution should be taken with contaminated needles or sharp instruments. Even though organisms routinely manipulated at Biosafety Level 2 are not known to be transmissible by the aerosol route, procedures with aerosol or high splash potential that may increase the risk of such personnel exposure must be conducted in primary containment equipment, or in devices such as a BSC or safety centrifuge cups. Other primary barriers should be used as appropriate, such as splash shields, face protection, gowns, and gloves. Secondary barriers such as handwashing sinks and waste decontamination facilities must be available to reduce potential environmental contamination.

River Proteobacteria Assignment - **APPENDIX TWO**

Name: ________________________

1. Record your assigned river, its temperature, and its pH based on information provided by the collectors during the first session of class.

2. Record your MacConkey plate counts, including sorting by the colors/lactose activity of each.

	# Purple/Pink Lactose (+)	# Light/White Lactose (-)
Plate Replicate 1		
Plate Replicate 2		
Plate Replicate 3		
Plate Replicate 4		
Plate Replicate 5		
Average		

3. Of the lactose (-) colonies tested for oxidase, how many were positive?

4. Using answers from questions 2 and 3, calculate the following in terms of cells/L in original river. Be careful with Pseudomonad value as it requires calculating and correctly applying the percentages observed in question (3).

Total Enterics/L in Original River Sample

Lactose (+) Enterics/L in Original River Sample

Pseudomonads/L in Original River Sample

5. What do the above distributions of Proteobacteria suggest to you about this site in terms of possible pollution impact? Is the water safe to drink? Why/why not?

6. Using instructor-provided controls and river lactose (+) isolates, complete the following phenotypic identification chart. Make sure you indicate the letter or number of your controls as indicated below.

	Control ______	Control ______	River Lactose (+) 1	River Lactose (+) 2
Sulfur				
Indole				
Lactose				
Glucose Acid/Gas				
Citrate				

7. Identify your instructor-provided controls; remember, these are graded all-or-nothing.

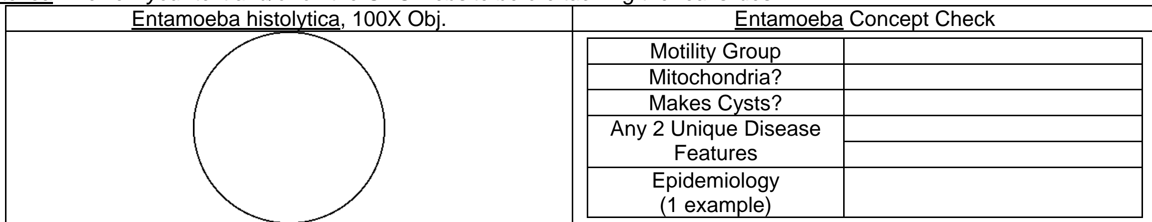
Control ______ is: Control ______ is:

8. For each river isolate, prepare a flow-chart and identify your organisms as closely as possible using the available tests. Use your text or other resources to find at least 2 other tests that could improve your identification.

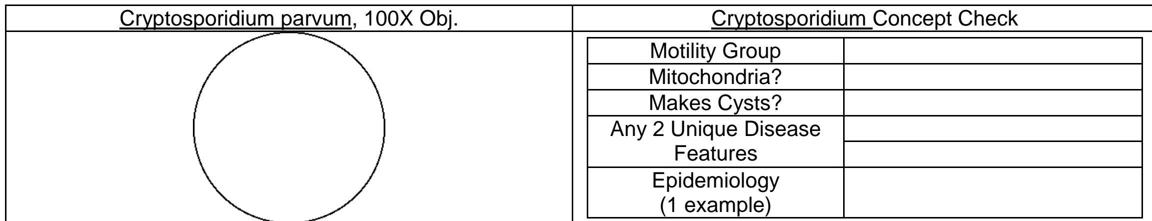
Virus/Protozoa Assignment - **APPENDIX THREE**

Name: ________________________

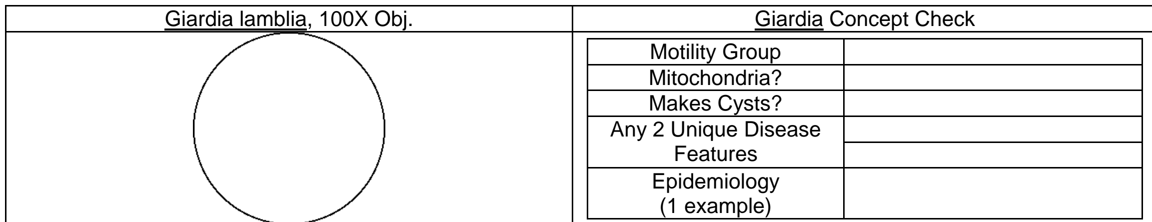
A. Protozoa: *Review your text and/or on the CDC website before tackling the real slides*



Entamoeba histolytica, 100X Obj.	Entamoeba Concept Check	
	Motility Group	
	Mitochondria?	
	Makes Cysts?	
	Any 2 Unique Disease Features	
	Epidemiology (1 example)	

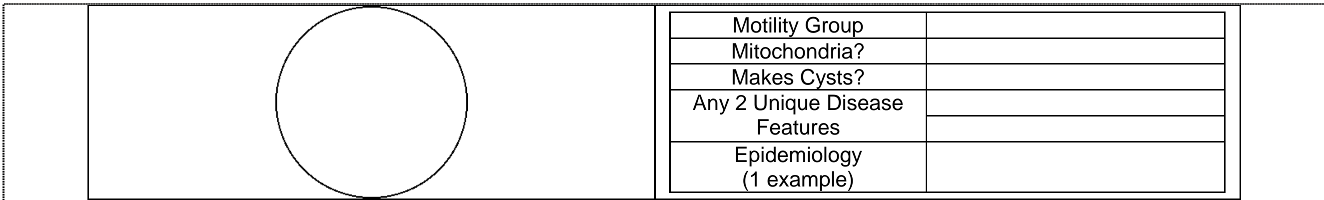


Cryptosporidium parvum, 100X Obj.	Cryptosporidium Concept Check	
	Motility Group	
	Mitochondria?	
	Makes Cysts?	
	Any 2 Unique Disease Features	
	Epidemiology (1 example)	



Giardia lamblia, 100X Obj.	Giardia Concept Check	
	Motility Group	
	Mitochondria?	
	Makes Cysts?	
	Any 2 Unique Disease Features	
	Epidemiology (1 example)	

Balantidium coli, 100X Obj.	Balantidium Concept Check



Motility Group	
Mitochondria?	
Makes Cysts?	
Any 2 Unique Disease Features	
Epidemiology (1 example)	

B. Microscopy Practical: Instructor's initials ______.

C. Viruses:
(1) Electron Micrographs: Using the CDC website (search for PHIL - Public Health Image Library section), locate and print 1 image of each virus; label and staple to this worksheet.

(2) Using your text and the CDC website (A-Z Index), complete the following table about each virus.

	Poliovirus	Hepatitis A	Norwalk
Genetic Material			
Enveloped?			
Any 2 Unique Disease Features			
Epidemiology (1 example)			

(3) Current Research: For 1 of these viruses (your choice), locate a primary research article (<5 years old) about this agent. For this assignment, go to www.asm.org and publications/journals, and select from J. of Clinical Microbiology, J. of Virology, or Clinical & Diagnostic Laboratory Immunology. Answer the following questions about this article:

a) Which virus is this article about?

b) Briefly describe 3 research procedures used in this article (i.e. 1 sentence per procedure).

c) Briefly describe 3 conclusions made by the authors of this article (i.e. 1 sentence per conclusion).

Partial Key for Virus/Protozoa Assignment - **APPENDIX FOUR**

All answers taken from a combination of course text and CDC Health Topics A-Z website; the disease features and epidemiology answers will vary considerably.

A. Protozoa:

Entamoeba Concept Check

Motility Group	Sarcodina/Amoeba
Mitochondria?	No - Anaerobic Metabolism
Makes Cysts?	Yes
Any 2 Unique Disease Features	Causes true dysentery - invasion of intestinal wall/inflammation
	Also invades liver and brain if untreated
Epidemiology (1 example)	About 100,000 people die/year worldwide

Cryptosporidium Concept Check

Motility Group	Apicomplexa/Sporozoa; non-motile in adult stages
Mitochondria?	Yes
Makes Cysts?	Yes, highly chlorine resistant
Any 2 Unique Disease Features	Mild, self-limiting in immune-competent people
	Serious, possibly fatal for immune-compromised, HIV/AIDS
Epidemiology (1 example)	Largest US waterborne epidemic, 403,000 in Wisconsin in 1993

Giardia Concept Check

Motility Group	Mastigophora/Flagella
Mitochondria?	No, true diplomonad
Makes Cysts?	Yes, moderately chlorine resistant
Any 2 Unique Disease Features	Acute forms - explosive, foul, watery diarrhea - no blood
	Can be acute, chronic, or present in asyptomatic carriers
Epidemiology (1 example)	Largest US waterborne epidemic, 403,000 in Wisconsin in 1993

Balantidium Concept Check

Motility Group	Ciliophora/Ciliate
Mitochondria?	Yes, and also have micronuclei and endosymbiotic bacteria
Makes Cysts?	Yes
Any 2 Unique Disease Features	Causes dysentery - invasion of intestinal wall/inflammation
	Less invasive beyond GI tract than Entamoeba
Epidemiology (1 example)	Poor reporting, most significant reservoir = pigs

B. Viruses:

	Poliovirus	Hepatitis A	Norwalk
Genetic Material	RNA, single stranded (+)	RNA, single stranded (+)	RNA, single stranded (+)
Enveloped?	No	No	No
Any 2 Unique Disease Features	Most symptoms in the GI tract - diarrhea	Symptoms - nausea, fever, dark urine, jaundice	Mild to moderate diarrhea, Usually self-limiting
	CNS infection/paralysis relatively minor outcome	Average symptoms last 28 days (range = 15-50 days)	Incubation 24-48 hours, symptoms 24-60 hours
Epidemiology (1 example)	1952 = peak year in US 52,000 paralysis cases	100,000 US cases/year (despite vaccine)	50% all gastroenteritis!! 23 million US cases/year

Instructor Version.
This class lab extends over four sessions that all should be run back to back.

Session One
In session one, students are provided with an extensive pre-lab lecture about Enterics and Pseudomonads, emphasizing key identification traits and respective roles in medicine, ecology, and bioremediation. Specifically, major reportable fecal-associated Proteobacteria are described: Escherichia coli (toxigenic hemorrhagic strains), Salmonella (typhimurium, enteritidis, and typhoid species), Shigella, and Campylobacter (only the latter is not a true Enteric and would not be retrieved using these lab methods). Lecture materials have not been provided in this presentation as all information and images were derived from the course text (4), Sherris Medical Microbiology - an Introduction to Infectious Diseases (6), and the CDC website.

Following lecture, I review the protozoa and virus assignment, emphasizing that students need to carefully manage their time during all sessions in such a way that enables them to complete all project components. Protozoa were selected to demonstrate all four motility-based subgroups: amoeboid Entamoeba histolytica, sporozoa Cryptosporidium parvum, flagellate Giardia lamblia, and ciliate Balantidium coli. Students should review their text and the CDC website prior to examining slides so they can distinguish pathogens from host tissues. In addition to simple scientific drawings, I require that students physically set up and show me one of the slides during session four, one of two times students are required to demonstrate physical skill using a microscope (the other being Gram Staining, earlier in the term).

Following lecture, each student prepares five replicates of river water (0.2 ml per plate) collected earlier that morning by student volunteers, as shown in Figure One (see additional information in Safety and Handling section). Alternatively, instructors may collect these samples without students up to 12 hours before class, refrigerating samples between collection and class plating. My class samples and compares Gentle Creek, a small stream that runs through several miles of farmland, with the Willamette River, a large river that runs through a few hundred miles of industrial and agricultural areas. Both areas are publicly accessible, popular for fishing, swimming, and boating. Water samples, collected in October and April, have been observed to range from 9-15°C and 4.5-6.5 pH. In general, students retrieve 5-30 colonies per MacConkey plate (a representative plate is shown in Figure One), which typically includes 3-10 morphologically different lactose + and lactose - variants (anecdotal observations suggest that counts vary according to the amount of recent rainfall). To achieve comparable numbers and variation, instructors may have to adjust student replicate numbers or select different river systems in their area. Given no other procedural obligations during session one, students are able to begin protozoa and virus assignments. Depending on the lag time between sessions, instructors or students should refrigerate plates after 18-24 hours.

Session Two
During this session, each student enumerates his/her plates in terms of lactose + vs. lactose - colonies, which many students have a difficult time judging. If this is the first time they are working with this medium, they should be provided either with plated controls (e.g. lactose + Escherichia vs. lactose - Salmonella) or review control images, available in most standard lab texts (e.g. Wistreich, 6). Following enumeration, each student then completes two tasks: Coliform sub-culture and Oxidase/Pseudomonad testing. For the former, each student selects two morphologically distinct lactose + Enterics and streaks to isolate each onto new MacConkey plates. Students are warned that they must come in to check and refrigerate their plates within 24 hours, repeating streak plating as needed until they have two pure isolates by session three (in our case, four days away). Although students have had to perform streak plating almost ten times already, 10% of my students fail to produce proper plates on the first attempt because they have forgotten some basic step in streak plating (e.g. used the wrong pattern or forgotten to flame between sectors). Thus, they are reminded what a "good" streak plate should look like in their procedures: homogenous colony types with some pure colonies that are well-isolated.

The second task is to sort 10 lactose - colonies using the oxidase test (see additional comments in Safety and Handling section). Oxidase testing effectively distinguishes Pseudomonads from Enterics (a positive and negative control are shown in Figure One); it is also worth mentioning to students that this test is medically important because Neisseria (e.g. causative genera for gonorrhea and meningococcal meningitis) are also distinctively oxidase positive. While oxidase is a strong indicator of Pseudomonas, there are a few Pseudomonads that will use lactose, albeit at low levels. In researching and developing this exercise, I actually could not find this information in basic sources like the course text and Shorter Bergey's Manual (1). I eventually located a useful table in the Manual of Clinical Microbiology (5), which reported that three of ten clinically relevant Pseudomonads variably used lactose at rates of 25% (vs. 100% for true lactose users like Escherichia). Given this information, it is possible that this exercise misses some lactose + Pseudomonads, and I make students aware of this fact while discussing the procedures. However, lactose + MacConkey colonies are challenging to interpret using oxidase tests because they are already dark purple. Given that students have not done or seen oxidase testing before, they run controls prior to assessing river colonies.

Since initiating this lab, class data suggests that 70-100% of all lactose - isolates are oxidase + Pseudomonads. Anecdotally, most students seem at first relieved to discover this information, which implies to them that there are fewer fecal-associated bacteria in the water. The more they come to understand Pseudomonads, however, the more they develop new and equal concerns about (a) the significance of these data in terms of potential industrial pollution selection; and (b) health issues concerning contact Pseudomonad skin infections. Instructors should thus be prepared to discuss these kinds of follow-up questions and may even want to consider additional extensions, some of which are described in the Possible Modification section.

Sessions Three and Four
During these two sessions, each student's primary goal is to use a basic array of Enteric-focused medical tests to identify two river lactose + Enterics and two assigned controls. Specific tests and media are extensively described in virtually all course texts and laboratory manuals (e.g. Wistreich, 6); thus, detailed descriptions of these procedures (sulfur, indole, motility, lactose, glucose, and citrate) are not included in this paper. Indeed, this set of activities represents nothing more than a simple variation of standard Gram Negative identification exercises, emphasizing the use of phenotypic keys

and illustrating alternative metabolic substrates and products. While I have, over the years, elected to pare down my array of class tests, other instructors could readily expand testing (e.g. adding urea, Voges-Proskauer, methyl red, a larger battery of carbon utilization tests, etc.). After presenting an overview of methods during a brief pre-lab lecture, students set up all tests using all samples during session three and then read them during session four. It should be emphasized that river lactose + Enterics seldom match anything on the key I provide or even in more extended resources (e.g. Bergey's, 1), a likely product of performing too limited testing and the fact that "natural" isolates may, in fact, represent new strains, species, or genera. As an environmental microbiologist trained in culture-independent methods, I believe it is important that students wrestle with unknown diversity, the implications of which are important for microbial ecology, applied microbiology, and emerging disease. Nevertheless, it is also important that students experience the satisfaction of identifying assigned controls that will match the table so as not to leave them entirely frustrated. While I grade controls all-or-nothing, I am flexible in my evaluation of river isolates given a logical defense. The final 30 minutes of class are devoted to the microscopy practical, which most students take less than 5 minutes to complete.

Safety and Handling Issues.
Local rivers contain, minimally, Enterics from untreated agriculture run-off (typically, students observe 10^{3-4} lactose + Enterics per L). River samples also contain Pseudomonads (on the order of 10^{3-4}/L) and lactose - Enterics (on the order of 10^{1-2}/L). It is also possible that water samples contain protozoa or viruses but we do not actively amplify or assay for these microbes. Consequently, students are required to wear gloves (we use non-latex given allergy issues) and clean hands while sampling and handling raw river samples. Following initial plating, extra river water and materials contaminated with river water are immediately autoclaved. MacConkey-retrieved isolates are handled as though they are pathogens and students are fully informed that provided controls include pathogenic genera.

Although all controls recommended in this procedure are available commercially, most are defined as biosafety level 2 (BSL2) pathogens and additional precautions must be taken. Instructors should review specific information about these agents and BSL2 facility definitions; an appropriate source is the CDC/NIH handbook "Biosafety in Microbiological and Biomedical Laboratories" (available on-line at www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm). Although BSL2 agents are moderate pathogens, their main mode of transmission is via contact, not air. Consequently students should use gloves, goggles, and lab jackets when handling these agents. A specific citation of the CDC/NIH description of BSL2 agents and facilities is included in the student procedures (Appendix One).

Finally, all oxidase test methods use tetramethyl-p-phenylenediamine dihydrochloride, a moderately toxic agent that should not contact skin. After comparing several different testing formats, I prefer oxidase disks (BBL™ Taxo™ N Discs) because they have a long shelf life and are easy to control in terms of safety/contact issues, particularly relative to the liquid/filter approach. The disks are also significantly more cost-effective than DrySlides™. After discussing safety issues with the class, I distribute pre-assembled petri dishes with oxidase disks and forceps to each student.

ASSESSMENT and OUTCOMES

Suggestions for Assessment.
Individual students each turn in completed copies of the River Proteobacteria Lab Analysis Worksheet (20 pts). Student teams turn in completed Waterborne Viruses and Protozoa Worksheet, which includes the microscopy practical (20 pts). Additionally, 20% of the lab midterm exam (20/100 total points) covers this lab exercise. The combined value of this lab unit is 20% of the lab assignment grade (60/300 total points).

Field Testing.
Since being developed in 1997, approximately 160 junior- or senior-level undergraduate Biology Majors have completed this curriculum. Most (50-60%) students were pursuing careers in the health sciences. The remaining students sought careers in secondary education and research (academic, government, or biotechnology).

Student Data.
We have completed assessment of lab curricula in General Microbiology, which serves a maximum of 16 students per term. Thirty-eight students rated this curriculum on a 10-point scale (10 = best; 1 = worst) in Fall 2003, Spring 2004, and Fall 2005, as summarized below:

Please Rate The Statement: This Lab…	Average Rating
Made Connections Beyond Microbiology	8.7
Improved My Awareness of Microbial Diversity	9.4
Enhanced My Interest In Scientific Research	8.6
Exposed Me To New Technology	7.9
Enhanced My Organizational Skills	7.9
Enhanced My Writing Skills	7.0
My Overall Rating Of This Lab Is	8.7

SUPPLEMENTARY MATERIALS

Possible Modification.
Given that this activity is an inquiry-driven adaptation of standard unknown identification exercises, there are many ways that this lab could be modified, three of which are presented here.

Modification One – Different or Expanded Sampling Approaches
In my current version of this laboratory exercise (run since 2003), I have pared down my sampling sites to two local river locations, comparing Enterics and Pseudomonads at a small and mostly agriculture-impacted river with those populations at a larger, agriculture- and industrial pollution-impacted river. When initially troubleshooting this exercise (2000-2002), I allowed each student team to design and execute its own river sampling procedures, my only requirements being that each team sample/compare at least three locations (notably on their own time and using their own transportation) and develop a reasonable hypothesis based on their selected sties. Student teams were extremely creative when given this opportunity, with the most elaborate and memorable project being a study wherein a student team selected and compared four locations along a single river - from its pristine headwaters all the way to its impacted terminus. Although I was very tempted to use this worthwhile and interesting study for my current class, there were two strong deterrents: (1) sampling required nearly 8 hours, over half of which was driving; and (2) headwater samples contained only 5-10 colonies using these retrieval methods – which would have been inadequate for subsequent individual student subculture analyses. Nevertheless, allowing student teams to design their own experiments is strongly encouraged, provided that instructors make efforts to assess plans and ensure that adequate MacConkey-derived colonies will likely be retrieved if dependent analyses and identification are to be carried out.

Modification Two - Anti-Microbial Sensitivity Testing

Given the extensive agricultural use of anti-microbial compounds, testing river isolates for drug resistance is an interesting and relevant follow-up lab. This extension involves propagating colony isolates in nutrient broth overnight and then preparing lawns for disk-based testing. However, a key problem I have encountered is the fact that many river isolates (in some classes, as many as 75%) fail to grow in nutrient broth. Consequently, I recommend heavily streaking isolates directly onto new MacConkey plates, and then placing anti-microbial disks directly thereon to assess for relative sensitivity. To retrieve more accurate and comparable data, instructors should attempt traditional Kirby-Bauer sensitivity testing (2), experimenting with other non-selective media (e.g. tryptic soy broth) prior to assessment on Mueller-Hinton agar.

Modification Three - Pseudomonas Identification and Bioremediation Assay
As with Enterics, it is also possible to identify river Pseudomonads and controls to the species level via sub-culture purification and an array of biochemical tests (e.g. commercially available Pseudomonad species include aeruginosa, fluorescens, fragi, and putida). Some recommended differentiation tests are summarized below, with more in the Bergey's Manual (1). Finally, an indirect bioremediation assay can be performed, as adapted from Luu et al. (3). After streaking isolates on minimal or nutrient agar and incubating 12-18 hours, 5 mg indole crystals are placed in the petri dish lid where they volatilize up to the media/growth zone. Degradation of this aromatic yields a variety of colored products - typically red, blue, or brown - and has been shown to correlate with the ability to break down chemically similar compound (e.g. trichloroethylene, chlorinated and/or aromatic compounds). A representative image of this assay is shown in Figure One, although these isolates were enriched from hydrocarbon-contaminated soil, not river water.

species	Denitrification	Gelatin Hydrolysis	Fluorescence	Pigment
aeruginosa	+	+	+	+ on King B only
fluorescens	+	+	+	-
putida	-	-	+	-
stutzeri	+	-	-	-
mendocina	+	-	-	+ on Nutrient
malei	+	+	-	-

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