MICROBIAL DIVERSITY

MBIO 3480

LAB MANUAL

2010

Lab manual is available as a pdf file on the website.

TABLE OF CONTENTS

Lab Number	Title	Page
	MICROBIAL DIVERSITY SCHEDULE	3
	GENERAL INSTRUCTIONS	5
	LAB STANDARD OPERATIONS PROCEDURE (SOP)	10
	WHMIS	14
	MICROBIAL SOIL DIVERSITY PROJECT	16
	EXPERIMENTS	
1	Nitrogen Fixation	27
2	Functional microbial diversity	35
3	Anoxygenic Phototrophic Bacteria: Photosynthetic Pigments	45
	Spectrophotometer 3100 Operation	51
4	Aerobic Anoxygenic Phototrophic Bacteria	53
	APPENDIX	
	Media and Solutions	61
	Pure Culture Methods	63
	Soil Collection and Transport Method	65
	Soil Microorganism Dilution and Plating Procedure	66
	Sample Calculation of the number of microorganisms per ml	67
	Outlier plate counts	68
	Percentage of copiotrophs and oligotrophs	69
	Hemocytometer instructions	69
	Automatic Colony Counter	71
	pH meter operation	71
	Pipette Operation	72
	Phase Contrast Light Microscope	74
	Anaerobic Chamber Operation	79
	Fluorescence Identification Test (FN slant)	79
	Cellular Morphology and Colony Characteristics	80
	Gram Stain	81
	KOH string test	81
	Motility by Direct Microscope Observation	82
	SAMPLE LAB EXAM	83

MICROBIAL DIVERSITY MBIO 3480 LAB SCHEDULE 2010

The class is divided into two sections alphabetically (in class notification and on website). Section 1 students start at 2:30 pm and section 2 students start at 4:00 pm unless otherwise noted.

DATE	WEEK	EXPERIMENT/PROCEDURE
Sept 16	1	Lab Introduction Microbial Soil Diversity Project: Introduction and Group Project Assignment **Microscope quiz - includes a 10 min written quiz and a practical quiz - slide set up and microscopic examination** - details below. Lab 1 Nitrogen Fixation Part I: Isolation of Free Living Nitrogen Fixing Bacteria
Sept 23	2	Microbial Soil Diversity Project: Experiment outline reviewed by the instructor during lab time Lab 1 Nitrogen Fixation Part I: Isolation of Free Living Nitrogen Fixing Bacteria and Part II: Isolation of Symbiotic Nitrogen Fixing Bacteria
Sept 30	3	Lab 2 Functional Microbial Diversity
Oct 7	4	**Section 1 students only (lab start time is 2:30 pm in room 204)** Section 2 students do not attend. Lab 3 Anoxygenic Phototrophic Bacteria: Photosynthetic Pigments **Section 2** (Lab room 302) Microbial Soil Diversity Project: Group experiment time. Not compulsory.
Oct 7	4	PROJECT: Marked experiment returned in class (Tuesday before lab). Group may set up experiment on their own time if project incubation is required. The majority of project experiment work should be **done during class time** over the next 3 weeks. This allows the TA to assist you with your experiment. Basic and requested lab supplies available in lab.
Oct 14	5	**Section 2 students only (lab start time is 2:30 pm in room 204)** Section 1 students do not attend. Lab 3 Anoxygenic Phototrophic Bacteria: Photosynthetic Pigments **Section 1** (Lab room 302) Microbial Soil Diversity Project: Group experiment time. Not compulsory.
Oct 21	6	Microbial Soil Diversity Project: Group experiment time. Lab attendance is not compulsory.
Oct 28	7	**Section 1 students only (lab start time is 2:30 pm in room 204)** Section 2 students do not attend Lab 4 Aerobic Phototrophs - carotenoid characterization **Section 2** (Lab room 302) Microbial Soil Diversity Project: If required, additional group experiment time. Not compulsory.
Nov 4	8	**Section 2 students only (lab start time is 2:30 pm in room 204)** Section 1 students do not attend Lab 4 Aerobic Phototrophs - carotenoid characterization **Section 1** (Lab room 302) Microbial Soil Diversity Project: If required, additional group experiment time. Not compulsory.
Nov 11	9	No lab, Remembrance Day
Nov 18	10	PROJECT PRESENTATIONS (see lab website for information, location and times)
Nov 18	10	**LAST DAY FOR LAB CLEANUP and REMOVAL OF LAB COATS.** If group number, initials or names found on anything, marks will be subtracted from the final lab mark.
Dec 2	12	LAB EXAM

Week 1 QUIZ Information - Eclipse **E100 microscope with slide phase condenser**
-10 min written quiz on Eclipse E100 microscope with slide phase condenser, short answer. See lab manual appendix, PHASE CONTRAST LIGHT MICROSCOPE OPERATION for ECLIPSE E100 with SLIDE PHASE CONDENSER, for information required for quiz.
-practical microscope operation (slide set up, viewing slide on phase contrast microscope such that a TA can easily see with only fine focus adjustment). Each group prepares one slide. Each group has only 5 minutes to get a good microscope view. If unable to get a good view in five minutes, need to prepare a new slide and go to end of line. Repeat until successful, a pass.

Report Due Dates

Date[a] Thursday unless otherwise stated	Description
Sept 23	Microbial Diversity Project Experiment Oultine. Bring to lab as checked in lab.
Sept 30	Microbial Diversity Project - Materials and Procedure
Oct 5 (Tuesday)	Lab 2 Functional Microbial Diversity DATA SHEET due by 2:30 pm (data sheet does not require an Honesty Declaration attached)
Oct 7	Lab 1 Nitrogen Fixation Report
Oct 14	Lab 2 Functional Microbial Diversity Report
Oct 28	Lab 3 Anoxygenic Phototrophic Bacteria: Photosynthetic Pigments Report
Nov 10 (Wednesday)	Lab 4 Aerobic Phototrophs
Nov 18	Microbial Diversity Project Group Report. Project Peer Evaluation: Each group member must hand in a separate peer evaluation.

[a]all reports due by 4:30 pm day requested.

GENERAL INSTRUCTIONS

Lab Instructor:	Dr. L. Cameron	Office: 414B Buller
Lab Demonstrators:	Jalil Nasiri	Lab: 304 Buller
	Talal Abboud	Lab: 423 Buller (during construction 301)
	Aniel Moya Torres	Lab: 304 Buller

WEBSITE: www.umanitoba.ca/faculties/science/microbiology/staff/cameron/

OR via University of Manitoba Microbiology Homepage:
http://www.umanitoba.ca/faculties/science/microbiology/labinformation.htm
Information available at the website: reference links, changes/corrections, additional information, data, marks

REGULATIONS

1. Lab attendance is compulsory.
2. Bring a permanent marker.
3. Students must wear a lab coat. There is no drinking or eating in the lab..
4. Students work in pairs for the majority of the lab. For the project ONLY, two pairs will work together.
5. Emails: subject must contain course number and subject, e.g. 3480 lab 1 report. If no subject given, email is deleted. Emails replies occur only during working hours. Email must include student name.

EVALUATION

1. The lab is worth 25% of the final mark:

Lab exam:	13%
Lab reports, quiz and possible assignment[c]:	7%
Lab project:	
Experiment Outline[a]	
Peer evaluation	0%[b]
Hypothesis, Material and Methods report	1%
Group presentation	2%
Group report	2%

[a] No marks allotted, however, marks may be subtracted

[b] No marks are allocated for the peer evaluation but you must hand in your peer evaluation or marks will be subtracted (0.5%). It is important to hand in your evaluation as I make the decision to subtract marks (see below) based on peer evaluations handed in.

If your peer evaluation final mark is below 75%, your group report and presentation mark will be reduced dependent on peer evaluation mark.. For an evaluation of 70-75% minus 10%, for evaluation of 65% -70% minus 15%, for evaluation of 60% - 65% minus 20%, continuing with every 5% peer evaluation decrease there is a further decrease of 5%.

[c] The value of each report and quiz or assignment may vary. All marks (reports and quiz) are totaled and brought to a mark out of 7%.

At the end of the practical lab you are expected to discard all your cultures (Petri plates, broth tubes, etc). Last day for lab clean up will be posted. Up to 2% mark will be subtracted from your final lab mark if this is not done. Marks will be also be subtracted if you do not remove labels from glassware put on discard trolley during term.

2. Students must pass the lab to pass the course (12.5% of the 25% lab mark).
3. Lab reports are to be handed in as stated in schedule by 4:30 pm of that day. ONLY hand in lab reports through slotted filing cabinet drawer located on the 300 level of Buller bldg. in the hallway across from room 302 entrance. Instructor and demonstrators do not accept lab reports. If handing in lab late, 10% of mark will be subtracted for each class day late. Marked lab reports will be returned to students the next week. A late report will not be accepted after that report has been returned to the class. If applicable, also hand in data, release forms, assignments through slot of filing cabinet. Data may be emailed. All reports, assignments, and quizzes not collected by the student are destroyed six months after end of term via confidential shredding.
4. The lab exam will be held at 2:30 pm, regular lab slot. The date is stated in the schedule. Exam must be written in pen (not pencil). Location will be posted on lab door ~1 week before lab exam.
5. Approximately two weeks prior to the lab exam, a brief outline of lab exam format and information content will be available on the website (also includes lab exam location).
6. You must notify the lab instructor no later than two school days after the missed lab. A Doctor's certificate is required for a missed lab exam. All deferrals will write the lab exam at a scheduled time set by the instructor. Failure to comply will result in a zero on your lab exam.
7. **Plagiarism (copying another student's lab report (present or previous year) or copying published literature without citing) is a violation of University regulations. Refer to the STUDENT DISCIPLINE BY-LAW in your student handbook (rule book) for action taken for plagiarism.**

WRITTEN REPORT PRESENTATION

[Before handing in your report review report to ensure that all information is included. When printing Excel spreadsheets make sure you have selected all information before printing. If you are using text boxes, they must be completely within the selected area or they do not print.]

1. All reports must have an Honesty Declaration attached at end of report - available as a pdf file on lab website.

2. If a Word document or Excel spreadsheet format has been provided, you must use it for your report write up.

3. Lab reports may be done as an individual effort or a group effort by the two students that carried out the experiment. The decision on the number of reports per group is totally dependent on members of the group. This decision may be changed any time during the term. Therefore for each lab report the group has the option to hand in one or two reports exclusive of what has been done before or after that particular report. Indicate on the cover page of the report if the report is a group report or an individual report. If handing in an individual report also include lab partner's name. Only ONE PROJECT REPORT is accepted per group.

4. Lab reports must be **typed.** Up to 10% of the mark subtracted for reports not typed. Diagrams acceptable hand drawn and labelled (must be PEN, pencil only acceptable for diagrams). No binders. Staple report left hand corner. Calculations must also be typed.

5. On the front page of the report state (Does not need to be separate):
   Course name and number
   Experiment number and Title
   Group # and section #
   Individual or Group name(s). If handing in an individual report, also include lab partners name.
   GROUP report or INDIVIDUAL report
   Date

6. Number pages.

7. Lab report information is to be presented exactly as requested in lab manual. Number sections in the SAME ORDER as the lab manual. No binders. Stapled left hand corner.

8. Include a sample of each calculation type requested.

9. If your experiment does not produce the requested data for your lab report, borrow broth or plate culture(s) from another group to allow you to record your own data. Do not borrow another group's data. If you cannot find another group with expected results, use a textbook or internet to record expected results but must reference information.

10. Cite reference in text of lab report and record full reference at end of lab report. When should you cite and reference. The following is a good definition of plagiarism that explains when you should cite a reference. "**The unacknowledged use of another person's work, in the form of original ideas, strategies, and research, as well as another person's writing, in the form of sentences, phases and innovative terminology**." (Spatt[1], 1983, p.438) This is done by using bracketed reference number that you used when listing references at end of lab report or by bracketing first authors name and date. Quote text unless you paraphrase completely in your own words. But remember, quotes should only be a small part of your work. If you are using the name year system, list the references alphabetically. Some examples are as follows (McMillan[2] 1997):

    Binder V. Hendriksen C, Kreiner S. 1985. Prognosis in Crohn's disease - - based on results from regional patient group from county of Copenhagen. Gut 26:146-50.
    Danforth DN, editor. 1982. Obstetrics and gynecology. 4th ed. Philadelphia: Harper and Row. 1316 p.
    Petter JJ. 1965. The lemurs of Madagascar. In: DeVore I, editor. Primate behavior: field studies of monkeys and apes. New York: Holt, Rinehart and Winston. p 2920319.

---

[1]Spatt, B. (1983). *Writing from Sources*. New York: St. Martin's Press.

[2]McMillan V.E. 1997. Writing Papers in the Biological Sciences. 2nd ed. Boston: Bedford Books: 1997. 197 p. and McMillan, V.E. 2001. Writing Papers in the Biological Sciences. 3rd ed. Boston: Bedford Books. 123 p.

If journal article assessed on the internet, site as journal (no need to state accessed online). However, if available only on the web, reference as follows:
Kingsolver JC, Srygley RB. Experimental analyses of body size, flight and survival in pierid butterflies. Evol. Ecol. Res. [serial online] 2000;2:593-612. Available from: Colgate University online catalog. Accessed 2000 Oct 3.

11. Personal or Commercial Electronic sources[2]:
Cite in-text by putting the following in parentheses, author's last name or file name (if no author's name is available) and publication date or the date of access (if no publication date is available).
At the end of report list
(i) author or organization
(ii) publication date or date last revised
(iii) title of Web site
(iv) URL site in angle brackets
(v) the date accessed.
Cameron, L. 60.344 Microbial Physiology Lab Information <http://www.umanitoba.ca/faculties/science/microbiology/staff/cameron/60_344.htm>. Accessed 2002 April 12.

12. **Bacteria Nomenclature Guidelines** [3]

- The bacterium's name consists both of the genus and species and possibly the subspecies. The genus name starts with uppercase letter. The species and subspecies start with lowercase letter, for example, *Aermonas hydrophila anaerogenes*. Never refer to a bacterium by the species name alone.
- Bacterial names are written in italics or underlined (if handwritten).
- The first time a bacterium's name is referred to in your lab report you must write the full name. After that you may abbreviate the genus name by using the first letter (capitalized and italicized). If your report contains two or more genera that start with the same letter it is best to spell out the complete name of each genus to eliminate confusion. To eliminate some of the confusion, some genera may be abbreviated with the first two or more letters but this is not common practice, for example, *Sal. cholerae* for *Salmonella cholerae* whereas *Shigella boydii* is abbreviated as *S. boydii*. If the bacterium's name also contains a subspecies both the genus and the species may be abbreviated, *Aermonas hydrophila anaerogenes* as *A. h. anaerogenes* (this is not standard nomenclature). *E. coli* is an exception to this rule. Since *Escherichia coli* has been extensively used in research, it is simply referred to as *E. coli* for the complete scientific name.
- The bacterium's name should not be preceded by an article.
- The bacterium's name is never written in plural form.
- Bacterial groups above the level of the genus are capitalized but not italicized, for example, Enterobacteriaceae.
- The ending of some scientific bacterium's name is occasionally changed to form a group common name. For example, pseudomonads, is not capitalized or italicized.
- The collective abbreviation for species is as follows:
    - one species: *Salmonella* sp.
    - more than one species: *Salmonella* spp.

[3]McMillan, V. E. 1997. Writing Papers in the Biological Sciences. Boston: Bedford Books. p 146-148.

Table presentation (if available, use lab website table format for report)

- Table number and title (legend) presented above the table body.
- Number tables using arabic numbers, even if only one table in a report.
- Include enough information in title to completely describe table, eliminating the necessity to search elsewhere in the lab report to understand information presented in table. Table title starts with an incomplete sentence. Additional complete sentences may be included to adequately describe the table, eg. number of days of colony growth and temperature, media type, microorganism source (this also applies to figures).
- If abbreviations are used in table, indicate what abbreviations mean as a footnote. Other footnotes may be required to clarify material in the table.
- Like information should be in columns making it easier to view the table.
- Data in columns is listed under the center of each heading. Align decimal points and dashes. If a number value is less than 1 always include zero before the decimal.
- Column or Row headings should be complete and self explanatory. A heading is a separate entity from the title. It cannot be assumed information given in the title is adequate for a heading. **The unit of measurement should only be included in the heading, not in column data.**
- Group related column headings under larger headings.
- If information is the same for each column or row do not include but treat as a footnote.
- Make the table as concise as possible but include all necessary information. For example, when presenting a table of bacteria colony characteristics it is important to state media type, incubation time and temperature as colony characteristics vary depending on these conditions somewhere in the table.
- Tables should be properly set up with a straight edge.

Figure presentation (graphs, diagrams, photographs, films)

- **All figure graphs must be computed generated using Excel** (detailed procedure given in the lab)
- Figures are to be numbered separate from tables, using arabic numbers. Include figure number even if only one figure, e.g. Figure 1.
- Following the figure number a figure title should be presented below graph. The figure title, like the table, starts with an incomplete sentence describing the graph. For example, do not repeat just the labels of the x- and y-axis but present in a descriptive manner. Additional sentences should be included if additional information is required to completely describe figure, for example, abbreviations explanation, any constant experimental conditions, etc.
- **All diagrams, photographs, and films are figures** and should be completely labelled.
- **For graphs:** Usually there is one dependent variable plotted and one or more independent variables plotted. The dependent variable is a function of the independent variable. It is accepted practise to plot the independent variable on the x-axis and the dependent variable on the y-axis. For example, the measurement of absorbance (dependent) with increasing concentration of protein (independent). The size of the graph should fit the plot(s). The overall size of graph should not be too large but should not be so small that information is obscured. Graph must be completely labelled (always include units). Use different symbols for each plot (not different coloured pens) on a graph. If more than one plot, explain symbols in legend or in a key included in the body of the graph. If required to label on graph area never write on plot line, eg. never circle peak area. Just write the peak maximum beside peak, if necessary use an arrow.

- **For other figures**. Do not write on figure data area, ie. gel lane, film surface, etc. Use small symbols or arrows to indicate what you want and explain in figure title. When labelling TLC, make sure all lanes, origin and solvent front are labelled. Make sure all essential information is included in the figure title such that when you look at the figure you understand the experiment that was performed and what the data means.

Note: When writing your lab reports you are frequently requested to present both a table and a figure for a given set of data, similar to keeping a research journal. This is not the accepted practice for papers published in journals or books. Usually either a table or a figure is presented for a given set of data and depending on nature of data, it may only be summarized in the text. How do you make a choice of data presentation? The aim is to effectively and efficiently demonstrate what you want to show, for example, correlations, comparisons, pattern, trends, etc.

LAB STANDARD OPERATIONS PROCEDURE (SOP)

Safety information relevant to the Microbiology department is available at http://umanitoba.ca/science/microbiology/WHMISworkshop.htm

PERSONAL SAFETY:

•You must wear a buttoned lab coat. If you forget your lab coat, lab coats are available in the lab – return to box after using.

Lab Coat Laundry instructions: wash separately from other clothes with detergent and bleach. When taking lab coat home for washing, carry separate (in plastic bag) from all other personal effects, i.e. not in your back pack.

•No personal effects (this includes outer clothing and back packs) are permitted in the lab, only essential lab supplies. There are cupboards available in the hall across from the lab for outer clothing and backpacks. Cupboards may be locked but locks must be removed each time after using. Please do not leave any of your belongings on the hallway floor.

•Long hair must be tied back. Keep your hands away from your hair.

•Wash hands with antibacterial soap (SWISH contains sodium lauryl sulfate (SDS) a detergent, coco diethanolamide, coco amido betaine, and copolymer of acrylamide) before leaving the lab. It is recommended that you wash your hands for 30 sec.

• Remove gloves using finger of opposite hand to peel off other glove by inserting at wrist, rolling off glove. Repeat with other hand. Dispose of gloves in Petri plate containers.

•No eating or drinking in the lab.

•Never mouth pipette. Always use a pro-pipette.

•Cover any cuts with a bandage (if necessary, available in the first aid kit).

•Students are advised to wear closed toed shoes.

•MSDS book located in lab room 302 by south hand wash station**.**

LAB ENVIRONMENT:

•Wash bench area before and after your lab work with BDD (Backdown Detergent Disinfectant containing nonyl phenoxy polyethoxy ethanol, alkyl-aryl ammonium chloride and ethyl benzyl ammonium chlorides). Never assume that the lab bench has been cleaned by the previous student.
•First aid kit present in lab (by south hand wash station).
•Know location of exits, fire extinguisher, eye wash, full body shower, and first aid kit.
• Know how to operate equipment before use. DO NOT use equipment unless you know exactly how to operate the equipment. The TA is always available to assist.
•Leave your bench area clean. All equipment and supplies should be returned to original location.

DISPOSAL:

•All biohazard disposable containers must be labelled with a biohazard label. After autoclaving the biohazard label is removed.
• All biohazards must be autoclaved. Biohazards include any surface that has come in contact with bacteria. The autoclave is monitored monthly to ensure all organisms are destroyed. The half filled bag from the Petri plate container is removed, untied, opened and placed in a large tray on the autoclave trolley. After autoclaving, the biohazard sticker is removed, bag tied and the bag is placed in a black plastic bag and tied before disposing. Individual bags from plastic lined buckets are carefully removed and placed in a larger autoclave bag (labelled biohazard). Closed while transported to autoclave room. The autoclave procedure is the same as above. After autoclaving the biosafety sticker is removed, bag closed. The bag is placed in a corrugated cardboard box pre-labelled as broken glass, taped shut for disposing by caretaker.
•**Petri plate containers**: Discard all non-sharp biologically contaminated items in the Petri plate container (large covered plastic lined bucket on the floor): culture plates, API strips, antibiotic strips and disposable gloves.
•**Plastic lined bucket**[4]: Any 'pointy' item must be disposed in bench top plastic lined bucket (not Petri Plate container), this includes pipetman tips, sticks, toothpicks, slides, disposable 1 ml and 10 ml pipettes, Pasteur pipettes, broken glassware, brittle plastic objects, Biolog plates, metal objects[a] (not needles or blades), etc. This includes all items that are 'pointy' regardless of biological contact.
•**Biological Spills**: Put on glove. Immediately wash container or test tube rack with BDD disinfectant. Put stack of paper towels on top of spill, pour disinfectant around and over. Do not press down. Collect soaked paper towels in Petri plate container. If spill includes broken glass or any sharp item put in plastic lined basin.
•**Glassware (unbroken)**: Remove tape and pen markings (use alcohol) from glassware before placing on discard trolley. Used glassware that has not contained bacteria should be rinsed and placed on the discard trolley. Rinsed test tubes (no biological contact) should be placed in tray provided on the discard trolley.

•**Chemical hazardous material**: Read the MSDS information available in lab or online at http://ccinfoweb.ccohs.ca/msds/search.html . Organic solvents must be disposed of in organic solvent container. The lab demonstrator will instruct proper disposal methods for labs that contain hazardous materials. These containers are disposed of through the university safety office. Never pour solvents down the sink. Use extreme care with flammable solvents. Alcohol used to flame spread rod should never be positioned within 40 cm of flame. Never put a very hot spread rod into a beaker of alcohol. The alcohol may catch fire.

---

[4]due to the multi-use nature of the teaching lab, all 'pointy' items will be treated the same as similar items contaminated with microorganisms.

Handle caustic (acids and bases) solutions with care. Never discard an acid or base greater than one molar down the sink. Discard in labelled glass containers provided. Use lots of water when discarding caustic solutions (< 1M). These materials are disposed of through the university safety office.
When handling stains or reagents, wear disposable gloves as the majority of stains or reagents contain hazardous material.

•**Biohazard sharps disposal**: Dispose of all sharps (needles, syringe tops, razors, scalpel blades) in specified container (red or yellow). Dispose of syringe with needle attached - do not take apart. Do not replace the needle cap before disposing (high frequency of accidents occur when replacing cap). Sharp's containers are autoclaved before disposing. You must dispose of the syringe top in the biohazard sharps container even if not used for biologicals as it is a perceived hazard by the general public.

•**General garbage disposal:** Nothing 'pointy' should be disposed in the general waste basket, such as pipets, tips, can top, toothpicks, sticks, etc. Nothing that has come into contact with biological material should be disposed in general waste container. No liquids, the caretaker does not know what the liquid is!

LABORATORY BIOSAFETY GUIDE

Soil contains mostly level 1 risk microorganisms but also contains level 2 microorganisms. Treat all isolated microorganisms from soil as if they were level 2 risk microorganisms. Assume all soil samples that you handle contain level 2 bacteria. Follow standard operations procedure, SOP (see above).

The University of Manitoba Biosafety Guide (Feb 2000) and Health Canada Laboratory Biosafety Guidelines booklets are available in your lab.

UM Biosafety Guide:
http://umanitoba.ca/admin/human_resources/ehso/media/BiosafetyGuideMarch05.pdf

Laboratory Biosafety Guidelines: http://www.phac-aspc.gc.ca/publicat/lbg-ldmbl-04/
MSDS (infectious agents: http://www.phac-aspc.gc.ca/msds-ftss/index-eng.php
There is no listing of level 1 agents in the guidelines or MSDS pamphlets

Risk group 1 bacteria are low individual and community risk and are unlikely to cause disease in healthy workers.

Risk group 2 bacteria are moderate individual risk and limited community risk. Bacteria in this group can cause human or animal disease but are unlikely to infect healthy laboratory workers. Effective treatment is available. Risk of spreading is limited.
CONTAINMENT LEVEL 1 (UM biosafety guide p. 11)
- microbiology lab with washable walls, countertops and hand wash sink
- established safe laboratory practices (hand washing and disinfection of countertops)
- general WHMIS safety training
- UM lab registration

CONTAINMENT LEVEL 2 (UM biosafety guide p.11)
- all of level 1 specifications
- biosafety permit
- biological safety cabinet (not required)
- biohazard sigage

- a written standard operations procedure
- MSDS for the infectious agent

LAB PROCEDURES SPECIFIC TO THIS LAB

1 Microscopes
Microscopes are stored in cupboards in the lab. Each student is responsible for handling of the microscope. Microscopes must be returned to the cupboard after use. Clean objective lens with 2-propanol. Make sure the light is always turned off after using microscope. Return microscope to cupboard. Refer to appendix for microscope handling.

2 Media/Stains
The majority of media (plates/tubes) are available in the lab (front shelf) throughout the week. Please contact demonstrator if any supplies are depleted. If a reagent has to be stored at 4 - 6°C, it will be located in the cold box. When handling stains, wear disposable gloves.

3 Student's cultures/plates
Keep in labeled Petri plate containers when storing or incubating. Incubate in 28°C incubators located in the lab unless otherwise specified.

4. The lab is open Monday to Friday from 7:30 am to 5:00 pm.

WHMIS

The Workplace Hazardous Materials Information System (WHMIS) is a system for safe management of hazardous materials. WHMIS is legislated by both the federal and provincial governments.

Under WHMIS legislation, laboratories are considered to be a workplace, and students are workers. By law, all workers must be familiar with the basic elements of the WHMIS system.

The WHMIS program includes:
1. Cautionary labels on containers of controlled products. Consumer products, explosives, cosmetics, drugs and foods, radioactive materials, and pest control products are regulated separately, under different legislation.
2. Provision of a Material Safety Data Sheet (MSDS) for each controlled product.
3. A worker education program

1. A. SUPPLIER LABELS

Controlled products must have a label of prescribed design which includes the following information:

PRODUCT IDENTIFIER - trade name or chemical name
SUPPLIER IDENTIFIER - supplier's name and address
MSDS REFERENCE - usually, "See MSDS supplied"
HAZARD SYMBOL - (see illustration on next page)
RISK PHRASES - describes nature of hazards
PRECAUTIONARY MEASURES
FIRST AID MEASURES

B. WORKPLACE LABELS

All material dispensed in a workplace container must be labelled with the **Product Name, Precautionary Measures** (simplified) and **Reference to Availability of MSDS**.

2. **MSDS**
Material Safety Data Sheets (MSDS) are available for each lab. Refer to binder located in each lab. Also main binders are located in the Microbiology preparation room, 307/309 Buller. MSDS are also available on the internet. The MSDS will provide: relevant technical information on the substance, chemical hazard data, control measures, accident prevention information, handling, storage and disposal procedures, and emergency procedures to follow in the event of an accident.

3. SAFETY
The Laboratory Supervisor will provide information on the location and use of safety equipment, and emergency procedures.

WHMIS

(Workplace Hazardous Materials Information System)

HAZARD CLASSIFICATION	SYMBOL
Class A - Compressed Gas	
Class B - Flammable and Combustible Material 1. Flammable Gas 2. Flammable Liquid 3. Combustible Liquid 4. Flammable Solid 5. Flammable Aerosol 6. Reactive Flammable Material	
Class C - Oxidizing Material	
Class D - Poisonous and Infectious Material 1. Materials Causing Immediate and Serious Toxic Effects 2. Materials Causing Other Toxic Effects 3. Biohazardous Infectious Material	
Class E - Corrosive Material	
Class F - Dangerously Reactive Material	

WORKPLACE LABELS

These labels are applied at the workplace on controlled products or containers of controlled products when

A. The original supplier label is missing or becomes unreadable.
B. The material is decanted or transferred from the supplier's container to another container.
C. The material is produced at the workplace for use within the workplace.

Workplace labels will include the following information

1. **Name/Identity of the Product**
2. **Safe Handling Information** - refers to any precautions that must be followed to minimize the risks of adverse health effect or injury. This also includes any personal protective equipment (PPE) e.g., type of gloves, eye or respiratory protection to be worn or other controls to be used through statements or pictograms (symbols)
3. **Reference to the MSDS.**

MATERIAL SAFETY DATA SHEET

A Material Safety Data Sheet (valid for three years) will include information relating to each of the following categories

1. Product Identification and Use
2. Hazardous Ingredients
3. Physical Data
4. Fire and Explosion Data
5. Reactivity Data
6. Toxicological Properties
7. Preventive Measures
8. First Aid Measures
9. Date and Source of MSDS

and any other hazard information of which the Supplier is aware or ought reasonably to be aware.

Fire / Medical Emergencies

555 from 474, 789, 975, 977 exchanges or
#555 from a cell phone (MTS or AT&T)

or

911 from other exchanges. If 911 is called you must also call Campus Security at **474-9341**

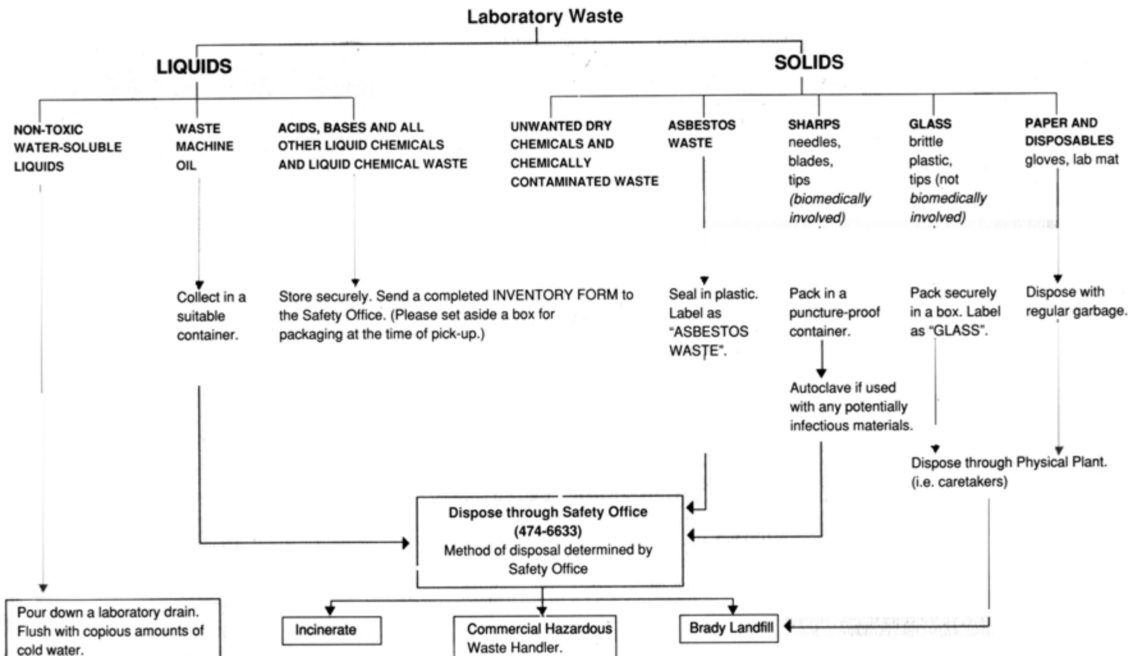
Chemical / Biohazards Emergencies

474-6633

(8:30 am - 4:30 Mon. to Fri.). If busy or after hours, call **555**

For further information on WHMIS, contact the Environmental Health and Safety Office at 474-6633.

Manitoba Worplace Health Hazard Regulation 53/88 requires that a complete and current chemical inventory is maintained at all times



NO CHEMICALS ARE TO BE LEFT FOR THE UNIVERSITY OF MANITOBA CUSTODIAL STAFF. Empty reagent bottles are to be rinsed and have the labels de-faced. All potentially infectious materials (*biomedically involved*) must be autoclaved or de-activated using a chemical sterilizing agent prior to disposal. Animal carcasses are to be incinerated. Radioisotope users should consult the University of Manitoba "Waste Disposal Chart For Radioisotope Users".

THE ABOVE CHART IS A GUIDE, MORE INFORMATION IS AVAILABLE THROUGH THE OCCUPATIONAL HEALTH & SAFETY OFFICE (474-6633).

October 1993

MICROBIAL SOIL DIVERSITY PROJECT

Handle soil bacteria with care using GOOD MICROBIOLOGY TECHNIQUE, as some soil bacteria are pathogenic opportunists for humans. All microorganisms isolated from the environment must be treated as Level 2 Biohazard.

PROJECT FORMAT

Project format is based on the American Society of Microbiology (ASM) recommendations for microbiology laboratory thinking

Interpersonal skills
Work effectively in a group such that all members of group share equal tasks. Carry out tasks in a timely manner. Each member of the group must submit a group peer evaluation after presenting work. The group peer evaluation pertains to both the written report and the presentation.

Cognitive processes
First decide what question you want to answer that satisfies the chosen project topic and objective. Next, develop an experiment to attempt to answer your question. Predict expected results. Follow an experiment protocol that allows you to answer your question.

Analysis skills
Collect all necessary data, making sure to record all experimental parameters. Present data in good table, figure or descriptive paragraph format as outlined in lab manual introduction. Analyze data and draw conclusions. Assess limitations of your experiment.

Communication and citizenry skills
Present results and findings in class. If possible, relate to human interaction with the soil from your backyard to agriculture.

PROCEDURE

Week 1

1. Form a group of four students, must be two groups combined. Select and sign up for one of the project suggestions. (Maximum of 14 groups)

   Section 1(select from the following list only)
   1. The effect of carbon availability on soil microbial diversity.
   2. The effect of nitrogen availability on soil microbial diversity.
   3. The effect of temperature on soil microbial diversity.
   4. The effect of NaCl on soil microbial diversity.
   5. The effect of flooding (water saturation) on soil microbial diversity.
   6. The effect of desiccation on soil microorganism diversity.
   7. The effect of motor oil contamination on soil microbial diversity.

   Section 2(select from the following list only)
   8. The effect of pH on soil microbial diversity.
   9. The effect of plant grow on soil microbial diversity (eg. grasses, legumes, non legumes)
   10. The effect of soil depth on soil microorganism diversity.
   11. The effect of oxygen tension (not soil depth) on soil microbial diversity.
   12. The effect of fertilizers on soil microorganism diversity.
   13. The effect of added carbon on soil microbial diversity.
   14. The effect of contamination on soil microbial diversity (herbicide, pesticide, detergent, household cleaners, etc.)

2. Before coming to next week's lab, complete the following experiment outline. Experiment details are not required but include all requested information. Outline must be checked in class by the instructor before proceeding to detailed write up of procedure.

MICROBIAL SOIL DIVERSITY PROJECT Experiment Outline
Available as a Word document on lab website.
(Type/write in point form - be concise. Hand written is acceptable but must use pen.)
Group Names: ____
Project Number/Name: ____
Soil Sample(s):

Soil Type	Soil Source

Soil Pretreatment - Method/Time (if applicable):

Varied Experimental Parameter (if applicable):

Procedure:
State only details that vary from MBIO3480 lab manual (see appendix and experiments especial experiment 2).

Sample analysis: Indicate the four methods selected by your group by circling letters in following list. If your group contains more than 4 group members, your group must increase number of methods or soil type. If group contains less than 4 group members, experiment complexity may be reduce, discuss with instructor.

a) Colony characteristics (if taking pictures turn off flash and remove cover of Petri plate)
- remember looking at diversity patterns, not just describing numerous colonies. Discuss dominant or unique colony types.
b) Growth time, r-selection (copiotrophs) compared to K-selection (oligotrophs).
c) Viable microorganism titer (all groups must use this method)
d) Total microorganism titer (hemocytometer)
e) Biolog Ecoplate Soil Community Analysis (contains 31 carbons sources in triplicate)

Basic Equipment and Supplies available for project (see schedule for time available).

Check all bench surfaces, shelves, cold box in your lab for supplies - majority of perishable supplies are stored in the 4°C cold box.

Basic equipment or media supplied	Number
balances/weigh spoons and paper	3/lab
sterile 1 and 10 ml pipettes and matching propipettors	as required
turntables, glass spreader and alcohol, vortexes	
6" sterile metal capped tubes	20/group
28°C and 4°C incubator	
pH meter	2/lab
colony counters (available in room 201 or 204)	
sterile milk dilution bottles	4/group
sterile saline, 200 ml/flask - use in conjunction with sterile test tubes to prepare all required dilution blanks other than initial 10-fold dilution of soil	1/group
sterile saline, 90 ml/milk dilution bottle - use only for initial 10-fold dilution of soil	6/group
T-soy agar plates plus cycloheximide, for most groups this is the only media type required	30/group[a]
plastic containers (~500 ml to 1 liter) for soil samples/plastic resealable bags	4 each/group
all basic supplies found in your lab	

[a]more plates available upon request

Addition requested supplies available in lab (see schedule for time available).

Hemocytometer If required, ask TA to demonstrate use of the hemocytometer during scheduled lab time.
Pipetmen/tips.
Biolog plates, dispensed/labeled per project number, available in Student Supply Cold Box (4°C). Make sure you only take your group's project Biolog Plates as limited supply.
Varied Temperature Incubator: Please see Dr. Cameron the day before you require incubators set at different temperatures other than available in the lab.
Other requested supplies: anaerobic chambers, unique solutions, chemical treatments (e.g. motor oil, fertilizers....), additional media, etc.

Week 2
In lab discussion of group project experiment outline with instructor. The project experiment outline must be completed before coming to lab – use Word format provided, hand written or typed. Discussion with instructor must be completed before leaving the lab.

Week 4 to Week 9

Basic and requested supplies are available in lab.
Group carries out experiment using their own time schedule. See lab schedule for project scheduled lab times. See appendix for soil sampling procedure, dilution and plating procedure, etc. If using Biolog Ecoplates, determining soil dry weight, pH, etc. see Lab 2 procedure.

See lab schedule for Date
PROJECT PRESENTATION
Presentation schedule and marking outline will be posted in class and on the lab website prior to presentations.
All students must attend their section's presentations.
Section 1 starts at 1:00 pm (location TBA)
Section 2 starts at 3:00 pm (location TBA)

Time
~10 min presentation (maximum), ~5 min discussion
Students will be stopped if they go past 12 min. Be sure to time your presentation.
All members must be involved in the presentation.
Students will also be marked on asking relevant questions as a group.

Equipment Available
Installed computer and data projector available in classroom. Bring PowerPoint presentation on USB memory key. All lecture room computers now have Microsoft Office 2007.

PowerPoint Slide Presentation
(Reference: UTS Newsletter, The University of Manitoba, Vol 4 No. 3 January 1996)
-Do not use too many slides - put only relevant information on an overhead.
-Use large clear font. Use dark colors on light background or light colors on dark background. The text must stand out. Text should be in lowercase, not all in capitals - much easier to read. All caps looks like you are shouting.
-Use bullets unless you want to show rank or sequence then use numbers.
-Need at least 30 point font for titles and no less than 20 point font for text. Tables and figures should be legible to everyone in the room.
-Limit amount of text on each slide or overhead (if not a figure or table) - the ideal is 6 lines with 6 words per line.
-Allow sufficient space around diagrams.
-Keep tables and figures simple and brief.
-Two or more simple slides is better than one complex slide.
-Do not use undefined acronyms on the overhead or slide.

-If the material you are presenting is not your own, cite on overhead or slide.
-At the end include a slide of acknowledgments (group members or others, if applicable or may be included on title page) and references used in presentation.

Presentation guidelines
(marking allocation will be posted on the web page)
-good idea to write out the complete presentation
-**never read directly from written presentation**
-practice, practice, practice
-use report method of organization
-try to engage the audience, establish eye contact with audience
-keep presentation concise, lots of details are not necessary
-talk about the information on the slide, if necessary indicate information on the screen with (laser) pointer all the while maintaining as much eye contact with the audience as possible
-do not read word for word the contents of the slide
-be prepared for questions, write out possible questions and test your ability to answer these questions before giving the presentation
-if you cannot answer a question, do not try to bluff, you are not expected to know everything
–try to relax and have fun

Reminder - all reports must have an Honesty Declaration attached. See lab website for Honesty Declaration Sheet.

PROJECT WRITTEN REPORTS[5]
Submit only one project report per group.
The same mark is given to all members of the group. However, different marks may be assigned if any member of the group has not participated equally as indicated by peer evaluation.

Microbial soil Diversity Project PROCEDURE GROUP REPORT (see schedule for due date)
Must use Word document format available on lab website for report. Record requested information.
Date:
Project Number:
Project Name:
Group Members (record names in full):
1 1. State the objective and predicted results. No explanation is required at this time.
1 2. Record requested information in the following table. Assume all other basic supplies are available. Complete table after writing procedure.

Basic Supplies	**Number supplied**	**Indicate number required greater than supplied (see lab manual)**
sterile saline, 200 ml/flask - use in conjunction with sterile test tubes supplied to prepare all required dilution blanks other than initial 10-fold dilution of soil	1/group	
sterile saline, 90 ml/milk dilution bottle - use only for initial 10-fold dilution of soil	6/group	
T-soy agar plates plus cycloheximide	30/group	

Additional Supplies (add if not listed)	**Check if required and/or indicate number required**
anaerobic chamber plus supplies	
Biolog Eco Plates/P200/tips	
Biolog 90 ml sterile 0.2% water agar solution	
Hemocytometer/P20/tips	
*Incubator (Temperature = ____________)	

*Request temperature incubators (other than 28°C) the day before using.

---

[5] McMillan, V.E. 1997. Writing Papers in the Biological Sciences. 2nd ed. Boston: Bedford Books. 197 p

All other additional requested supplies are available in lab. Special supplies such as Biolog plates, agar plates, solutions on bench or in student cold box labeled with your project number.

4.5 3. Record requested experiment procedure information requested.
First read appendix or other experiments in your lab manual before answering this question to ensure that you do not duplicate experiment procedures.

a) Soil Sample Type(s) and Source(s):

b) Incubation Time and Temperature:

c) Method of Sample Collection:
Used MBIO 3480 Lab Manual Appendix procedure (state yes or no):
If applicable, state sample collection method changes from MBIO3480 appendix (if no change, state NA):

d) If applicable, list Varied Experimental Parameter
(e.g. pH values, soil type, soil depth, C-source, etc):
1.
2.
3.

e) If applicable, list treatment or pretreatment (state time and incubation temperature). If necessary, include all necessary concentration calculations. If this section does not apply to your group, state NA.

f) Record requested information in the following table.

Sample Analysis method**	Identical (state yes or no). If not used by your group, state NA.
Viable microorganism titre*	yes
Growth time*, r-selection (copiotrophs) compared to K-selection (oligotrophs)	
Total microorganism titre (hemocytometer)	
Biolog Ecoplate Soil Community Analysis **	
Colony characteristics***	

*Total microorganism titre and Growth time must be done by all groups identical to MBIO3480 procedure - plating dilution range requested in duplicate. This includes stated incubation time of the agar plates. When analyzing your results always compare bacteria titre not plate counts.
**For Biolog Ecoplate procedure refer to MBIO3480 lab manual, lab 2. You must use one Ecoplate per sample parameter, giving triplicate data.
***If colony characterization applies to your group you must answer yes above and describe exactly how you are going to collect and analyze colony characteristic data in part g.

CONTINUED ON THE NEXT PAGE . . .

g) For sample analysis methods that are not identical or details are not given in the MBIO3480 lab manual (no in the above table), record sample analysis method. Record only changes or additions made to the MBIO3480 lab manual procedure. Do not duplicate lab manual, just cite. Procedure must be an organized or marks subtracted. For this section you require only experiment procedure, do not include sample calculations here as required below in part 4.

1.5 4. In the following table, include all data manipulation **formulae** (no numerical calculations required). This must be separate from procedure.

Sample Analysis method	Formula (if not applicable, state NA)
Viable microorganism titre	
Growth time, r-selection (copiotrophs) compared to K-selection (oligotrophs)	
Total microorganism titre (hemocytometer)	
Biolog Ecoplate Soil Community Analysis	
Colony characteristics	

___
8

PROJECT REPORT (see schedule for due date)
Do not double space text. Create your own word document, word format not provided.
(i) Each student must submit a separate **group peer evaluation** (see form), do not attach to project report, do not attach to other group member's evaluation
(ii) Submit **one typed report per group**
(iii) Remember to number pages
(iv) Report is modeled on a journal article, not a typical lab report presentation
(v) Must follow format requested
(vi) Do not use personal language
This is a third year project. You must use recent journal articles (last 10 years) in your report. Good place to search for available articles is the ASM site http://journals.asm.org/ or highwire site http://highwire.stanford.edu/cgi/search/ . At the ASM site all papers (except last year) are available. Or search UM E library http://www.umanitoba.ca/libraries/elibrary/index.shtml using Goggle Scholar (excellent source.) The majority of papers will use molecular biology techniques to investigate microbial diversity **- incorporate this into your report.**

0.5 **Title**
-identifies major finding or point of view
-precise to allow accurate identification by indexes
-informative and concise
-accurate, do not overstate your findings
-organize title around key words
-include taxonomic names if pertinent
-avoid abbreviations and use terms familiar to your desired audience

1.5 **Introduction (maximum one page)**
-write from general to specific
-include information that gives background to your experimental findings
-discuss other relevant research, acknowledge research paper authors when discussing their work At least one relevant research article should be as recent as possible. For example, in 2005, Stub's group showed....
-information on techniques used (what specific information is obtained and how)
-be concise and specific to your area, presentation is prepared for your instructors and classmates who know the general area
-keep it short, maximum one page
-end the introduction by summarizing results in one sentence
-remember to cite references including a recent article

1.0 **Materials and Methods**
-include subheadings
-present in paragraph form ONLY using a separate paragraph for each different method
-include enough information that would allow a classmate to repeat the experiment (scientific experiments must be reproducible)
-if procedure found in lab manul, just cite
-any variation on lab manual procedures must be included here
-organize information
-describe any equipment, reagents, media, environmental conditions, etc. that differs from lab manual

-be concise, do not include unnecessary details
-do not include results

2.5 **Results**

-write in paragraph format, citing inserted figures and tables in text
-use past tense
-present results in an organized manner
-divide results into sections with appropriate headings or subheadings
-figures and tables must be inserted in text (similar to a journal article) not on a separate page and definitely not just appended.
-use text to present and summarize results, but do not analyze results in the result section
-do not include a table or figure of information that can be easily stated in one or two sentences
-all information must be relevant
-When analyzing viable cell count always compare bacteria titre using plate counts, not the plate counts..
-DO NOT INCLUDE FORMULAE OR CALCULATIONS IN THE RESULT SECTION, include in appendix as requested.

1.5 **Discussion (maximum of one page)**

-write from specific to general
-write with confidence
-be clear, concise and organized
-analyze results citing figures or tables and referring to your data, numerical if relevant
- explain what your results mean (if more than one explanation is possible, select the most likely and explain why)
-discuss your findings relevant to research done in this area (must cite literature)
-do your results support your hypothesis (predicted results)
-state limitations (errors)

2.5 **Appendix**

-include all data collected not presented in result section eg titration plate counts, Biolog data collection sheets, colony and cell physiology data, etc.
-in plate count data for viable bacteria/ml put an asterisk by each plate count that is significant (used to calculate titre, bacteria/ml)
-include formula and a numerical sample of each calculation type
Note: calculations must also be typed and data presented in an organized manner
-statistical analysis is not required, but include if wanted Note: cannot use ANOVA analysis when comparing only two samples, use general 2-sample comparison calculator, found at the same site as the ANOVA test. http://statpages.org/#Comparisons

0.25 **Acknowledgments**

-state what each project member contributed to the experiment for both the report writing and the project presentation.

0.25 **References**

-include a list of cited reference as outlined in general introduction

10

PEER EVALUATION (due same day as written project report)
Each member of the group must submit a separate completed group peer evaluation form (available on the next page or on the website). Do not attach to report.

MICROBIAL DIVERSITY GROUP PROJECT PEER EVALUATION FORM
Hand in INDIVIDUALLY through filing cabinet slot (same location as reports) in hallway across from the entrance to room 302 Buller. Do not attach to report.

Date: ________________

Student Name: ______________________ Student Number: ________________

Other group member names	**PEER CRITERIA EVALUATION** See footnotes for criteria[b] and rating scale[c].							Total
	1	2	3	4	5	6	7	

[b] criteria:

1. Student participated in the organization of the project tasks.
2. Student effectively carried out assigned lab work.
3. Student effectively participated in project report.
4. Student effectively participated in project presentation.
5. Student interacted well within the group and respected other group members.
6. Student contribution was essential for the completion of the project with respect to research, talent and thoughtful ideas.
7. Student used time productively and met deadlines.

[c] excellent = 5, good = 4, average = 3, fair = 2, poor = 1

Notes:
i) Do not base evaluation on friendship or personality conflicts.
ii) Evaluations will not be seen by group members.
iii) Your evaluation is a valuable indicator to help assess contributions in a fair manner.

LAB 1 NITROGEN FIXATION

Nitrogen fixation, the conversion of atmospheric nitrogen into organic nitrogen, is performed by numerous soil microorganisms (Table 1) and plant symbiotic nitrogen fixers. This lab focuses on *Azotobacter* and the symbiotic nitrogen fixer, *Rhizobium*. Each group will isolate *Azotobacter* from the environment and identify to the species level. The experiment is designed to eliminate all other bacteria. Secondly your group will isolate a pure *Rhizobium* species from a root nodule.

Table 1. A selection of Free-Living Nitrogen fixing Organisms[6]

Oxygen requirement	Chemoorganotrophs	Phototrophs	Chemolithotrophs
Aerobic	*Azotobacter spp.*	*Cyanobacteria*	*Alcaligenes*
	Azomonas		*Thiobacillus (some species)*
	Klebsiella		*Streptomyces thermoautotrophicus*
	Beijerinkcia		
	Bacillus polymyxa		
	Mycobacterium flavum		
	Azospirillum lipoferum		
	Citrobacter freundii		
	Acetobacter diazotrophicus		
	Methylomonas		
	Methylococcus		
Anaerobes	*Clostridium spp.*	*Chromatium*	Archaea:
	Desulfovibrio	*Thiocapsa*	*Methanosarcina*
	Desulfotomaculum	*Clorobium*	*Methanococcus*
		Rhodospirillum	
		Rhodospeudomonas	
		Rhodomicrobium	
		Rhodophila	
		Rhodobacter	
		Heliobacterium	
		Heliobacillus	
		Heliophilum	

Azotobacter (Bergey's Manual Group 4, Gram Negative aerobic bacteria)

[6]Madigan, M.T., Martinko, J.M., & J. Parker. 2000. *Nitrogen Fixation.* In: Brock Biology of Microorganisms. 9th ed. Upper Saddle River: Prentice Hall. p. 634.

Azotobacter are large, gram negative, catalase positive ovoid cells that can be isolated from soil and water. Bacteria occur singly, pairs (most often), clusters, or in chains. *Azotobacter* use nitrate, ammonia and some amino acids. Mannitol, a less readily fermentable C-source, permits the growth of the majority of *Azotobacter* species by keeping the medium aerobic. If a more readily fermentable sugar such as glucose or sucrose was used, the medium would quickly become anaerobic favoring anaerobic nitrogen fixers. The members of *Azotobacter* form distinctive thick walled smaller spherical resting cells known as cysts from vegetative ovoid (egg shape) shaped cells. The cytoplasm contracts and a double cell wall is formed. The cysts are resistant to desiccation but not to heat treatment. Azotobacter species (Table 3) are differentiated mainly on pigmentation and carbon utilization.

Elimination of other bacteria is based on the manipulation of media, environmental conditions and known characteristics of nitrogen fixing bacteria. Anaerobes cannot tolerate the presence of oxygen. Phototrophs require light. The elimination of other nitrogen fixing aerobes relies mainly on cellular characteristics – only a few nitrogen fixing aerobes form cysts: *Azotobacter*, *Beijerinckia, Methylomonas* and *Methylococcus*. Azotobacter is the only ovoid bacteria except for some species of *Azomonas.*

Table 3. Differential Characteristics of *Azotobacter* genus[7].

characteristic	*Azotobacter armeniacus*	*Azotobacter beijerinckii*	*Azotobacter chroococcum*	*Azotobacter nigricans*	*Azotobacter paspali*	*Azotobacter vinlandii*
motility	+	-	+	-	+	+
yellow-green fluorescent[a] water soluble pigment	-	-	-	-	+	+
green water soluble[b] pigment	-	-	-	-	-	+
brown-black water soluble pigment	-	-	-	+	-	-
brown-black to red violet water soluble pigment	+	-	-	+	-	-
rhamnose carbon source utilization	-	-	-	-	-	+
mannitol carbon source utilization	+	+	+	+	-	+

[a]use FN slant,
[b]water soluble pigments diffuse into the Ty medium, check color of medium around colony. Sometimes it is helpful to look at the agar plate from the side to check for water soluble pigments.

[7]Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T., & S.T. Williams. 1994. Group 4 Gram-negative aerobic rods. Bergey's manual of determinative bacteriology, 9th ed. Philadelphia: The Williams and Wilkins Company. p 135

Rhizobium (Bergey's Manual Group 4, Gram Negative aerobic bacteria)

Rhizobium are small short motile rods. Cells are pleomorphic (x, y, star and club shaped) under adverse conditions or when present in root nodules. *Rhizobium* uses a wide range of carbohydrates and organic acid salts as carbon source as long as there is a nitrogen source available. This group of soil bacteria is important to agriculture as they cause the formation of nodules on the roots of leguminous plants. Inside the nodules (anaerobic conditions) *Rhizobium* carries out symbiotic nitrogen fixation with the plant host. All species show host affinities. Differentiation of species is largely due to host specificity (Table 4).

Table 4. Differential Characteristics of *Rhizobium* genus[8].

host plant		*Rhizobium galegae*	*Rhizobium leguminosarum*			*Rhizobium loti*	*Rhizobium meliloti*
latin name	common name		*biovar viceae*	*biovar trifolii*	*biovar phaseoli*		
Pisum sativum, vicia hirsuta & Vicia sativa	Pea, broad bean and Vetch	-	+	±	±	-	-
Phaseolus vulgaris	Bush and Pole Bean	-	-	-	+	±	-
Trifolium repens	Clover	-	±	+	-	-	-
Lotus corniculatus	Lotus	-	-	-	-	+	-
Medicago sativa	Alfalfa	-	-	-	-	-	+
Macroptilium atropurpureum	Purple Bush bean - tropical	-	-	-	±	±	-
Galega orientalis, Galega officinalis	Oriental goat's rue	+	-	-	-	-	-

+ nodulates, ± sometimes nodulates and - does not nodulate

[8] Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T., & S.T. Williams. 1994. Group 4 Gram-negative aerobic rods. Bergey's manual of determinative bacteriology, 9th ed. Philadelphia: The Williams and Wilkins Company. p 169

PROCEDURE Students work in pairs for all of the following labs.
All *Azotobacter* mannitol and rhamnose medium contains 50 µg/ml cycloheximide (see appendix).

PART I: ISOLATION OF FREE LIVING NITROGEN FIXING BACTERIA
Note: For this lab and all subsequent labs, if you are unable to record data on specified day store plates at 4°C until you can record data (short a time as possible). However, motility may be affected.
Prior to coming to lab:
1. Day of lab or day before lab, collect a fresh soil sample (need only several grams). The best source of soil sample for this lab is waterlogged, low oxygen tension, fairly alkaline soil. Most of Manitoba soil is slightly alkaline.

Week 1
1. Add a small scoop of soil to 50 ml *Azotobacter* mannitol medium in a 250 ml flask.

2. Allow to stand for 1 week at 28°C in the dark until formation of a pellicle. If you do not know what a pellicle is, find out before coming to lab next week.

Week 2
Day 1
3. Streak two *Azotobacter* mannitol medium agar plates for single colonies using a loopful from the pellicle. Care must be taken when streaking, as Azotobacteraceae grow rapidly forming mucoid colony on *Azotobacter* mannitol medium agar (nitrogen free). Incubate at 28°C in the dark.

Day 2 ....
4. Growth should occur after one or two days - may take longer. If there is little growth on Friday, check plates on Monday. If you do not have isolated colonies, re-streak on *Azotobacter* mannitol medium agar. Do not discard your original plates, store in cold box.

5 Check cell morphology - must select a colony of large ovoid cells for further characterization. Do not proceed until you have a selected a colony of large ovoid cells. May need to go back to your original plates and re-streak on *Azotobacter* mannitol medium agar.

6. **Perform the following identification tests (see appendix for procedures):**
Motility (use only a freshly grown *Azotobacter* mannitol medium plate)
Gram stain (confirm with KOH string test) - must be gram negative
Colony water soluble pigment - streak on Ty agar plate. Water soluble pigments diffuse into the medium, check color of medium around colony. Sometimes it is helpful to look at the agar plate from the side to check for water soluble pigments.
Fluorescent pigment production on FN slant (after growth store slant at 4°C until next scheduled lab day when the TA will assist you viewing presence or absence of fluorescence. Remember that the culture is most likely dead after UV exposure.)
Growth in *Azotobacter* medium agar (mannitol)
Growth in *Azotobacter* medium broth (rhamnose)
Cyst formation (at least 7 days growth on *Azotobacter* mannitol medium agar) - must be present

PART II: ISOLATION OF SYMBIOTIC NITROGEN FIXING BACTERIA
Week 2

Prior to start of lab:

1. Day before or morning of lab collect a legume plant with roots that contain nodules. Root nodules appear as outgrowths on roots ranging from very small rectangular shape to large circular shape. It is best to put entire plant in a plastic bag and slightly moisten if soil is not already damp. If the soil is compact, it may be difficult to dig up a legume plant with intact root nodules. Take care when removing the soil from the roots. Also, as the plant gets older the root nodules fall off the roots easily. Possible legumes to collect: bush beans, peas, alfalfa, clover, vetch. Legume plants will be supplied in lab if you unable to find one.

2. Bring plant with root nodules to lab.

In lab procedure:

Note: Add enough solution (alcohol, bleach, or water) to the Petri plate to cover root nodules or as much as possible.

> Ethanol and bleach washes - traditional method for plant tissue sterilization. Both ethanol and bleach remove/kill any surface microorganisms (bacteria and fungi) without damaging the plant tissue.

1. Remove the root nodules (2-5 depending on size*) from legume plant. Record the color and shape of the nodule. Place root nodules in a Petri dish containing 95% EtOH for 30 seconds.
   *if bean, you only need two
2. Drain off ethanol. Remove remaining ethanol with a sterile Pasteur pipette. Add 10% bleach solution (sodium hypochlorite, NaOCl). Incubate for 1 min.

3. Drain off 10% bleach solution. Again remove remaining bleach with a sterile Pasteur pipette. Wash nodules with at least 3 changes of sterile water in petri plate. Make sure that bleach solution is completely removed.

4. Using sterile forceps crush the nodules to release *Rhizobium* from the nodules. There should be enough sterile water remaining in the bottom of the Petri plate to ensure a THICK suspension of *Rhizobium* released from nodules.

> As general dogma, whenever you are asked to streak an agar plate, it is implied that you streak for single colonies.

5. Using the phase contrast microscope (oil immersion objective, Ph3 or 4 condenser setting) microscopically examine crushed nodule solution for bacterium, recording shape, arrangement and motility.

6. Streak Ty agar plate for single colony.

7. Incubate plates at 28°C in the dark. After 4 days (Monday) record *Rhizobium* colony characteristics on Ty agar plate. Require a minimum of 5 characteristics, see appendix for details.

LAB 1 NITROGEN FIXATION REPORT (Must use Word Format available on lab website.)
Date:
Group #:
Group names:
Group or Individual Report:
Indicate group members name if individual report:

Part I: Isolation of Free Living Nitrogen Fixing Bacteria

3.5 1. In your MBIO3480 all bacteria but *Azotobacter* are eliminated from the soil sample. In the following table state the experimental procedure(s) that eliminate the listed bacteria or bacteria groups.

Table 1. Experimental procedures permitting the isolation of *Azotobacter* genus from soil.

Group or microorganism eliminated.	Experiment procedure.	State reason eliminated with respect to experiment procedure.
Eukaryotic fungi		
Non-nitrogen fixing bacteria		
Phototrophs		
Anaerobes (require two procedures)		
All chemoorgantrophs and chemolithotrophs listed in Table 1 except *Azotobacter*, *Beijerinkcia, Methylococcus* and *Methylomonas*		
All chemoorgantrophs and chemolithotrophs listed in Table 1 except *Azotobacter* and some species of *Azomonas*		

0.5 b) Many of the bacteria listed in Introduction Table 1 are eliminated by more than one *Azotobacte*r isolation experiment procedure. State an additional elimination procedure (other than stated in the above table) for *Streptomyces thermoautotrophicus*.

CONTINUED ON NEXT PAGE

3 2. Record the following *Azotobacter* identification test results in table. It is possible that not all tests will agree with the bacteria you select. If a test results differs, give your result and state the expected results in brackets.

Table 2. Identification test results required to identify soil isolated *Azotobacter* to the species level.

Identification Test	Test result and description (must include color, shape, etc. if applicable)
cell shape	
gram stain	
motility	
fluorescent water soluble pigment (FN slant)	
water soluble pigment (Ty plate)	
cyst formation	
growth in *Azotobacter* medium (mannitol)	
growth in *Azotobacter* medium (rhamnose)	

Conclusion: The bacterium isolated is ______________________________.

CONTINUED ON NEXT PAGE

Part II: Isolation of Symbiotic Nitrogen fixing Bacteria – *Rhizobium*

2 5. Record the following *Rhizobium* identification test results in table.

<table>
<tr><td colspan="6">Table 3. Characterization of Rhizobium isolated from legume root nodule.</td></tr>
<tr><td colspan="6">Host plant (common name):</td></tr>
<tr><td rowspan="2">Cellular Morphology</td><td>Shape</td><td colspan="2">Arrangement</td><td colspan="2">Motility</td></tr>
<tr><td></td><td colspan="2"></td><td colspan="2"></td></tr>
<tr><td rowspan="2">Colony Characteristics on Ty
Incubation time: ____
Incubation temperature: ___</td><td>Size</td><td>Color</td><td>Margin</td><td>Elevation</td><td>Light reflection</td></tr>
<tr><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td colspan="6">Bacteria Name (genus, most likely Rhizobium species[a] and if applicable biovar):</td></tr>
</table>

[a]do not base your answer on ± sometimes nodulates, must be + nodulates

1 6. After isolation of *Rhizobium* from the root nodule it is streaked on Ty agar - state nitrogen source utilized by *Rhizobium.*

___
10

LAB 2 FUNCTIONAL MICROORGANISM DIVERSITY

Biolog™ Functional Diversity

It has been shown that Biolog plates are a sensitive method to measure functional potential[9] in the soil. Albeit, with cautions discussed by Gamo and Shoji, 1999 and other researchers. The inoculum density, bacteria cell reserves of carbon and incubation conditions greatly influence the Biolog plate results. The concentration of substrate in the Biolog wells is greater than encountered in most soils and depending on environmental source the C-sources available may be very different. The population in the wells after growth does not reflect the original population just amplifies bacteria best suited to the growth conditions and nutrients supplied by the Biolog plate. However, Biolog data is excellent for comparison of effects of various environmental parameters on a soil community or allowing the researcher to following change of microbial diversity with time.

Each well contains the redox dye tetrazolium violet (clear). The assay is based on the microorganism(s) ability to oxidize the carbon source and in doing so irreversibly reduces tetrazolium violet to a purple insoluble formazan. Color development requires sufficient cell density, $\sim 10^8$ cells/ml[10]. In your lab you will use a Biolog™ EcoPlate that contains 31 varied carbons sources and 1 water control in triplicate (Table 1). The selection of carbon sources is based on high consumption by soil microorganisms. http://www.biolog.com/pdf/eco_microplate_sell_sheet.pdf assessed July, 2008).

Table 1. Classification of Biolog™ EcoPlate carbon sources.

saccharides & derivatives	oligo-/poly-saccharides	sugar alcohols & derivatives	amino acids & degradative product[a]	organic acids & derivatives	detergent (non-ionic)
α-D-lactose	cyclodextrin	erythritol	L-arginine	pyruvic acid methyl ester	Tween-40[b]
D-xylose	glycogen	mannitol	L-asparagine	glucosaminic acid	Tween-80[c]
D-cellobiose		D,L-α-glycerol phosphate	L-phenylalanine	D-galactonic acid γ-lactone	
β-methyl-D-glucoside			L-serine	D-galacturonic acid	
glucose-1-phosphate			L-threonine	2-hydroxy benzoic acid	
N-acetyl-D-glucosamine[d]			glycyl-L-glutamic acid	4-hydroxy benzoic acid	
			putrescine	hydroxybutyric acid	
			phenylethylamine	ltaconic acid	
				α-ketobutyric acid	
				D-malic acid	

[a]putrescine breakdown product of the amino acid ornithine by removal of CO_2 [b]polyoxyethylene sorbitan monopalmitate [c]polyoxyethylene sorbitan monooleate [d] amide between glucosamine (saccharide) and acetic acid

---

[9]Smalla, K., Wachtendorf, U., Heuer, H., Liu, W-T, Forney, L. 1998. *Analysis of BIOLOG GN substrate utilization patterns by microbial communities.* Appl. Env. Micro. 64:1220-1225.

[10]Gamo, M, Shoji, T. 1999. A method of profiling microbial communities based on a most-probable-number assay that uses Biolog plates and multiple carbon sources. Appl. Env. Micro. 65: 4419-4424.

The data is used to calculate % functional diversity for each set of data.

$$\% \text{ functional diversity} = \frac{\text{number of positive carbon source wells}}{\text{total number of carbon source wells (31)}} \times 100$$

Statistical Analysis

VasarStats website http://faculty.vassar.edu/lowry/VassarStats.html to calculate One-Way ANOVA for 3 independent samples.

ANalysis Of VAriance (ANOVA) is used to determine if soil type has any effect on the microbial community diversity as assayed using BIOLOG Ecoplate. ANOVA statistical analysis is used since we are comparing more than two samples at a time. ANOVA is expressed as F-ratio. F-ratio = standard deviation of the group/expected variation of the group. If there is no effect (supports the null hypothesis) this value should theoretically be 1. If there is an effect due to soil type the value should be greater than one. How much greater depends on the significant level.

The simplest way to interpret results is to look at the P value which is the probability of the null hypothesis being true, that is, the difference between the sample groups is a random event. If the probability is less than 0.05 probability (95% confidence, scientifically accepted level) or 0.01 probability (99% confidence), then there is a good chance that the difference observed is due to the variable being studied, in our case, soil type.

PROCEDURE

Week 3

The operation of the P200 pipetman will be demonstrated in the pre-lab. The appendix also gives detailed instructions on P200 operation. Make sure you know how to read the volume setting. Do not turn above 200 μl. Use the P200 to measure 150 μl. If you are not familiar with Pipetmen operation, confirm with the TA that you have the correct setting and know how to use the pipetman before pipetting samples.

1. Soil samples provided in lab: clay, sand, compost (high organic). Soil samples were collected as described in lab manual appendix. Each group uses only one type of soil sample. See table for type of soil sample your group uses.

Soil Type	Group Number
sand	1,4,7,10,13,16,19,22,25,28
clay	2,5,8,11,14,17,20,23,26,29
compost	3,6,9,12,15,18,21,24,27,30

2. Determine% Moisture of your soil sample (Wall & Londry, 2002[11]).
(a) Label the outside of the aluminum dish with your name and soil type (do not use masking tape).Weigh and record weight of aluminum dish.
(b) Put a heaping spoon of your soil in weigh dish. Weigh and record weight to one decimal place.
(c) Place soil sample in tray provided. The soil samples will be dried for ~16 h at 100°C.
(d) **NEXT DAY** the tray containing the samples will be available in the lab after 11:00 am. As soon as possible weigh and record the weight.
(e) Determine % moisture content as moist weight of soil minus dry weight of soil divided by moist weight of soil times 100%.

3. Determine pH of soil (Wall & Londry, 2002[6]):
(a) Measure 5 g of soil (original sample) into large screw capped test tube containing 20 ml 0.01 M $CaCl_2$ solution. Replace cap and shake to mix.
(b) Allow the soil to settle. $CaCl_2$ solution promotes the settling of the soil components with little effect on pH.
(c) Read pH of supernatant with pH meter (refer to appendix for operation of pH meters). Remember to turn off pH meter and rinse probe with distilled water before returning holding solution (pH 4 buffer).

[11]Wall, M., Londry K. 2002. 60.228 Microbial Ecology Laboratory Manual. Department of Microbiology, University of Manitoba.

4. <u>Prepare soil dilutions:</u>
   Add 10 g of your soil sample to 90 ml sterile 0.2% water agar solution (10^{-1} dilution). Shake <u>vigorously</u> for 2 min. Using a sterile 25 ml pipette, transfer 10 ml of the 10^{-1} dilution to 90 ml saline solution (10^{-2} dilution). Mix by shaking. Using a sterile 10 ml pipette, transfer 10 ml of the 10^{-2} dilution to 90 ml saline solution (10^{-3} dilution). Mix by shaking.

5. <u>Inoculate BIOLOG EcoPlate</u>:
   Remove the EcoPlate from sterile foil container. Use the P200 pipetman to transfer exactly 150 µl (0.15 ml) of saline diluted sample to each well (96 wells) of a Biolog™ EcoPlate. The dilution will form a soft gel within seconds. Be sure to shake the sample dilution frequently during inoculation of microplate. Work as quickly as possible to reduce contamination. Replace microplate cover, put inside a large plastic zip bag, seal and incubate at 28°C for 96 hours. A bucket of water has been placed in the incubators to ensure a moist environment.

6. **Monday:** Read Biolog Ecoplate color development from plate top to ensure class consistency. To determine a positive well compare color density in all wells to control well, A-1 average. All wells lighter than A-1 are negative. If there is a noticeable purple color, highlight well. Do not highlight wells with small purple flecks, they are negative. Record all requested information on data sheet. For each replica record the number of positive wells. Discard Biolog plates in plastic lined bucket located on front table.

7. **Hand in ONE COPY of your group's MicroPlate™ EcoPlate DATA SHEET Tuesday by 2:30 pm.** Data does not require an Honesty Declaration attached. Make sure to include all requested information. Class data will be collected, and available on lab website as soon as possible for ANOVA statistical analysis. MicroPlate™ EcoPlate DATA SHEET available as a Word document on lab website. Save file before adding requested information.

MicroPlate™ EcoPlate DATA SHEET (available as Word document on lab website)
Group #: _____________
Group names: ____
Date: ___________________________
SOIL TYPE: ______________________
SOIL % moisture content: ____________ (**must include % moisture value**, however, sample calculation is only required for lab report not class data collection)
SOIL pH: ______________
Record number of positive well: replica 1______ replica 2_______ replica 3 _____

|----------replica 1 ---------------|---------replica 2------------------|----------replica 3----------------|

BIOLOG Microbial Community Analysis
EcoPlate™

A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine
B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine
C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine
D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine
E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine
F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid
G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine
H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine

FIGURE 1. Carbon Sources in EcoPlate

% moisture calculation (only required for lab report not class data collection):
[must be on this page]

http://www.biolog.com/pdf/ECO%20MicroPlate%20Sell%20Sheet.pdf accessed July, 2010 (source of EcoPlate data sheet)

LAB 2 REPORT (Use Word Format and Excel Format available on lab website.)
See Lab 2 appendix for excel functions and graph procedure.

1.5 1. a) Include your group's completed MicroPlate™ EcoPlate data sheet (Word Format, typed). Record requested information on data sheet and highlight the carbons sources utilized. Include footnote to explain your method of highlighting if highlighter not used. Include requested % soil moisture calculation.

2 2. a) Include a complete Class Data Excel Worksheet. This includes class % functional diversity values, average % functional diversity, average pH, average % moisture content, and standard deviation for each parameter. Show requested group % functional diversity calculation below table. Print all requested information to fit one page - calculation must be on the same page as the table.

3 b) Using average class data present an Excel figure. The graph must be a bar plot. Plot average % functional diversity & average % moisture content on one y-axis and pH on the other y-axis for sand, clay and compost on x-axis. Each bar graph should have a standard deviation bar. See lab 2 appendix for details on how to set up figure. Also see basic figure presentation in general instructions. Print figure on a separate page.

1 Also answer question on this worksheet.
QUESTION: Only taking into consideration graphical presentation of class data indicate whether soil type has a significant effect on % microbial functional diversity with respect to soil type combinations. Hint: Must consider standard deviation bars on graph.
Answer YES or NO for each, no other answer accepted:
SAND with respect to COMPOST: __________
SAND with respect to CLAY: _____________
COMPOST with respect to CLAY: ___________

1.5 3. Use ANOVA analysis of variance for independent samples to determine soil microorganism % functional diversity P-value for class data. See Lab 2 appendix for VassarStats instructions. Clearly write requested information on printout - **no other location in report acceptable** otherwise marks subtracted.
(i) Include a copy of **printable report** (Vassar Stats) of statistical analysis of class data.
(ii) On printout (must be here) state data source and define values entered for sample 1, sample 2 and sample 3.
(iii) On printout define P-value.
(iv) On printout by P-value state experiment conclusion (one sentence) with respect to P value result. Conclusion must be specific to experiment performed.

__
10

LAB 2 APPENDIX
MS EXCEL
There are a variety of methods to carry out Excel operations. Only one way is described here.
Hints:
(i) Right click on whatever you want to change and select appropriate item from the pull down menu.
(ii) **Make use of copy and paste, once you have designed a formula, just copy and paste for like calculations. This makes % functional calculations easy.**
(iii) When determining mathematical functions, spaces are not considered. eg. AVERAGE for a column of numbers with blank spaces
(iv) Use the CNTRL key to select scattered data, not in row when setting up graphs
Functions are described using Excel 2003 but are similar in 2007
CELL FORMULAE NOTATIONS
-start equation with an = sign, do not leave spaces between entries
-use mathematical brackets wherever required
* multiply
/ divide
+ add
- minus
^power

SUM
Put the cursor in the cell where you want to record SUM . Select AUTOSUM button (∑). The program buttom automatically selects cells, if this differs from what you want, use the cursor to select numbers you want to add.. Press ENTER. Or use pull down menu - select Insert, function, statistics, then SUM. Click OK. A pop-up menu appear, use or just move out of the way if necessary. Using your mouse click the first or last1 cell of data set. Hold down button and scroll down to the last or first cell in the data set. Release button. Click OK on pop-up menu or press enter. The SUM appears in your selected cell. If required, repeat for remaining data sets.

AVERAGE
Put the curvsor in the cell where you want to record average. Select paste function button (f_*), then statistics (or defaults to most recently used), then AVERAGE. Or use pull down menu - select Insert, function, statistics, then AVERAGE. Click OK. A pop-up menu appear, use or just move out of the way if necessary. Using your mouse click the first or last1 cell of data set. Hold down button and scroll down to the last or first cell in the data set. Release button. Click OK on pop-up menu or press enter. The average value appears in your selected cell. Repeat for remaining data sets.

STDEV (standard deviation).
Put the cursor in the cell where you want to record standard deviation. Select paste function button (f_*), then statistics (or defaults to most recently used), then STDEV. Or use pull down menu - select Insert, function, statistics, then STDEV. Click OK. A pop-up menu appears, use or move out of the way if necessary. Using your mouse click the first or last cell of data set. Hold down button and scroll down to the last or first cell in the data set. Release button. Click OK on

pop-up menu. The standard deviation value appears in your selected cell. Repeat for remaining data sets.

BAR GRAPH ON TWO AXES CHART

Changes to graph may be made at any time by right clicking or double clicking the appropriate area of the chart.

Excel 2003 Instructions

1. Select CHART from INSERT pull down menu.
2. Click next button. Defaults to data range tab.
3. Select series location, row (most likely) or column. This depends on how you set up your spreadsheet. Put cursor in data range box. If there is information already entered in box, delete before proceeding. Hold down CNTRL key, put cursor on first entry of average % functional diversity, click, put cursor on second entry of average % functional diversity, click, and repeat for last % functional diversity. Release mouse button. Range is now entered in the box.
4. Select SERIES tab.

   Series 1 (Functional Diversity)

   Put cursor in category (x) axis labels: box. Hold down CNTRL KEY, put cursor on first entry, ie sand, click, then clay, click and finally compost, click). X-axis labels are now entered. Put cursor in Name box. Type in name - average % functional diversity or select column header box (this gives the correct name in the legend box).

   Series 2 (pH)

   Click **Add** under series box - a new series is added to the box. Put the cursor in the Values box. Delete information in box. Put the cursor in spreadsheet and select pH data as above. Put cursor in name box. Type in name - pH or select column header box. Click

   Series 3 (% moisture)

   Add under series box - a new series is added to the box. Put the cursor in the Values box. If required, delete information in box. Put the cursor in spreadsheet and select % moisture data as above. Put cursor in name box. Type in name - % moisture or select column header box.
5. Click next. CHART OPTIONS menu appears. Under default TITLES tab, enter title and y-axis label (% functional diversity and % moisture content only as you are going to make a second y-axis for pH). Press NEXT. Comments: Unfortunately the title defaults to the top of the graph. After you are finished setting up graph, just select and move below graph. Mostly likely you will need to move the graph up. May require resizing. Best to reduce font size to 10 point. Change background to white by double click background (or right click). Keep legends box. Or right click and clear whatever you do not want.
6. Keep the default - graph in spreadsheet.
7. Click FINISH.
8. All data is plotted on one y-axis. Often pH value is relatively small compared to % functional diversity and moisture content. Put the cursor on any pH bar and right click. Select FORMAT DATA SERIES. Select Axis tab. Click OK. Select Secondary Axis

should be inserted but there is no label. Put the cursor on an empty space in the graph, right click. Click CHART OPTIONS. Under default TITLES tab enter name of secondary y-axis.

9. Insert STANDARD DEVIATION bars for average % functional diversity data. Right click a bar of the series (average % functional diversity)on the graph to select all bars in that series and to bring up menu. Click FORMAT DATA SERIES. Select Y ERROR BARS tab. Select display BOTH. Select CUSTOM. Put the cursor in the + box. On the spreadsheet hold down the CNTRL key and put cursor on first entry of standard deviations, click, second, click and third, click. Range is now entered in the box. Put the cursor in the - box. Repeat the same standard deviation data selection. A standard deviation bar length is equal to + and - standard deviation, ie 2x SD. Click OK. Standard deviation bar appear on the appropriate columns. Repeat for pH and % moisture standard deviation using the appropriate data.
10. Right click on any % functional diversity bar. Select Format Data Series from menu. Click Format Data Series. Select Options tab. Adjust overlap and gap width by scrolling up or down until bars do not overlap. Press OK.
11. Select chart. Print chart on separate page.

Excel 2007 Instructions

1. Select Insert Menu. Select 2-D Column, ie first type, Clustered Column.
2. Chart Tools, Design is now selected. Select Data. Select Data Source box appears. If any values are in the top box, chart data range, delete, you do not use this box. Under Legend Entry Series, select Add. Cursor defaults to Series Name, type % functional diversity or select cell containing name. Delete default information in series values box - insert functional diversity class average data select sand value, hold down CNTRL key, select clay value and compost value. Press OK. In the horizontal category axes labels, 1, 2 and 3 should be listed. Select Edit as need to change to sand, clay and compost. Axis range box appears, select sand name cell, hold down the CNTRL key, select clay, then compost name boxes. Click OK. You return to main box. Repeat process adding pH and % moisture information. Click OK.
3. All data is plotted on one y-axis. Often pH value is relatively small compared to % functional diversity and moisture content, therefore, must include second y-axis. Put the cursor on any pH bar and right click. Select FORMAT DATA SERIES. Select Secondary Axis. This inserts a secondary axis for pH. Unfortunately it may change the primary axis range to -10 instead of zero. You need to correct this. Right click on the primary axis. Change minimum range to fixed and type in zero. The problem with two axes is that the bars are no longer aligned side by side - see below (#7) for instructions to correct this.
4. Go to Chart Tools, Layout, Axis Titles to add axis titles to primary and secondary axes.
5. Got to Chart Tools, Layout Chart Title to add Figure # and Title. Defaults to top and font is large. Move box below graph. [You also need to reduce size of font - select title text box then select format under chart tools and reduce font size A ▾.] OR select first chart layout under Chart Tools, Design and edit to fit required presentation. Legend defaults to below graph, this is fine, just increase chart box height to accommodate title and legend, move and change font size to fit.

6. Insert STANDARD DEVIATION bars for average % functional diversity data. Select % functional diversity bars by clicking on one bar. Select Layout, Error Bars ▾, More Error Bars Options, select Custom under Error Amount, click Specify Value, a Custom Error Box appears, remove all default information in boxes. In the positive error box select (while holding down the CNTRL key) in same order as entered bars, sand, clay and compost select average SD for each. Repeat with same values for negative error box. This should work - sometimes Excel gives an error message, but just make sure you have completely deleted default information in the boxes. Repeat for pH and % moisture. Again you may need to reset y-axis to zero by right clicking on the vertical primary axis to remove default negative value, see above.
   OR right click a bar of the series (average % functional diversity)on the graph to select all bars in that series and to bring up menu. Click FORMAT DATA SERIES.
7. Bar alignment adjustment: Right click on any pH bar. Select Format Data Series from menu. Adjust series overlap by scrolling side to side until bar overlap is reduced. I found 0% separated overlapped and 500% (maximum) gap width is fine. Press OK. Right click on any % functional diversity bar. Select Format Data Series from menu. Adjust series overlap. I found -70% separated overlapped and 250% gap width is fine.
8. Print table and calculations on one page. Select Page Layout, Print Area ▾, set print area, then select area. Check to make sure all information to be printed is included. If the entire chart box is not within the selected area, it will not print. Print to fit ONE portrait page - make sure page layout is set to 1 page for both width and height. Select chart. Print chart on separate page. As default chart prints on spreadsheet if inside area selected to print, to change go to chart location, found under Chart Tools, Design, Move Chart Location to separate sheet. Print to one page.

ANOVA Statistical Analysis of Class Data (VasarStats website)
home page: http://faculty.vassar.edu/lowry/VassarStats.html
link also available on lab website

1. The link is to the VassarStats homepage. There is a Browser alert on this paper. If you encounter problems, follow instructions. Select ANOVA from left hand column.
2. Select One-Way ANOVA for up to five samples.
3. **Read instructions** on this page. Scroll down to Setup. Enter 3 as the number of samples. Enter 3 in space after independent samples. Leave default setting as Correlated samples weighted.
4. Go to Data Entry. Copy paste each series of Excel data in three boxes, one for sand, clay and compost % functional diversity respectively. You need to delete empty lines after the last value in the series; cursor should be beside the last value not below. Just use the backspace key. If applicable, remove any other empty lines in your data.
4. Click Calculate button.
5. Once you click calculated data is entered. Click Printable Report. Print for report. Caution that P value is reasonable for your data - a P value of 1 is very unlikely indicating an error has been made entering required information.

LAB 3 ANOXYGENIC PHOTOTROPHIC BACTERIA: Photosynthetic Pigments

Both the purple and the green sulfur bacteria are anoxygenic phototrophic bacteria. Light energy from photons is absorbed by the bacteriochloropyll pair which in turn becomes a strong electron donor for electron flow generating the production of ATP. The reaction is complete when cytochrome c_2 donates an electron back to the bacteriochlorophyll pair, i.e. cyclic photophosphorylation. The electron donor for autotrophy, CO_2 reduction to cellular material (photoautotrophic assimilation) comes from sulfide (H_2S), although elemental sulfur, thiosulfate ($S_2O_3^{2-}$) and Fe^{2+} may be used by various species for electron donation. The H_2S in the process is oxidized to elemental sulfur and then sulfate (no longer stored).

Purple non-sulfur bacteria are predominately photoheterotrophic with development dependent on simple organic substrates such as alcohols, fatty acids, hydroxy- and keto- acids which are photo assimilated or serve as electron donors for carbon dioxide assimilation. In addition, most species can also grow photoautotrophically using carbon dioxide and hydrogen or hydrogen sulfide.

Most often ammonium salts are the nitrogen source. However, many phototrophic bacteria fix nitrogen.

The phototrophic bacteria are divided into seven subgroups[12] by virtue of their physiological properties and content of carotenoid and photosynthetic pigments. In this lab we will examine the photosynthetic pigments of three subgroups.

Subgroup	Category	Location	Characteristics	Example genera
1 Purple sulfur bacteria	Photoautotrophic	anoxic aquatic where H_2S accumulates	-oval or rod shaped motile cells -elemental sulfur stored inside the cell -cultures purple or red purple	*Chromatium*
3 Purple nonsulfur bacteria	photoheterotrophic or photoautotrophic or aerobic/anaerobic growth in dark (chemolithotrophic or chemoorganotrophic)	aquatic environment with low H_sS, with or without oxygen	spiral motile cells purple or red culture	*Rhodospirillum*
5 Green sulfur bacteria	photoheterotrophic or photoautotrophic	anoxic aquatic where H_2S accumulates -most tolerant of high sulfide and low light	-straight or curved non-motile rods -sulfur deposited outside the cell -cultures are green or brown. Non-motile rods found often in chains	*Chlorobium*

[12]Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T., & S.T. Williams. 1994. *Rhizobium, Anoxygenic phototrophic bacteria*. Bergey's manual of determinative bacteriology, 9th ed. Philadelphia: The Williams and Wilkins Company. p 353.

The Winogradsky column

The Winogradsky column is a habitat-simulating device for the enrichment of photosynthetic bacteria. In the thick mud of the Winogradsky column, the purple and green sulfur bacteria often develop as well defined colonies, visible to the naked eye. The nonsulfur purple bacteria, the least tolerant of sulfide and most tolerant of low oxygen tension are found in the water column. In this lab the Winogradsky column will be used as the source of anaerobic phototrophic bacteria for isolation of phototrophic pigments. Bacteria are harvested from the lower part of the Winogradsky column, high sulfur and anaerobic, select only green and purple sulfur bacteria. *Rhodospirillum rubrum* that has been grown in reduced light to promote the production of photopigments will also be used in the lab.

Photosynthetic Pigments

Bacteriochlorophylls absorption maxima change upon extraction[13] (Table 1). Purple bacteria have either bacteriochlorophyll a or b and the green sulfur bacteria have predominately either bacteriochlorophyll c, d or e. The purple non sulfur bacterium *Rhodospirillum* has only bacteriochlorophyll a. The color of the bacteria is also influenced by the presence of carotenoids.

Table 1. Absorption maxima (nm) for bacteriochlorophylls.

Bacteriochlorophyll	Absorption maxima (nm)	
	whole cells	acetone extracts
BChl a	375, 590, 805, 830-911	358, 579, 680*, 771
BChl b	400, 605, 835-850, 986-1035	368, 407, 582, 795
BChl c	457-460, 745-755	433, 663
BChl d	450, 715-745	425, 654
BChl e	460-462, 710-725	459, 648

* usually only a shoulder peak

[13]The phototrophic way of life by Jörg Overmann and Ferrau Garcia-Pichel http://www.mbl.ku.dk/mkuhl/ambio/materials/The%20Phototrophic%20Way%20of%20Life.pdf accessed July 2007

PROCEDURE
Week 4& 5
Week 4(Section 1 students only start at 2:30 pm **in room 204 Buller**)
Week 5 (Section 2 students only start at 2:30 pm in **room 204 Buller**)
CAUTION: NO BURNERS, NO FLAMES (solvents in lab).
This is a long lab - come prepared to stay the three hours (waiting is required for sonicator and spec4000 analysis).

1. Each group should collect one culture of *Rhodospirillum rubrum*[a] and one Winogradsky column sample[b]. **Return all leftover *Rhodospirillum rubrum* cultures to rack on supply bench, do not discard.**
   [a] *Rhodospirillum rubrum* was grown for 4 weeks at room temperature (24°C) in Van Niel's yeast medium (large screw capped tube filled almost to top). Tubes were placed in a north facing window in cardboard containers (reduced light).
   [b] Winogradsky sample was collected by pouring off the contents of a large Winogradsky column. The purple and green colonies remained in column as they adhered to the lower inner surface of the container. The bacteria were suspended in pond water and labeled Wingradsky sample (contains both green and purple sulfur bacteria). Winogradsky sample was filtered through cheesecloth to remove large particle debris.

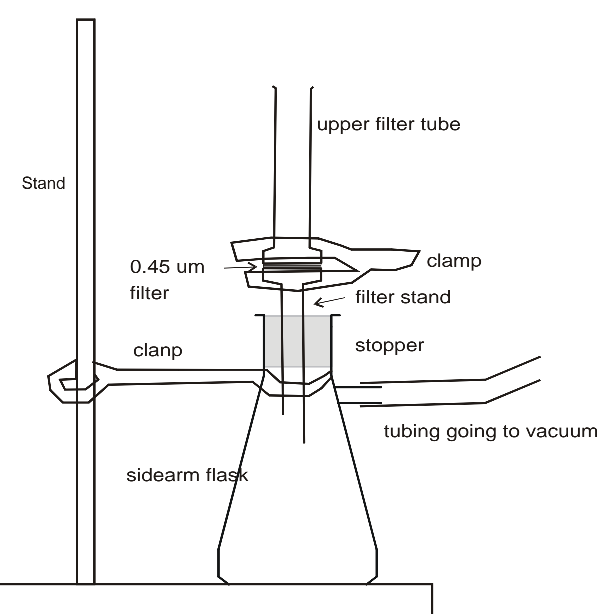
Part I: Spectrophotometric absorbance scan of live purple non-sulfur bacteria, *Rhodospirillum rubrum*.
[Part I may be done at any time during the lab ideally when the line-up for the spec3100 is short.]

1. **Before** going to the spec remove 1 ml *Rhodospirillum rubrum* culture and transfer to a 5 inch test tube (May need to dilute with Van Niel's yeast medium - if concentration is too high 350 nm peak must be below 3 abs- should be light red, take from top before shaking, dilute with yeast medium if necessary). Add 0.1 ml 50% sucrose. Place a small piece of parafilm over the top and mix by inverting 3 to 5 times. The sucrose reduces background interference at the lower wavelengths.

2. Scan your sample on the spec3100. A TA will help you scan your sample (320 nm to 900 nm). The blank (reference) is uninoculated medium. Refer to spec3100 operation for procedure details. Your scan data is collected as a Biochrom Excel spreadsheet. Live *Rhodospirillum rubrum* Biochrom Excel spreadsheet will be available on lab website.

Part II: Extraction of photosynthetic pigments
CAUTION: Wear gloves throughout procedure. Acetone is a hazardous chemical. NO burners, no flames.
1. Carry out the extraction procedure for two samples, (i) *Rhodospirillum rubrum* and (ii) Winogradsky sample (contains purple and green sulfur bacteria).
2. Collect 4 glass filters (this is not the glass filter stand), one for each sample in duplicate. Set up vacuum filter apparatus making sure to place the Whatman GF/C glass filter (0.45 μm) on the glass filter stand. Vacuum filter 1 - 5 ml of each sample. Add 1 ml at a time. If filtration slows (only 1 drop coming through the filter every 10 sec), do not add any more sample. Vacuum filter your sample until all the liquid in the upper filter tube is gone. If this is taking too long, turn off vacuum and pour off remaining liquid in upper

tube. Turn on vacuum and continue to filter until no liquid remains in upper tube. Carry out this procedure twice for each sample, *Rhodospirillum rubrum* and Winogradsky.



3. After turning off the vacuum and dismantling the vacuum filter apparatus, remove the filter with forceps. Place filter in a 2ml Eppendorf tube with the filter top (cells) facing inwards. Use the forceps to arrange the filter around the inside edge of 2 ml Eppendorf tube (larger than basic Eppendorf tubes) creating a hole in the middle. Repeat with second sample filter on opposite inside, (two filters of the same sample per tube). Add 1.8 ml extraction solvent (19:1 acetone:water) in the center of the tube. After you are finished filtering samples, take apart apparatus, rinse and put on discard trolley.

4. Most likely the sonicator* is located in room 313, room location will be confirmed in pre-lab. The TA will help you with the sonication of your samples. Put your sample on ice for at least one minute prior to sonication (bucket of ice available outside sonicator room). Sonicate for 30 seconds using the small probe. Place sample on ice for a minimum of 15 seconds. Again, sonicate 30 seconds. Slowly remove filter paper allowing as much liquid as possible to remain in Eppendorf tube and discard in organic waste container in the fumehood.
CAUTION: must wear earphones when sonicating and close lab door.

Sonication: mechanical disruption (sheering) of cells by ultrasound (20-50 kHz). High-frequency sound waves (ultrasound) are generated by inserting a probe into the sample (see lab websiste for picture). Samples sonicated for short intervals with placement on ice between pulsing to dissipate heat generated by ultrasound.

5. Micro-centrifuge your sample for 5 min to pellet debris.

6. For each sample carefully transfer ~0.9 ml supernatant to two Eppendorf tubes (one for concentrating and one for ultraspec analysis). Discard pellet in organic waste container in the fumehood.

Part III: Spectrophotometric absorbance scan of acetone extracted Winogradsky sample purple and green sulfur bacteria.

1. A TA will assist you with the scanning of your two extracted samples using the specphotometer3100 (320 nm to 900 nm). The blank (reference) for the scan is acetone:water (19:1). Your scan data is collected as a Biochrom Excel spreadsheet,

Acetone extracted samples rubrum Biochrom This Excel spreadsheet will also be available on lab website.

2. Do not discard sample after scanning. Return sample to eppendorf tube for TLC procedure. Cover samples with foil. With time, light destroys the pigments.

Part IV: Thin Layer Chromatography (TLC) Protocol
The lights must be dim. Pigments quickly disappear in the presence of light.

1. For each sample, one 0.9 ml Eppendorf tube, evaporate excess solvent using the air lines (blue Pipetman tip attached to air line via rubber tubing). Turn air on at lowest possible setting before placing in tube, check on your arm. Place tip just above liquid or touching the surface. Watch carefully to prevent loss of sample due to splattering. Air is not usually used to evaporate sample as there is the possibility of sample degradation. Why? What gas should be used to evaporate sample? Also there are often problems with air lines containing water, be watchful. Evaporate sample to approximately 50 µl.

2. Spot the entire amount in 5 µl aliquots, allowing spotted sample to dry between application. Spot sample 2 cm from one end and 2 cm apart. Do not mark the gel to determine this - just estimate visually. You may use the air line in the lab to dry sample (tubing with Pasteur pipette attached) between applications but this is risky as the air line may contain rusty water. Be ready to pull away quickly.

3. The TLC chambers have been set up in the fumehood at least 2 hours prior to the lab to allow the atmosphere to equilibrate with solvent. The TLC solvent is acetone:petroleum ether 1:9 (v/v).

4. Place your TLC plate in the chamber with the sample end down. **The cover is slippery, take care.** The sample spots must be above the solvent sitting in the bottom of the chamber (sample is lost if below solvent). Coordinate with other groups to minimize the opening of the chamber - as each chamber holds four TLC plates, 4 groups should place plates into the chamber at the same time. Adding and remove TLC plates should be done as quickly as possible. Run the TLC plate for 20 min.

5. Remove plate. Immediately mark running front of solvent by nicking the silica gel on the TLC plate. Clearly write your group number (see posted class list for group # if not sure as must be accurate) in the space above the solvent front. Group # must be large enough to be seen on photograph of TLC. See TA to have a photograph of your TLC taken. TLC photograph jpg files will be posted on lab website.
   Note: The pigments (light sensitive) lose intensity when exposed to light - work quickly.

6. Discard solvent tubes in container located in the fumehood.

References used for lab protocol, not required for lab report writeup.
Millie, D.F., Paerl, H.W. & J.P. Hurley. 1993. Can. J. Fish. Aquat. Sci. Vol. 50:2513-2527.Bowles, N.D., Paerl, H.W. & J. Tucker. 1985. Can. J. Fish. Aquat. Sci. Vol. 42:1127-1131.

LAB 3 REPORT

Data presentation and analysis Biochrom Excel spreadsheets and TLC jpg files available on lab website. Do not include Excel scan absorbance values in report, just requested figures. Refer to appendix for instructions for Excel scan figure presentation. Refer to lab manual general report instructions for figure set up.

7 1. Present absorbance scan figures for each of the following. Label and include requested information for each figure in figure title. See Table 1 lab introduction.

a) Live *Rhodospirillum rubrum* culture. Label absorbance peaks (four) that correspond to bacteriochlorophyll present by inserting textbox with peak nm by peak maximum (refer to biochrom chart data for numerical peak nm). In figure title state bacteriochlorophyll present. If the number of peaks differ from expected state missing expected peak(s) that characterize bacteriochlorophyll present.
Note: some peaks are very small but present, possibly as a shoulder peak.

b) Acetone extract of *Rhodospirillum rubrum*. Label absorbance peaks (three) that correspond to bacteriochlorophyll present by inserting textbox peak nm by peak maximum.

c) Acetone extract Winogradsky sample. Label absorbance peaks that correspond to bacteriochlorophylls present by inserting textbox peak nm by peak maximum. In figure title state what sulfur bacteria groups are present, beside each in brackets correlate to bacteriochlorophyll present and corresponding peak maximums.
For example: green sulfur bacteria, (bacteriochlorphyll ...; ..., ... nm)

3 2. Present a completely labelled figure of TLC silica gel plate using your jpg file available on lab website. Insert jpg file in a Word document and resize (if necessary) to ~1/2 page Indicate bacteriochlorophyll* spot(s). All the remaining pigments belong to **ONE** group, label. Indicate visible color of spots if not printed in color. Remember to include a figure title such that the TLC photograph is completely described.

* Just using the TLC data from experiment performed in this lab, you cannot label type of bacteriochlorophyll unless pure sample.

—
10

BIOCHROM SPEC3100
Each group scans three samples, LIVE saved as one Biochrom Excel spreadsheet and two ACETONE extracted saved as one Biochrom Excel spreadsheet (combined). Biochrom will be available on lab website**.**

Keypad and Display
Arrows to navigate. Enter or downward arrow to select
Mode: select measure mode or recall set-up pages, enable selection of post run routines
Function: to access instrument utilities.
Set ref: set reference at all wavelenghts in the mode selected (subtracted from sample absorbance)
Print: output to printer or PC (automatic if Auto-print selected) - do not need to use as auto-print to computer selected.
C: to clear a numeric entry (In nucleic acid or multi-wavelength, C enables set reference in order to restart another experiment)
Run: starts measurement (may set reference automatically if in non-basic modes. Sample number and cell position are automatically incremented.
Stop: ends current activity or returns to main menu

Two modes:
Basic (simple layout): Absorbance, %Transmission, Concentration, Nucleic Acid, Protein
Non-basic (graphical layout with status bar, messages that need to be done
Wavescan Procedure (Mode key goes back, Enter key goes forward)

Wavescan Procedure (Mode or End key goes back, Enter key goes forward)
NEVER ADD REAGENTS WHILE CUVETTE IS IN SPEC HOLDER.
1. Turn on computer, start Biochrom software, BioDC icon. Press run button must have popup menu open before running scans. Leave this open the entire time you are scanning.
2. Turn on spec3100 15 min before using, switch at back (by plug cord).
3. Screen defaults to Basic. Using the arrow key move to select applications tab.
4. Defaults to Wavescan as first. Press **enter**. Press enter to select set up. Enter wavelength range, 250 - 310 nm. Use down arrow key to move and use number key to enter number. Keep other defaults. If you want to check a box, use right arrow. Move all the way down and new menu comes up.
5. Put cuvets in spec3100, first reference in position 1 (blue holder), then samples in positions 2, 3, 4...... counterclockwise (check number of holder), make sure light goes through correct side of cuvet. (1 cm pathway).
6. Press **run.** First reads reference blank then switches to sample 1. Open a new Excel spreadsheet. When the scan is complete it automatically prints data to BioDC software. When data is completely transferred copy paste to to new Excel spreadsheet or appropriate column in Excel spreadsheet (template). Save file in 3480 Section folder as group ___#__ live or group ___#___ acetone. Press Run again, sample 2 is scanned, copy paste to new Excel spreadsheet for each group live sample. If acetone sample label columns (absorbance) Rhodospirillum rubrum and Winogradsky in one Excel file. Each group should have two Excel files.
7. When you are finished remove all sample, turn off spec3100 and computer.

Biochrom EXCEL absorbance scans Instructions
(Instructions for 2007 version, adapt if you still have 2003 version)

Each group includes ONLY three figures in lab report: Live *Rhodospirillum rubrun*, Acetone extracted *Rhodospirillum rubrun* and Acetone Extracted Winogradsky sample. Collected absorbance data is not included in report since the scan range is so wide. Repeat the following instructions for each figure.

Chart instructions (there are numerous ways to carry out the same thing in Excel, only one version given below).
First Select all data, wavelength and absorbance. If you do not do this, Excel may state too much data.
Select insert. Select scatter, select smooth lines. If your lucky, all data entered and plot done, if so, skip pertinent instructions below.
Go to Chart Tools, Design. Select Select Data - select data source pop menu. Select Add. Cursor defaults to Series name. Type name. Move cursor to series X values, select wavelengths, 350 nm to 900 nm - data entered. Move cursor to series Y values, delete information and select absorbance data -data entered. Press OK.
Resize x-axis to fit nm range, right click axis value, select format axis change to fixed and put in appropriate range values, low 350 nm and high 900 nm. Press close. Repeat if required for y-axis.

Go to Chart Tools, Layout.
Select Axes Titles ▾ to enter axes titles. [May need to reduce size of font - select axes text box then select Chart Tools, Format and reduce font size A ▾.]
Select Chart Title ▾ to enter figure # and title. Excel options for location are limited. Select default (may already be present) at top of figure. Enter Figure # and title (include all requested information. Move box below graph. [You also need to reduce size of font - select title text box then select format under chart tools and reduce font size A ▾.] When moving figure title to bottom of chart, it is good to increase the size of the chart box and reduce the size of the graph, need to select each before changing size.
Since you have only one plot on each figure graph, delete legend.
Remove horizontal lines on chart by right clicking on line, select delete.
Uses text boxes to label ONLY bacteriochlorophyll peaks (nm), as requested in report - place at peak and reduce font size to 9.
Select chart to PRINT.

LAB 4 AEROBIC ANOXYGENIC PHOTOTROPHIC BACTERIA

by Vladimir V. Yurkov and Julius Csotonyi[14,15,16]

The obligate aerobic phototrophs differ from all other anoxygenic photosynthetic bacteria in their incapability of anaerobic photosynthesis or of photoautotrophy. They are primarily heterotrophic but can reap limited energy from aerobic photoheterotrophy. Although these bacteria produce considerably less bacteriochlorophyll (BChl) *a* than do their anaerobic relatives (only synthesized in the dark), they synthesize abundant carotenoid pigments. Some of the carotenoids and all of the BChl are bound to antenna complexes, which are composed of a reaction center (RC) surrounded by a light harvesting complex I (LHI), and sometimes an additional light harvesting complex II (LHII). BChl *a* absorbs at 380 nm, 590 nm, and at various positions between 800 and 870 nm, depending on the specific structure of the RC, LHI and LHII of the organism while carotenoids absorb in the 400 – 550 nm range (Table 1). The function of BChl and carotenoid pigments in these pigment-protein complexes is to transduce light energy into chemical energy for use in metabolism. The majority of the carotenoids, however, are dispersed throughout the cell wall and plasma membrane. There is some debate over their function, but it is argued that they serve as antioxidants, protecting the cell from oxidative damage incurred from reactive oxygen species generated during growth in aerobic illuminated conditions. They also shield the cells directly from intense sunlight by absorbing energetic blue light.

In this lab you will investigate an example of aerobic phototrophic bacteria with respect to cellular morphology, color, photosynthetic apparatus and number of light harvesting complexes, pigments and the environments that they inhabit. To accomplish these objectives, you will cultivate the bacteria (Table 1), employ microscopy, perform thin layer chromatography and spectrophotometry (on whole cells, organic extracts and separated pigments), and determine the absolute concentration of major carotenoid pigments in extracts (Table 2).

The Beer-Lambert Law formula, $A_m = \varepsilon bc$, is used to determine the mM concentration of carotenoid in the culture. Where A is the maximum absorbance of the light absorbing substance, $\varepsilon_C = 128\ mM^{-1}cm^{-1}$ (extinction coefficient of carotenoids in acetone:methanol extract), $\varepsilon_B = 76\ mM^{-1}cm^{-1}$ (extinction coefficient of BCla in acetone:methanol extract), c is carotenoid concentration of the substance in the solution (mM) and b is1 cm (pathway of solution UV light passes through). Given the concentration of the original 250 ml culture and the protein concentration (mg/ml), it is possible to determine biomass-specific pigment production by the bacteria (nmoles/g cells) in the original culture. Since the original culture volume is 250 ml and the cell extract is concentrated to 250 µl, there is also a dilution (D) of 1/1000 to be consider for biomass-specific pigment production by the bacteria (nmoles/g cells).

---

[14]Yurkov, Vladimir V, Csotonyi, Julius T. 2003. Aerobic anoxygenic phototrophs and heavy metalloid reducers from extreme environments. Recent Res. Devel. Bacteriol. 1:247-300.

[15] Rathgeber, C, Beatty, T, Yurkov, V. 2004. Aerobic phototrophic bacteria: new evidence for the diversity, ecological importance and applied potential of this previous overlooked group. Photosynthesis Research. 81: 113-128.

[16]Yurkov, V, Beatty, T 1998. Aerobic anoxygenic phototrophic bacteria. Micro. Mol. Biol. Rev. 62: 695-724. [ASM journal - link on lab website.]

$$\text{Biomass-specific pigment} = \frac{[\text{Pigment}_{culture}]}{[\text{Protein}_{culture}]} \times D$$

Table 1. Bacterial characteristics of the viable aerobic anoxygenic phototrophic bacteria, *Erythromicrobium ramosum* strain E5		
Cell color and Major *in vivo* Absorption Peaks (nm)	Source and Optimum Growth Conditions: Salinity (%), pH, Temp (°C)	Morphology
Reddish-orange. Carotenoids: 466, 478 BChl *a* : 380, 590, 798, 832, 868	Freshwater thermal spring. Salinity: 0; pH: 7-8.5; Temp: 25-30°C	Pleomorphic: ovoid to long thin rods (0.7-1.0 x 1.3-2.6 m); reproduces by branching or binary fission.

Erythromicrobium ramosum contains 20 different carotenoids (figure 1) all C_{40} that are classified into four groups. Of these *E. ramosum*[17] carotenoids, there are ten that predominant, listed below in order of increasing polarity. The most polar carotenoids spend the longest time in the polar silica gel relative to the non-polar solvent - ie move the shortest distance from the origin on the TLC plate. Relative polarity of predominant carotenoids in *Erythromicrobium ramosum*: Least Polar (travels greatest distance on TLC) ⇒ β,β-carotene ⇒ spirilloxanthin ⇒ bacteriorubixanthinal ⇒ zeaxanthin ⇒adonixanthin ⇒ caloxanthin ⇒ trihydroxy-β,β-carotene-4-one ⇒ nostoxanthin ⇒ tetrahydroxy β,β-carotene-4-one ⇒ erythroxanthin sulfate ⇒ Most Polar (travels shortest distance on TLC).

Table 2. Characterization of the major carotenoids of *Erythromicrobium ramosum* E5.		
Major Carotenoids	Carotenoid color	Absorbance maximum (nm) for acetone extracted carotenoids
adonixanthin	reddish purple	515
caloxanthin	orange	454, 477 (max)
nostoxanthin	yellow	454 (max), 477

[17] Yurkov, V, Beatty, T 1998. Aerobic anoxygenic phototrophic bacteria. Micro. Mol. Biol. Rev. 62: 695-724. [ASM journal]

Front (non-polar)
beta,beta-carotene
spirilloxanthin
bacteriorubixanthinal
zeaxanthin
adonixanthin

caloxanthin

trihydroxy – beta,beta, carotene
nostoxanthin

tetrahydroxy-beta,beta, carotene -4-one

erythroxanthin sulfate

origin (most polar)

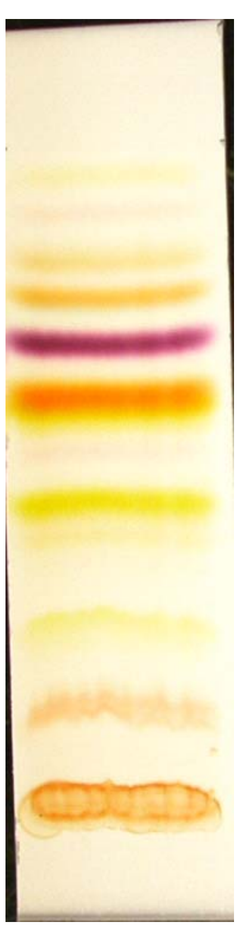


Figure 1. TLC using solvent petroleum ether:anhydrous ether:acetone:methanol 40:10:15:3 (v/v/v/v) of *Erythromicrobium ramosum* E3 acetone:methanol (7:2) extracted carotenoids.

The color of the aerobic anoxygenic phototrophic bacteria may be deduced by the composition of the carotenoids present taking into consideration that the color of the bacteria is due to reflected light (visible color) not the light absorbed by the carotenoids (Table 3). For example, if the aerobic anoxygenic phototrophic bacteria major carotenoids are in the lower wavelength range, blue-violet to violet the bacteria will be yellow.

Table 3. Color in relationship to wavelength.

pigment color	wavelength (nm)	opposite visible color on color wheel
red	780-622	green
orange	622-597	blue
yellow	597-577	violet
green	577-492	red
blue	492-455	orange
violet	455-390	yellow

PROCEDURE (Room 204 Buller)
Week 7 & 8
Week 7(Section 1 students only start at 2:30 pm)
Week 8 (Section 2 students only start at 2:30 pm)

Preparation of Acetone:Methanol Pigment Extract

250 ml cultures of *Erythromicrobium ramosum* strain E5 was grown with shaking at 28°C for 2 days in the dark. The culture was centrifuged at 12000 rpm for 20 min, the supernatant discarded and resuspended in 250 ml 10 mM Tris-HCl, pH 7.8. Again centrifuged at 12000 rpm for 20 min and supernatant discarded. The pelleted cells were suspended in 5 ml of acetone: methanol (7:2, v/v) by vortexing for 1 min. The solvent suspended cells were transferred to a small screw capped centrifuge tube and put in a dark fridge overnight. Next day, centrifuged and supernatant collected as pigment extract. The pellet consists of lysed cell debris. The volume was evaporated to dryness using a rotary vacuum or N_2 (bubbling nitrogen gas through the pigment extract). The sample was suspended in 6 ml absolute ether: petroleum ether (2:1 v/v). Mixed with 6 ml saline and ether layer collected. The aqueous layer containing aqueous soluble components that could interfere with TLC separation is discarded. The ether extract is again evaporated to dryness and suspended in 250 µl acetone:methanol (7:2, v/v) and store at -20°C until lab day.

Protein Determination

One ml of each 300 ml culture was added to an Eppendorf tube for protein determination. Centrifuged 1 min. Resuspended in 0.5 - 1 ml 10 mM Tris-HCl, pH 7.8. Sonicated 30 sec on off. Centrifuged at 14000 rpm for 10 min to pellet debris. The supernatant (cell extract) collected. The protein concentration (mg/ml) of cell extract was determined spectrophotometrically: [protein] mg/ml = (1.45 OD_{280} - 0.74 OD_{260}) x dilution factor. The protein concentration of the original 250 ml bacterial cultures will be available on lab blackboard and lab website.

STUDENT LAB STARTS HERE - This week's lab is in **Room 204 Buller**
The lights in the lab should be dimmed but still bright enough to see clearly.

2. Each group should collect one 5 ml *Erythromicrobium ramosum* strain E5 broth culture and one pigment extract (250 µl). Cover pigment extract with foil if not already done. Keep covered as much as possible during lab.

   E. ramosum E5 was grown in rich organic medium (RO) with rotation at 28°C in the dark for 2 days. Broth was inoculated with 10% culture.

All solvents must be discarded in solvent waste container not down the sink. One waste container located in fumehood and the other by Spec3100.
Supplies provide are one each/group.

3. **Live *Erythromicrobium ramosum* strain E5 5 ml broth culture**:

   (i) **Streak plate** *Erythromicrobium ramosum* E5 culture on RO agar plate. DO NOT USE THE BUNSEN BURNER. Sterile loops are provided (4/group). Label clearly and place in tray available on the supply bench. The TA will move the cultures to the 28°C in room 302 (your usual lab). Incubate for 4-5 days at 28°C in the dark. Record colony characteristics.

(ii) Preparation of *in vivo* (live cells) spec scan sample. Using a P1000 transfer 1.5 ml bacteria culture to an Eppendorf tubes. Micro-centrifuge at room temperature for 1 min. Remove supernatant with P1000. Using the P1000 resuspend the each pellet in 1000 µl 10 mM Tris-HCl buffer, pH 7.8 by carefully pipetting the solution up and down on the pellet. Add 0.1 ml 50% sucrose to the combined tube. Mix by inverting until homogeneous. Do not create bubbles. A TA will help you scan your samples (320 nm to 600 nm). For *in vivo* bacteria prep the blank (reference) is 1 ml 10 mM Tris buffer, pH 7.8 + 100 µl 50% sucrose. Biochrom Excel scan data will be available on lab website. Carotenoid peaks must be present on plot, if cells are too concentrated they must to be diluted with buffer and re-scanned.

> Remember the P1000 maximum volume is 1 ml. When transferring 1.5 ml just add 07.5 ml twice for each tube .

4. ***Erythromicrobium ramosum*** **strain E5 pigment acetone extract (250 µl):**

(i) **Thin Layer Chromatography (TLC) Protocol**
Using a P200 apply 25 µl aliquots of the pigment extract sample (total 250 µl) at a time as a thin line 2 cm from TLC end and about ½ cm from each side. To establish a straight line just put a small nick on each side 2 cm from end. Slowly apply sample as **continuous line** as you want as narrow a line as possible. Allow applied sample to dry before adding more sample. You may use the air line in the lab to dry sample (tubing with Pasteur pipette attached) between applications. There is the possibility of water in the tube or air line, turn on at least 30 sec before using. Repeat until the entire 250 µl pigment extract has been loaded. Take your time. The TLC chambers have been set up in the fumehoods at least 2 hours prior to the lab to allow the atmosphere to equilibrate/saturate with solvent. The lid is sealed with vasoline. The chamber has a large sheet of chromatography paper place in the solvent to increase the surface evaporation of solvent. The TLC solvent is petroleum ether:anhydrous ether:acetone:methanol 40:10:15:3 (v/v/v/v). Open and close chamber quickly to ensure continuous solvent vapor saturation. Place your TLC plate in the chamber with the sample end down. The sample spots must be above the solvent sitting in the bottom of the chamber (sample is lost if below solvent). Coordinate with other groups to minimize the opening of the chamber (opening of chamber increases run time). Run the TLC plate until 1 - 2 cm from top. Using a pencil, quickly mark the solvent front. Clearly write your group number (see posted class list for group # if not sure as must be accurate) in the space above the solvent front. See TA to have a photograph of your TLC taken. TLC photograph jpg files will be posted on lab website.

> When adding and removing sample from quartz cuvette tube take care not to scratch the inside of the cuvet.

(ii) **Spec3100 Analysis of dominant carotenoids**:
After the TLC has dried and photograph taken scrape the **dominant carotenoid** pigment spots into an Eppendorf tube (labelled with color, should be either orange, purple red or yellow). May be easier to first scrape onto a piece of paper then pour the scraped sample into an Eppendorf tube. Also scape the **next dominant carotenoid** into a labeled Eppendorf tube (color and distance migrated). Add 1 ml acetone:methanol (7:2) to each to elute the pigment from the silica. Vortex 30 sec. Microcentrifuge for 5 min. Transfer the supernatant to a clean labelled Eppendorf tube (bacteria, color and distance migrated). Do

not transfer any of the silica pellet. Discard TLC plate in plastic lined bucket on discard trolley. With the assistance of the TA use the Spec3100 to scan (320 - 600 nm) for each selected pigment extract. Reference (blank) is acetone:methanol (7:2). Both extracted pigments must be done at the same time as your Biochrom Excel spreadsheets combined before saving. Biochrom Excel spreadsheet will be available on lab website.

LAB 4 Aerobic Anoxygenic Phototrophic Bacteria REPORT
(Must use Work document available on lab website and insert Excel figures where requested.)
See Lab 4 appendix for Excel absorbance scan figure instructions.

1 1. Record bacteria colony characteristics in the following table.

Table 1. Colony characteristics of *Erythromicrobium ramosum* E5 on rich organic (RO) agar. Incubation time: ____ Incubation temperature: ___				
Size	Color	Margin	Elevation	Light reflection

2 2. Present a completely labelled figure of TLC silica gel plate. Insert jpg file available on lab website into report word document - may need to resize (~½ page). Label two carotenoids selected for spectrophotometer analysis with carotenoid name and color (if not printed in color). Refer to lab introduction.

2 3. Insert (copy/paste into Word format) Excel figure of *in vivo* (live) aerobic anoxygenic phototrophic bacterial absorbance scan. Resize to fit ~1/2 page. Label absorbance peaks that correspond to carotenoids present by inserting textbox of peak nm by each carotenoid peak maximum. Refer to Introduction table 1 for expected peaks.

4 4. Insert two Excel wavescan figures for the two selected TLC extracted carotenoids from *Erythromicrobium ramosum* E5 into Word document. Put both figures on ONE page, resizing if necessary. Label absorbance peak(s) that corresponds to the carotenoid isolated by inserting text box of peak nm by peak maximum(s). In each figure title also include carotenoid color and carotenoid name. Refer to Introduction table 2 for expected peaks.

Continued on next page

2 5. a) Include requested information in the following table.

Table 2. Carotenoid analysis of anoxygenic phototrophic bacteria, *Erythromicrobium ramosum* strain E5.			
[protein][a] mg/ml: ________________			
carotenoid name (two selected for spectral analysis)	maximum absorbance peak (nm)	Absorbance value at maximum	specific pigment production[b] (nmoles/g cells)

[a]protein concentration of original 250 ml culture.
[b]use only major peak if more than one associated peak, ie maximum absorbance peak First determine the number of mmoles pigment per culture and gram protein per culture or each per liter. Then calculate nmole/g. Remember that mM = mmoles/liter.

b) Show one sample calculation for biomass specific pigment production (nmoles/g cells):

___
10

Lab 4 appendix.

Biochrom EXCEL absorbance scans
(Instructions for 2007 version, adapt if you still have 2003 version)

Each group includes ONLY three figures in lab report: Live *Erythormicrobium ramosum E5*, two extracted *Erythormicrobium ramosum* carotenoids. Collected absorbance data is not included in report since the scan range is so wide. Repeat instructions for each figure.

Refer to Lab 3 Absorbance scan Excel instructions – changing instructions to fit this experiment:
-absorbance range 320-600 nm
-label requested carotenoid peaks not bacteriochlorophyll

APPENDIX

MEDIA AND SOLUTIONS

The composition of the media is relevant to the selection of different microorganisms. It is important to know the function of each media component as related to microorganism growth requirements. There are two main types of media; defined (all media components precisely defined, eg. *Azotobacter* medium) and complex (undefined media components added, eg. CT medium).

Review of the following microorganism categories.
Energy Source:
chemotroph - chemical energy source
phototroph - light as the primary energy source
Carbon Source:
autotroph - CO_2 as principle carbon source
heterotroph (organotroph) - organic material as principle carbon source

Four major nutritional/energy categories
Photoautotrophs - light as energy source and CO_2 as principle carbon source
Photoheterotrophs - light as energy source and organic compound as principle carbon source
Chemolithotrophs (lithos, Greek meaning rock)) - (in the dark) chemical energy source is reduced inorganic compounds, such as NH_4^+, NO_2^-, H_2, reduced sulfur (e.g., H_2S, S, $S_2O_3^{2-}$), CO, or ferrous iron and CO_2 as principle carbon source
Chemoorganotrophs - chemical energy source and organic compound as principle carbon source. Often a single organic source supplies both the carbon and energy source.

Microorganism
mineral requirements
-minerals are important components of microorganisms
-light metals, the monovalent cations Na^+, K^+ and divalent cations Ca^{2+} & Mg^{2+} are enzyme cofactors, parts of ion pumps and maintain osmotic balance. Mg^{2+} also stabilizes ribosomes, nucleic acids and cell membranes.
-heavy metals (Cu, Zn, Fe, Mn and Co) are required at low concentrations (Trace Metals) for growth and metabolic function by most microorganisms. Some organisms also require Mo or Ni.
-heavy metals serve many functions for example, enzyme reaction cofactors, enzyme prosthetic groups and redox centers
-many media (defined media must) have a buffer system added to maintain the required pH, since phosphates do not harm bacteria, a phosphate buffering system is usually used. It is composed of both mono- (more acidic) and di-basic (more alkaline) depending on pH required. For some media only one or the other phosphates are added. The cation associated with the phosphate is usually potassium or sodium.

Note: All plates inoculated with soil or natural source contain 50 µg/ml cycloheximide.

Cycloheximide inhibits the growth of fungi by inhibiting protein synthesis of 80S eukaryotic ribosomes but not 70S prokaryotic ribosomes unless otherwise specified.

T-soy (tryptic soy) agar: 15 Bacto tryptone, 5 g Bacto soytone, 5 g NaCl, 15 g Bacto agar per liter distilled water. Final pH 6.8 For **semi-soft** agar plates add 5 g/l agar.
Bacto tryptone - Pancreatic digest of the protein. Source of all nutrients; nitrogen, carbon, energy, minerals, and vitamins.
Bacto soytone - Enzymatic hydrolysate of soybean meal. Source of all nutrients; nitrogen, carbon, energy, minerals, and vitamins.

Ty agar plates: 5 g tryptone. 3 g yeast extract, 0.5 g $CaCl_2.2H_2O$ per liter. For agar plates add 15 g/l.

Azotobacter medium -mannitol agar: 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$*, 0.05 g $FeCl_3.6H_20$*, 0.05 g $CaCl_2.2H_2O$*, 0.05 g $NaMoO_4.2H_2O$*, 15 g mannitol* per liter distilled water. pH 7.4 - 7.6 For agar plates add 15 g/l.
*autoclaved separately and added to cooled salts

Yeast Medium (Van Niel's): 1.0 g K_2HPO_4, 0.5 g $MgSO_4$, 10.0 g yeast extract, tap water to 1 liter. Adjust pH to 7.0-7.2 before bringing up to final volume. Add $MgSO_4$ separately as a stock solution after autoclaving.
yeast extract: Source of all nutrients; nitrogen, carbon, energy, minerals, and vitamins.

Aerobic anoxygenic phototrophic bacteria medium:
Rich organic medium (RO): 0.3 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.3 g NH_4Cl, 0.3 g KCl, 0.05 g $CaCl_2.2H_2O$, 1 g sodium acetate, 1 g yeast extract, 0.5 g Bacto peptone, 0.5 g casamino acids, 2 ml trace elements and 2 ml vitamin solution per final volume 1 litre distilled water. Autoclave. The pH should be ~5 keeping the the cations and phosphates from precipitating. This media preparation breaks the accepted practice of setting the pH prior to bringing to final volume and autoclaving. For RO medium, after autoclaving add 6 ml sterile 0.5 M NaOH to bring to pH 7.8.
Trace elements (adjust 500 ml distilled water to pH 3.0 with distilled water and add (per litre): 0.3 g $FeSO_4.7H_2O$, 0.005 g $CoCl_2.6H_2O$, 0.003 g $MnCl_2.4H_2O$, 0.005 g $ZnSO_4.7H_2O$, 0.002 g $NiCl_2.6H_2O$, 0.001 g $CuCl_2.2H_2O$, 0.002 g H_3BO_3. and 0.003 g $Na_2MoO_4.2H_2O$
Vitamin solution (per litre): 0.4 g thiamine, 0.4 g nicotinic acid, 0.08 g biotin, 0.02 g vitamin B_{12}. For agar plates add 14 g/l Difco agar.
casamino acids: mainly a nitrogen source, although some carbon

PURE CULTURE METHODS

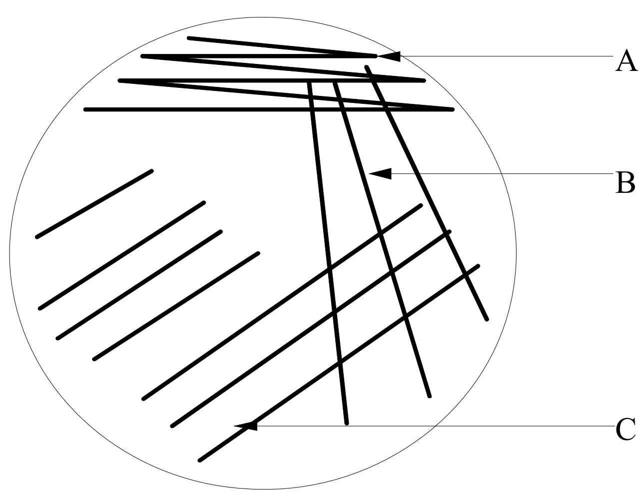
Sterilization: All materials that come into contact with the pure culture must be sterilized. A variety of ways are used to sterilize liquids, containers, and instruments; autoclaving (steam at 15 lb/in), exposure to radiation, and filtration.

Aseptic transfer technique: This technique involves avoiding any contact of the pure culture, sterile medium, and sterile surfaces with contaminating microorganisms. This is accomplished by work area cleaned with BBD, the transfer loop sterilized by heating with a Bunsen burner before and after transferring, and the work performed quickly and efficiently to minimize the time of exposure during which contamination of the culture or laboratory worker can occur. The steps for transferring a culture from one container to another are (a) flame the transfer loop and allow to air cool, (b) open and flame the mouths of the culture tubes/tubes, (c) pick up some of the culture growth and transfer to fresh medium, (d) flame the mouth of the cultures vessels and reseal them, and (e) re-flame the inoculation loop. Similar technique is used to transfer culture from a Petri plate (only the Petri dish is not flamed) and to transfer cultures using sterile pipettes (the pipette canister is flamed after removing top and then flamed again after removal of pipette before replacing the top). It is essential after removal of sterile caps, plugs, or pipette canister tops that they are kept in your hand sterile side down before replacing. DO NOT place on bench area.

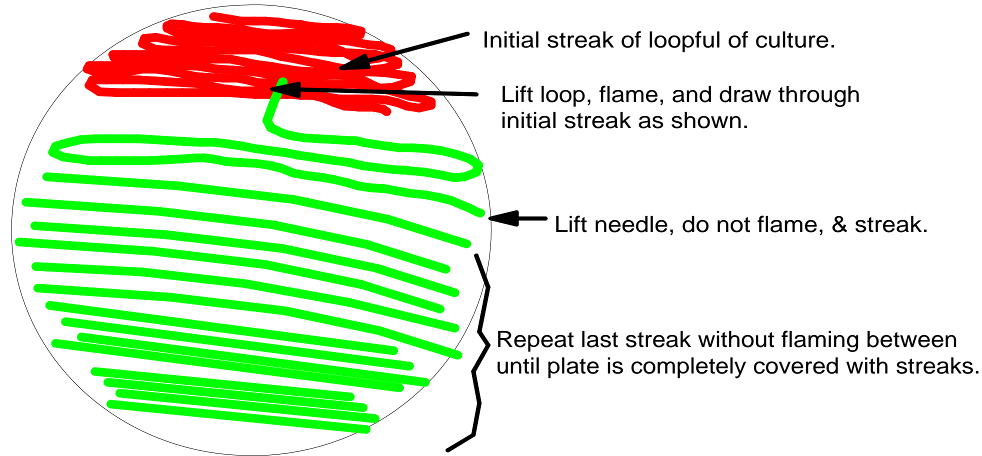
Isolation: Pure cultures of microorganisms are isolated by several different methods; streak plate, spread plate and pour plate. All the methods involve separation of single bacterium on solid (agar) media where it grows into a colony (clone) which is a pure culture of a single microorganism type. The following two methods are used in the Diversity Lab.
Spread Plate: In the spread plate method a drop of microbial suspension, usually 0.1 ml, is placed on the center of an agar plate and spread over the surface of the agar using a sterile glass rod. Usually culture dilutions are plated to obtain an appropriate dilution permitting separated single colonies.
Streak Plate: In the streak plate method a loopful of bacterial cells is streaked across the surface of nutrient agar plate. The method of streaking established a dilution gradient so that single colonies develop. Streak Plate Method A The procedure involves (refer to diagram) streaking the first streak (A) from the broth. B and C are done with a sterile loop. Flame the loop between A and B, B and C. Cool the loop after flaming by briefly touching it to an unused part of the agar surface each time just before streaking. Use the edge of the loop to form thin lines. Do not streak with the loop flat against the agar. Do not cut into the agar. Hold the lid of the Petri late at an angle over the surface of the plate when making each streak. Streak Plate Method B The method of streaking is the same as Method A however, the pattern of streaking is changed. Many methods for obtaining a pure colony by the streaking technique are available, with only two shown in the following figure. The objective of streak plate is to obtain single colonies. Whatever method works consistently is a good method.

Petri Plate Labeling:
Label plate in small print using permanent ink or grease pencil around edge of the bottom of the plate. Plates should be labelled with name or initials, date, bacterial species, dilution if applicable, and any other important information. Incubate plates upside down.

Streak Plate Method A



Streak Plate Method B



SOIL COLLECTION AND TRANSPORT METHOD

1. Soil sample should be collected as short a time prior to lab as possible. If soil sample is collected a day or two before the lab just store away from light at cool room temperature (~15-20°C) thus keeping the temperature similar to location. Be creative in your location of the soil sample as class data is collected. Your sample should be representative of the area you select. This is done by taking a random soil sample (van Elsas & Smalla 1997) as specified by ISO (International Organization of Standardization; Geneva).

2. Method selected for this lab is simple random samples. Divide the field site (use approximately 1 square meter) into a grid-like pattern.

3. Randomly take 10 soil samples (~10 g each) and put into a large clean sealable container (zip-lock bags or sealable plastic containers available in lab). Seal lid. Shake vigorously to mix. Since you do not have a scale in the field use a large spoon (~15 ml) to select your samples. Take care to collect the same size of sample each time. If your experiment does not vary soil depth, select a depth of approximately 2 cm. A sample of 100 g soil should be statistically representative of the area.

4. Record sample soil location; area size selected, i.e., one square meter; type of soil (clay, sand, organic, etc).

5. If your sample is collected prior to the day of the lab, store at 4°C (fridge) until start of lab. However, ambient temperature is acceptable.

References:

van Elsas, J.D,, Smalla, K. 1997. Methods for sampling soil microbes. In: Hurst, J.C. ed. Manual of Environmental Microbiology. Washington: ASM Press. p 383-390.

Dandurand, L-M.C., Knudsen, G.R. Sampling microbes from the rhizosphere and phyllosphere. In: Hurst, J.C. ed. Manual of Environmental Microbiology. Washington: ASM Press. p 391-399.

SOIL MICROORGANISM DILUTION AND PLATING PROCEDURE
for
Cultureable Viable Microorganism Titer (microorganisms/g wet weight soil or /ml)
Colony characterization
Comparison of copiotrophs and oligotrophs

1. Dilute soil sample 10-fold (10^{-1}) by adding 10 g soil to 90 ml sterile saline, vortex vigorously for 1 to 2 min. Let sit for 5 min before diluting further. Be sure to shake just before transferring aliquot of dilution.
   [90 ml sterile saline provided by prep room services]

2. Continue 10-fold dilutions by transferring 0.5 ml of dilution to 4.5 ml sterile saline (10^{-2} to 10^{-7} dilution). The range of dilutions prepared and plated depends on your sample but it is a good idea to plate a wide range of dilutions to ensure that you obtain adequate separated colonies.
   [You must prepare your own 4.5 ml saline dilution tubes using sterile pipettes, sterile 6" metal capped test tubes and 200 ml flask of sterile saline provided.]

3. Spread plate **in duplicate*** 0.1 ml of each dilution (must plate full range of dilution) of soil sample on nutrient agar containing cycloheximide.
   a) Aseptically transfer 0.1 ml of dilution to center surface of agar plate. Never lift the lid of agar plate completely off the plate or place on a bench surface. Best method is with the lid tilted above the plate.
   b) Dip hockey stick spreader in bottle of alcohol.
   c) Flame the spreader until alcohol ignites. Immediately remove spreader from flame and wait until alcohol completely burns off. Cool slightly.
   d) Open lid keeping it tilted over the plate and touch spreader to surface of plate that does not contain culture drop. If spreader is still hot, it will kill bacteria. Still holding the lid tilted over plate, move the plate around spreading bacteria evenly over agar surface. (Use turntable to rotate plate if available or use spread microorganisms evenly over the surface of the plate.)
   e) Allow plates to dry right side up at room temperature for several minutes before incubating upside down at 28°C (standard temperature, this may vary depending on experiment).

4. Examine plates day 2 and day 8 (if comparing oligotrophs and copiotrophs). Record results as required for your experiment, e.g. titre, colony characteristics at two days.

*if desired, you may plate in triplicate to allow some statistical analysis

SAMPLE CALCULATION of the number of microorganisms per ml

Notes: per ml is equivalent to per gram wet weight soil

Data for example calculations using the following sample data

Dilution plated	Number of colonies	
	Plate 1	Plate 2
10^{-2}	TNTC	TNTC
10^{-3}	320	316
10^{-4}	34	27
10^{-5}	2	3

TNTC = too numerous to count

Terms

Plating factor = reciprocal of volume plated

Dilution factor = reciprocal of dilution for significant counts

Significant plate counts = the sum of the plate counts at significant dilution divided by number of plates. Often more than one dilution has significant plate counts. It is important to use all significant plate count data. There are several ways to deal with data that has more than one significant plate count dilution. Significant plates counts is 30 colonies per plate to as many colonies as you can accurately count. See outlier plate count section that follows.

Number of plates = number of significant plates

<u>Calculation</u> Use Scientific format for titre value to significant decimals.

Do not average an average value as it incorporates error in your calculation (not statistically accurate). Use one of the following methods to calculate bacteria titer.

<u>Bring all significant counts to the same dilution</u>:

$$\text{Bacteria/ ml} = \frac{\text{significant plate counts}}{\text{number of plates}} \times \text{dilution factor} \times \text{plating factor}$$

$(320 + 316 + 340)/3 \times 1/10^{-3} \times 1/10^{-1} = 3.25 \times 10^{6}$ microorganisms/ml, since the smallest number of significant figures for plate counts is two, the answer is 3.3×10^{6} bacteria/ml

<u>Or calculate the titer for each significant plate count and average.</u>

Microorganisms/ml = significant plate count x dilution factor x plating factor

$320 \times 1/10^{-3} \times 1/10^{-1} = 3.20 \times 10^{6}$ microorganisms/ml

$316 \times 1/10^{-3} \times 1/10^{-1} = 3.16 \times 10^{6}$ microorganisms/ml

$34 \times 1/10^{-4} \times 1/10^{-1} = 3.4 \times 10^{6}$ microorganisms/ml

Average all values: $(3.20 \times 10^{6} + 3.16 \times 10^{6} + 3.4 \times 10^{6})/3 = 3.25 \times 10^{6}$ microorganisms/ml, since the smallest number of significant figures for plate counts is two, the answer is 3.3×10^{6} bacteria/ml

Outlier plate counts

Unlike plating a pure bacterial culture, plating soil is more difficult. In theory there should be a difference of 10-fold colony counts between dilutions; this is difficult to obtain for soil dilutions since the bacteria may not be evenly distributed. Soil particles containing numerous bacteria may get carried over in the dilutions causing outlier data.

What is an outlier? Any plate count that does not follow a pattern, is an anomaly. It is important to not include outlier plate counts in titre calculation. The following is an example of outlier data. For Plate 1 10^{-6} dilution and Plate 2 10^{-7} dilution do not include plate count values in titre calculation. State outlier values as footnote in your data table.

Dilution plated	Number of colonies (0.1 ml of each dilution plate	
	Plate 1	Plate 2
10^{-2}	TNTC	TNTC
10^{-3}	320	316
10^{-4}	44	57
10^{-5}	2	24
10^{-6}	45	1
10^{-7}	0	33

TNTC = too numerous to count

Percentage of copiotrophs and oligotrophs

$$\text{\% of copiotrophs} = \frac{\text{microorganism titer on day 2}}{\text{microorganism titer on day 8}} \times 100$$

$$\text{\% of oligotrophs} = \frac{\text{microorganism titer on day 8 minus microorganism titer on day 2}}{\text{microorganism titer on day 8}} \times 100$$

HEMOCYTOMETER INSTRUCTIONS

for total soil microorganism titer determination (includes viable, non-viable, or viable but not cultureable)

1. Select a microscope with a 40x objective. The hemocytometer is supplied in a sealed container submerged in ethanol. A special cover slip is also submerged in the ethanol. Careful when handling. Each hemocytometer holds two samples, inlet either side. Remove and wipe hemocytometer and coverslip with a kimwipe.
2. Transfer ~10 µl of solution (most likely 10^{-2} to 10^{-4} dilution) using a P20 to V-shaped grove of the hemocytometer*, cover lengthwise with cover slip and allow sample to settle for 1 minute then count.
3. Place on the microscope. Microscopically count microorganisms (rods to cocci ie regular shaped) using 40x objective. Adjust the condenser level, usually part way down. Ignore irregular size particles (not microorganims).
4. See figure below for diagram of center part of ruled slide consisting of a 5 by 5 **double lined** grid. There is a smaller single line grid inside each double lined square but is used only to help you count. Each row consists of 5 doubled lined squares. Count the number of microorganisms in rows 1, 2, 4 and 5 (omit row 3) - total of 20 squares. If microorganisms overlap rows, make sure you do not record them twice.
5. After using hemocytometer, return slide and cover slip to ethanol container. Replace cover and put on supply bench shelf.

Hemocytometer Titer Calculation:

Total the number of microorganism in rows 1, 2, 4 and 5, ie total of 20 squares.
Divide total number of microorganisms by 20 to get the number of microorganisms in one square.
The volume of each square is 0.02 cm x 0.02 cm x 0.01 cm depth. This is equivalent to 4.0×10^{-6} ml since 1 cubic cm is equivalent to 1 ml.

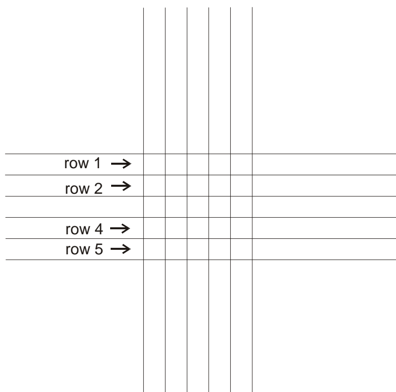
$$\text{Microorganisms/ ml} = \text{number of microorganisms in one square} \times \frac{1 \text{ ml}}{4.0 \times 10^{-6} \text{ ml}}$$

This is for the diluted sample. Multiply this number by dilution factor of sample counted.

Example calculation:
10^{-2} dilution of soil sample was counted using the hemocytometer.
Hemocytometer microorganism counts: row 1 (5 squares) contains 19 microorganisms , row 2 contains 18 microorganisms, row 4 contains 16 microorganisms and row 5 contains 17 microorganisms.

$$\frac{(19 + 18 + 16 + 17)}{20} = 3.5 \text{ microorganism/square or per } 4 \times 10^{-6} \text{ ml.}$$

$$3.5 \times \frac{1}{4.0 \times 10^{-6} \text{ ml}} = 8.8 \times 10^{5} \text{ cells/ml} \times 10^{2} = 8.8 \times 10^{7} \text{ cells/ml}$$



Row 1, magnification of row 1 above. Example of microorganism distribution (22 microorganisms)

Figure . Hemocytometer counting area.

AUTOMATIC COLONY COUNTER

There are several makes of automatic colony counters in this department. All automatic colony counters work on the same principle. The counter registers a count every time you touch the colony with the counter probe as long as the L-shaped probe in inserted into the agar at the edge of the plate. This completes th electrical circuit through the agar from the L-shaped probe to the counter probe (needle shaped probe) touching the colony.

Operation

1. Push or flip the power switch to turn on counter.
2. Press the button on the counter that resets the counter to zero.
3. Place agar culture plate on counter and remove cover.
4. Insert L-shaped probe into the agar at the edge of the plate.
5. Count colonies by touching each colony with the counter probe tip (needle shaped probe).
6. Remove plate, replace lid.
7. Remember to turn off power switch when you are finished counting.

Notes:
(i) Use a marker to divide the plate into sections or use the grid on the automatic colony counter to facilitate counting.
(ii) The counter also comes with a magnifying glass but it is not required unless you are counting very small closely spaced colonies.

pH METER OPERATION (AB15/15+ FISHER SCIENTIFIC)

Prior to lab the pH meter has been standardized using standard pH solutions, pH 4 and pH 7. Students are not required to do pH Setup.

1. Turn on Power by pushing button left back (if not already turned on).
2. Remove the electrode from storage buffer, rinse electrode with distilled water and blot dry.
3. Immerse the electrode in sample solution, if stirring sample make sure the electrode is slightly off to the side (do not hit electrode with stirring bar). Check that the display screen shows that you are the measure mode (upper left corner).
4. Take pH reading after the display screen shows the STABLE icon (centre right) or the pH read-out has stabilized. Note: Stable icon only displays if turned on in setup.
5. Distilled water rinse and blot-dry (do not wipe) electrode between each measurement.
6. When finished using the pH meter, repeat electrode distilled water rinse and blot dry. Replace electrode in storage solution (pH 4 standard or KOH solution).
7. Turn off pH meter if no other lab group's require the pH meter.

PIPETMAN OPERATION
(Excerpted from Gilson pipetman operation manual.)

In your lab, you use only the P200 pipetman. If you look at the top of the plunger it states the size of the pipetman.
P20 measures accurately from 2 µl to 20 µl.
P200 measures accurately from 20 µl to 200 µl.
P1000 measures accurately from 100 µl to 1000 µl.
Never turn the pipetman above the maximum volume; 20 µl for P20, 200 µl for P200, and 1000 µl for P1000 as this breaks the pipetman. The scale on the pipettor is read different for each type - refer to Figure 5 for an example of how to read the scale.

1. Setting the volume: The required volume is set on the digital volumeter by turning the knurled adjustment ring (Figure 3, 2-A). The volumeter display is read from top to bottom in µl for P20 and P200 and ml for P1000 (Figure 3, 2).

2. Place a disposable tip on the shaft of the Pipetman. Press on firmly with a slight twisting motion to ensure an airtight seal. Depress the push-button to the first positive stop (Fig. 3, 3-A). While holding the Pipetman vertical, immerse the tip 2-4 mm into the sample liquid. Release the push-button slowly to draw up the sample (Fig. 3, 3-B). Wait 1 to 2 seconds, then withdraw the tip from the sample.

3. To dispense the sample, place the tip end at a 10-45° angle against the inside wall of the vessel and depress the push-button SMOOTHLY to the first stop (Fig 3, 3-C). Wait 1 to 2 seconds and then depress the push-button completely to expel any residual liquid (Fig. 3, 3-D). With the push-button fully depressed, carefully withdraw the Pipetman, sliding the tip along the inside wall of the tube. Release the push-button. Remove the used tip by depressing the tip ejector button (Figure 3, 1-F).

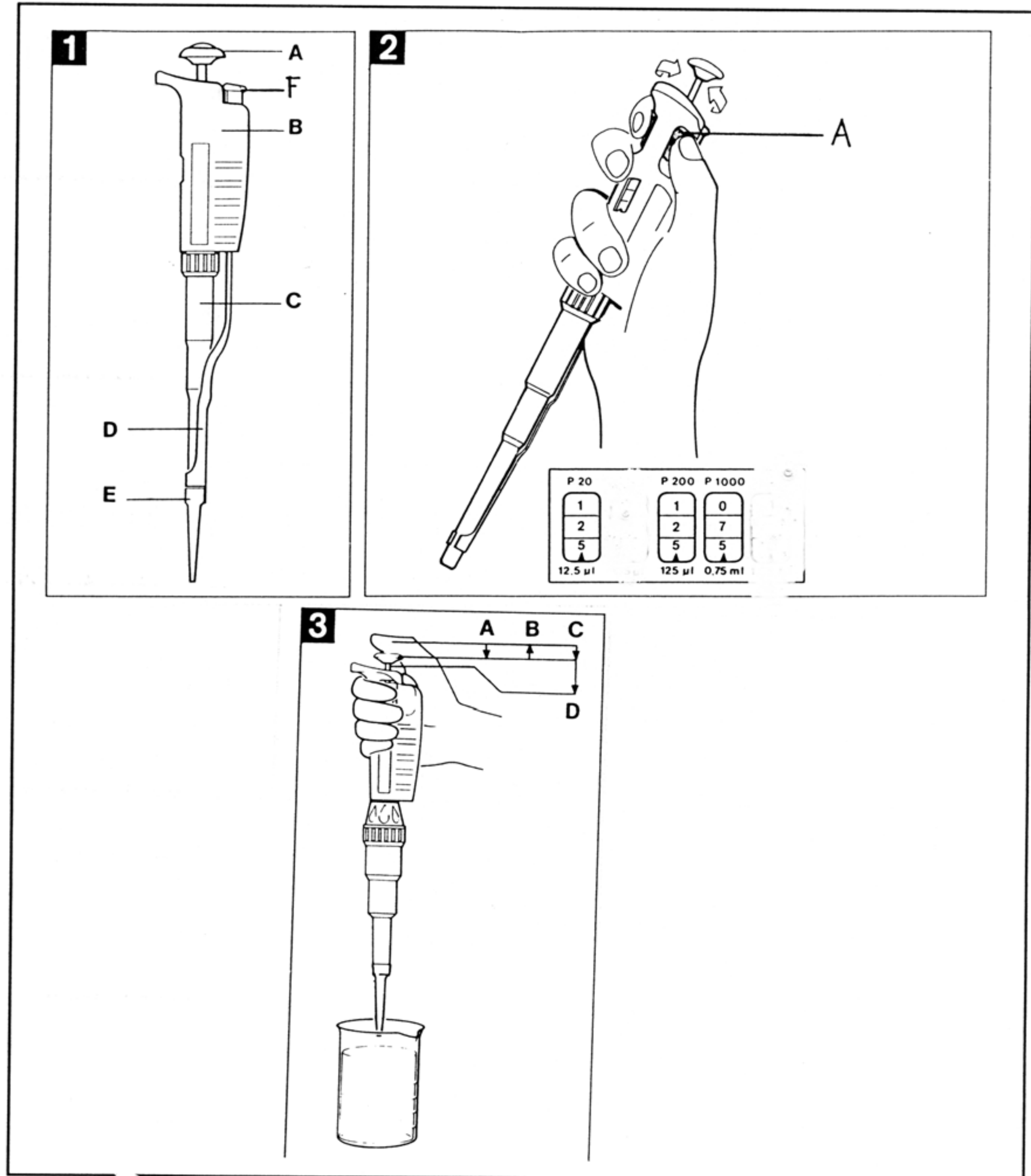


Figure : Gilson pipetman operation.

1-E, tip; 1-F, ejector button; 2-A, knurled adjustment ring; 3-A, 3-B, 3-C, and 3-D as discussed in operation of push-button.

PHASE CONTRAST LIGHT MICROSCOPE OPERATION for ECLIPSE E100[18] with SLIDE PHASE CONDENSER

Light waves go through viable bacteria almost unchanged. As a consequence unstained bacteria are very difficult to observe using a bright field light microscope. The phase contrast microscope has a phase plate in the objective lens (Ph3 DL x100 1.25 oil immersion) and a matching Ph 3 or Ph 4 condenser making viable cells visible by both retarding light waves and reducing amplitude of waves. This increases the contrast between the cell and the background. The phase contrast objective lens used in this lab is a dark phase; the bacteria appear dark in a bright field. The type of phase chosen depends on the type of material being observed. In our case, the DL phase objective is best suited for viable cells (bacteria) which differ little in refractive index from the background.

Microscope location and handling

The Phase contrast microscopes are in the left bench cupboards (facing south). It is the student's responsibility to remove the microscope for use and replace (slide removed and cleaned) when finished. Always carry the microscope with two hands, one holding arm and other under base. If you have difficulty using the microscope, get help, either the TAs or lab instructor. If a light is burned out or the microscope does not work, leave the microscope on top of one of the side benches or the center bench with a note stating problem.

Operation

Note: when recording the magnification of microscope view remember the eye piece lens is x10.

1. Prepare a slide**. For the oil immersion objective all viable cell slides require a cover slip**. Only stained slides do not require a cover slip. If viewing viable cells, it is best to place the cells inside a **permanent pen circle** to facilitate focusing the microscope.
2. Turn on the light (less intense for lower magnification and higher intensity for the phase contrast oil immersion lens). The light switch is located on the left side of the microscope with a separate brightness control knob.
3. Place the slide on the stage and swing the **20x objective into position**. Push the slide condenser to blank position (whiteout). The **coarse adjustment knob is only located on the left side** of the microscope. While looking at the stage (not in the microscope eyepieces) raise the stage as high as possible using the coarse adjustment knob turning away from you. The highest position of the stage does not come into contact with the 20x objective. Focus on black circle line by lowering the coarse knob, turning towards you. Fine focus on line.
4. Adjust the interpupillary distance (distance between eyepiece tubes) to fit your eyes.
5. Adjust the condenser level to as high as possible (lever down) for viable microorganisms (Phase). May need to lower for brightfield if stained cells.
6. Place 1 to 2 drops of oil on coverslip (vial cells).
7. Swing phase contrast oil immersion objective (Ph3 DL 1.25 x100) into position. Push the slide condenser to Ph3 or Ph4.

---

[18]Nikon Microscope Eclipse E100 instruction manual. Nikon Corporation. 2009.

8 Use the metal lever on the front of the slide condenser, to adjust the opening of the aperture. For the new Eclipse E100 microscope the setting is predetermined - just set to whatever is the magnification of the objective you are using. The aperture diaphragm should not be used to control brightness, use the light intensity knob for brightness. The aperture diaphragm controls the numerical aperture of the illumination (ideally 70% to 80% of objective numerical aperture (100x /1.25).

9. Adjust the diopters to suit your eyes using Ph3 DL 1.25 x100 oil objective. Holding the top of each eye piece turn clockwise until it stops (standard setting) -lowest setting of each eye piece - works if you have perfect vision in both eyes without further adjustment. First focus on specimen in the usual manner using both eyes and focus knobs. Next focus one eye at a time with respective eyepiece diopter not the focus knobs (turning top of eyepiece counter clockwise, ie., raises eye piece).With the two diopter system, the microscope is parfocal (all objectives in focus at the same time) for all users regardless of the individual's eyes.

   Note: ideally the diopter is adjusted after first focusing with the 40x objective and returning to 20x objective to adjust each eyepiece diopter. As only two objectives on your microscope adjust diopters using only the Ph3 DL 1.25 x100 objective.

10. Slight adjustment of the condenser may be necessary to fine focus.

11. Remove slide. Clean microscope objective lense with 2-propanol and turn off light. Return the microscope to the cupboard.

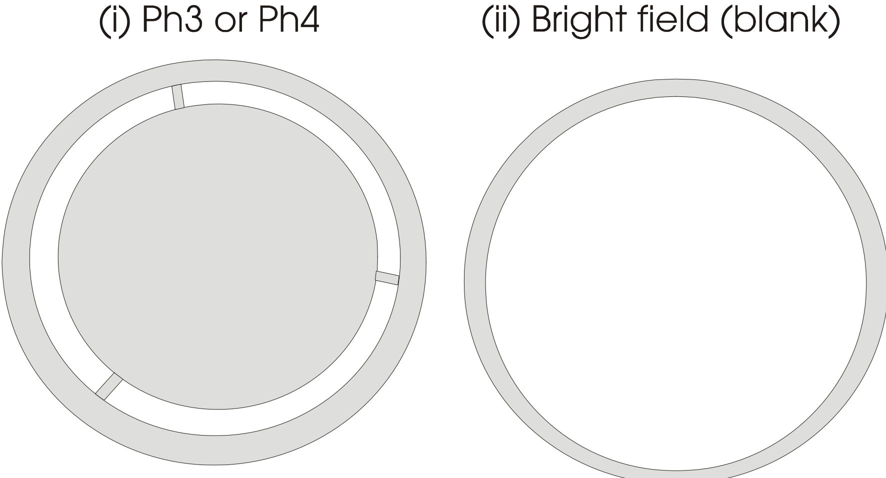


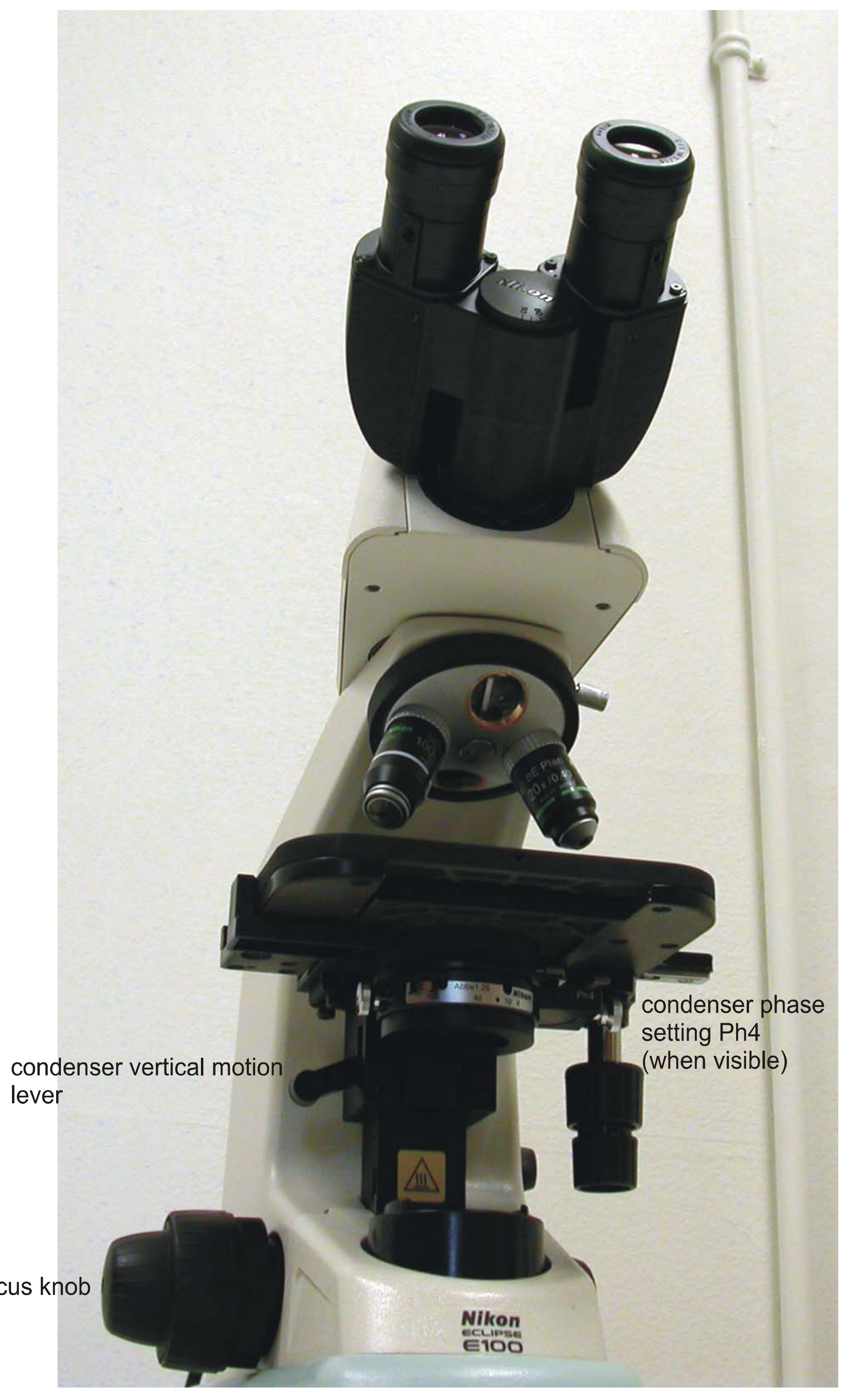
Figure 1. Phase Contrast Microscope -appearance of the opening of the (i) Ph4 or Ph3 phase and (ii) bright field on the slide condenser. For Ph 4 or Ph3 light only enters the microscope through the clear ring (white in diagram). When the slide condenser is pushed to the blank side there is no reduction of light, ie.,completely open. When using the bright field microscope you regulate the intensity of the light with the light switch.

Hints:
a. Use a permanent marker circle on slide to put bacteria in and to focus.
b. To initially find the approximate height of the stage, put the oil immersion objective into position and slide in position raise the stage with the coarse focus knob until slide or coverslip is 1 or 2 mm from objective. Then follow above procedure.
c. It is not always necessary to focus using the lower magnification objective first. Once you become familiar with the phase contrast microscope, it should become easy to focus and set up your microscope using the oil immersion lens.
d. Bacteria cannot be viewed using the X20 objective, you need to use the oil immersion lens (X1000 total magnification). Eyepiece magnification is always X10.
d. Whenever you move the stage up, always look at the stage, never through the eyepiece. Turning of coarse focus knob towards you lowers the stage, do this while observing specimen.

TIPS for x1000 Ph4 phase contrast setting:

- if you do not use enough oil, a poor microscopic view will occur
- fully rack up the condenser for viewing viable cells
- set the diopters to match your eyes
- light turned up to maximum for viewing viable cells
- use a pen marking to focus on -circle on slide which also serves to contain loopful of bacteria

Trouble Shooting:
No light – check that that cord is fitted tight to the microscope.
Blurred focus – check condenser fit up tight facing front, check that the blue filter (if present) is level in holder, check that the light casing is fitted correctly in base.


condenser phase
setting Ph4
(when visible)
condenser vertical motion
lever
fine focus knob
Nikon
ECLIPSE
E100
course focus knob

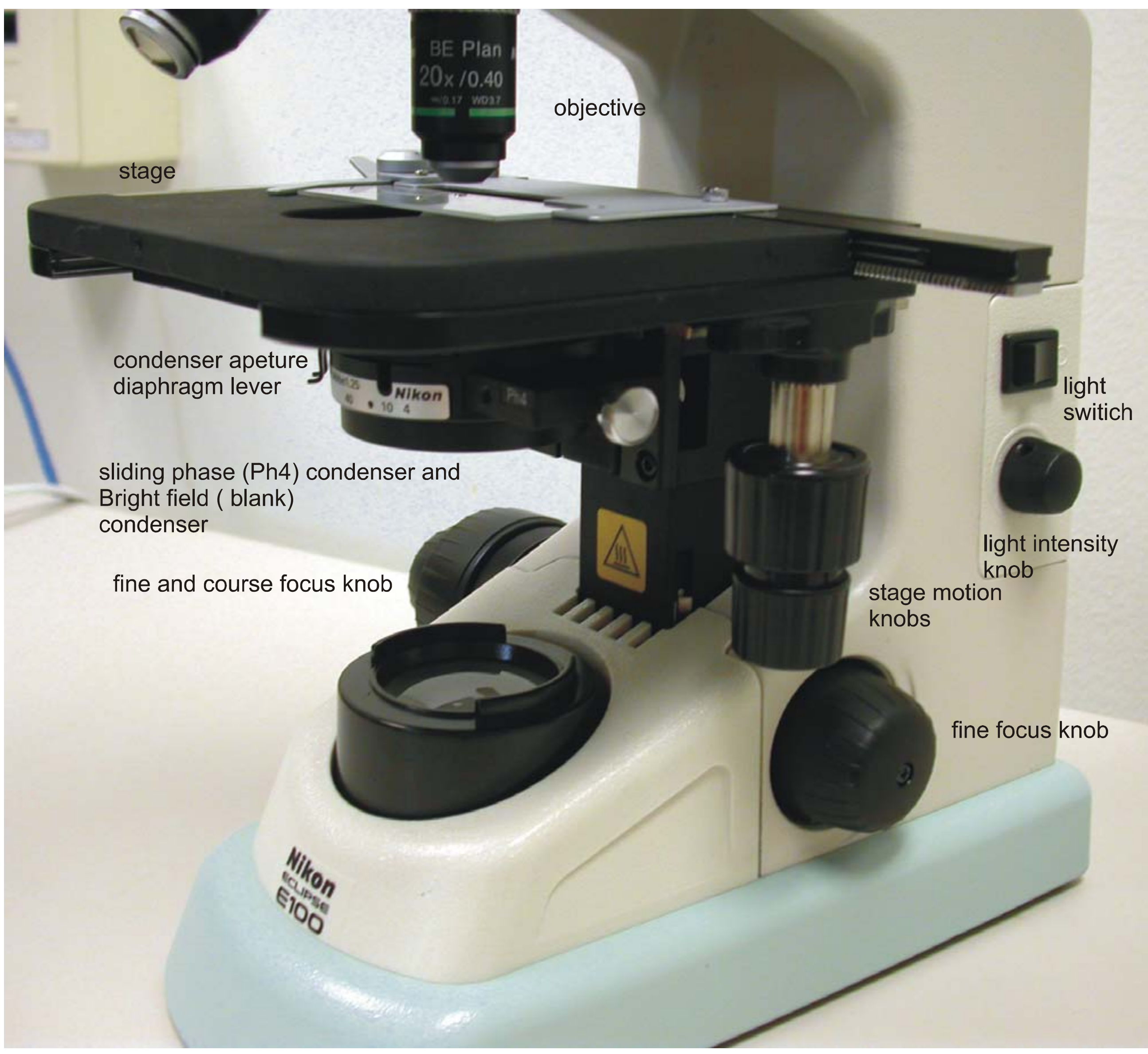


Figure 3b. Nikon Esclipse E100 with slide condenser, blank side or Ph3 or Ph4.

ANAEROBIC CHAMBER OPERATION

Principle: An anaerobic environment consisting of hydrogen and carbon dioxide gas is created using the GasPac system in a sealed jar. Anaerobic conditions are achieved rapidly, usually within 2 hours.
Components: anaerobic jar, palladium catalyst beads, GasPac hydrogen + carbon dioxide generator envelopes, and GasPac disposable anaerobic indicator. Oxygen and hydrogen in the presence of palladium catalyst beads are convert to water.

Procedure:

1. Use fresh catalyst pellets or pellets that have been rejuvenated* by heating at 160°C for 2 h. Remove cover. Turn cover over and unscrew safety screen containers from underside of lid. Place fresh catalyst pellets in containers. Replace the safety screen. Turn right side up and shake slightly to remove any pellets that may fall through the screen.
   * Pellets that have been exposed to H_2S, Cl_2 and sulfur gases should be discarded as the gases will poison the palladium catalyst.
2. Cut GasPac envelope along dotted line only at the upper right hand corner, as indicated on each envelope. Place GasPac envelope upright in anaerobic jar. Add 10 ml water to GasPac envelope.
3. Place inoculated agar plates into the anaerobic jar and add anaerobic indicator (placement such that can be easily viewed).
4. During incubation the atmospheric conditions within the jar should be easily visible. When the reaction occurs, it will be evidenced by condensation of water on inner side of jar, by warmth of lid above catalyst, and by change in indicator from blue to white (reduction of methylene blue).

FLUORESCENCE IDENTIFICATION TEST (FN slant)

Principle: Fluorescein is an organic luminescent pigment that upon excitation with ultraviolet light emits a green-yellow fluorescence. Fluorescence of colonies occurs due to the presence of cationic salts such as magnesium sulfate in the test medium.

Procedure:

1. Inoculate the medium by stabbing the deep with a heavy suspension of the culture and then streak the slant.
2. Incubate at optimum temperature for 24 to 48 hours. Store at 4°C student cold box until the following week. The TA will assist you with viewing the FN slant for presence or absence of fluorescence.
3. Place your tube along with an uninoculated tube on a UV light transilluminator. Make sure you cover the tubes with a plexiglas lid before turning on the UV light. The plexiglass cover protects your eyes, do not turn the UV light on before covering the tubes.
4. Turn on UV light in a dark room. Compare your culture tube to control (un-inoculated slant, return to shelf) for presence of fluorescence.

Interpretation: A bright yellow-green glow constitutes a positive test.

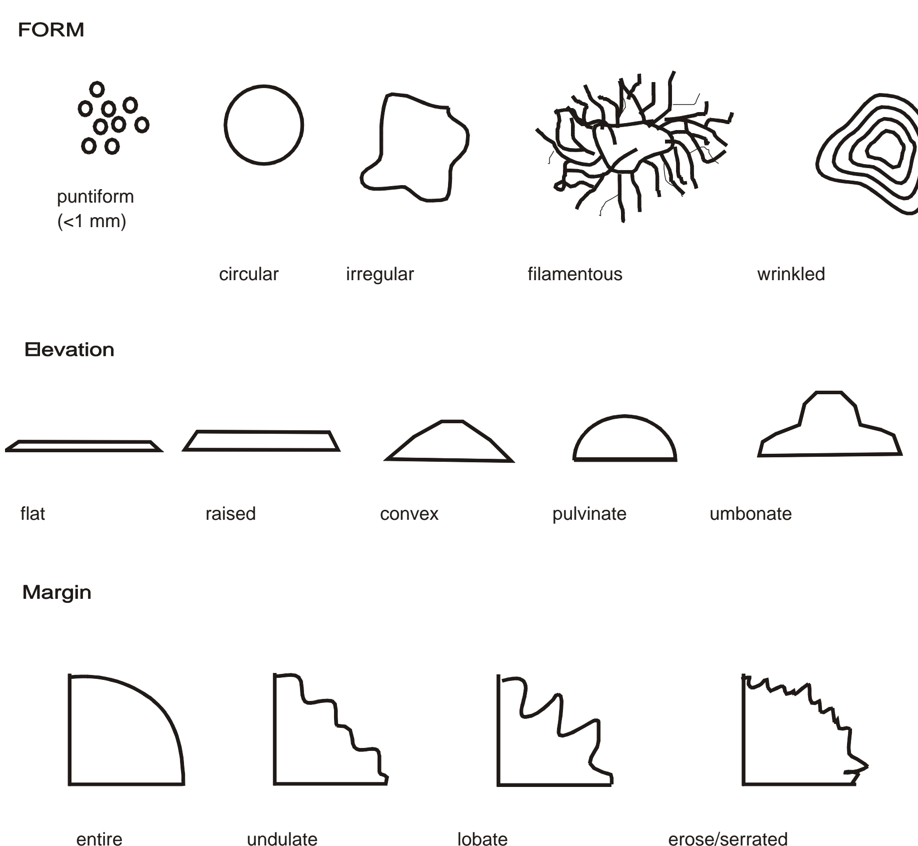
CELLULAR MORPHOLOGY

Microscopically observe and record apparent size (small, medium or large), shape and arrangement of a fresh sample culture. Also determine cell motility when asked for cellular morphology (not required for project unless you want to).

COLONY CHARACTERISTICS

Briefly outline colony characteristics; color, size (diameter, mm), shape/elevation, contour/margin, consistency, and opaque or translucent. When describing colony characteristics always include size (mm) and four other characteristics. Always state temperature and time of incubation for any colony description. If using colony characteristics as a group project sample analysis, possible comparative suggestions; take pictures, record relative presence of types of colonies...

Bacterial Colony Characteristics



Texture/Consistency

Use a sterile needle to determine texture by touching the colony. The following some terms that define texture:
brittle „ dry and crumbly
butyrous „ butter„like
membraneous „ resembles membrane/skin
muccoid „ wet looking, slimy

Surface qualitites

opaque (no light passes through) or translucent (light passes through)
dull or shiny

GRAM STAIN

Introduction: One of the most important and widely used procedures for differentially characterizing bacteria is the gram stain. Bacteria are divided into two groups, based on whether they retain or lose the 'primary stain' (crystal violet) after mordanting with iodine, treatment with alcohol and counter staining with safranin. Gram positive coccoid artifacts may be present in your stained sample. This is an artifact present in the safranin strain, not a contaminant in your bacteria sample.

Procedure:

. Prepare a smear of bacteria (from a culture not more than 24 hours old) on a slide. Fix the smear. Stain the smear with 3 to 5 drops of crystal violet solution for 1 min. Wash with water for a few seconds.
2. Apply 3 to 5 drops of Gram's iodine solution and let sit for 1 min. Wash slide with water.
3. Decolorize with 3 to 5 drops of alcohol-acetone until free color has been washed off (approximately 5-15 sec). Wash slide with water and blot dry.
4. Counter stain smear for 10 sec with 3 to 5 drops of safranin. Wash slide and blot dry.
5. To view a stained cell, use the oil immersion objective and set the slide condenser to the blank side (empty hole for brightfield microscopy).
6. **Confirm results** by doing the KOH string test (procedure follows).

Interpretation: Those organisms that retain the crystal violet appear dark blue or violet and are designated gram positive; those that lose the crystal violet and are subsequently stained by the 'counter-stain' (safranin) appear red and are designated gram negative.

[19]KOH STRING TEST: Confirmation of Gram stain

Introduction: Dilute alkali solutions (3% KOH) lysis gram negative cell walls while the cell walls of gram positive bacteria are not disrupted. When gram negative bacteria are lysed (5 to 60 seconds) the DNA is released causing the mixture to become viscous (1).

Procedure:

1. Place a drop of 3% KOH on a glass slide.
2. Using a loop remove a visible amount of fresh bacteria from a colony(s) on a T-soy agar plate. 3. Stir bacteria into KOH. Mix continuously in a 1 to 2 cm area on the glass slide for a maximum of 1 minute.
4. Frequently raise the loop 1 cm off surface to test if the mixture is becoming viscous and has the ability to "string out".

Interpretation:

Gram negative: Mixture becomes viscous and "strings out".

Gram positive: After 1 minute the mixture is not viscous and does not string out.

---

[19] Powers, E.M. (1995) Efficacy of the Ryu Nonstaining KOH technique for rapidly determining Gram reactions of food-borne and waterborne bacteria and yeasts. In Applied and Environmental Microbiology. 61:3756-3758.

MOTILITY BY DIRECT MICROSCOPE OBSERVATION
Note: motility tubes are not available for this lab.
Introduction: In order for a bacterial culture to show motility, (i) it must be flagellated, (ii) must be young and actively growing in the correct culture conditions, and (iii) treated gently such that the cells retain their flagella.

Procedure: Observe motility using a drop of fresh bacterial culture that was grown at optimum temperature. To observe motility use the phase contrast oil immersion objective and use either a hanging drop slide or a basic slide with a cover slip.

SAMPLE LAB EXAM: Microbiology MBIO 3480 MICROBIAL DIVERSITY

Note: spaces provided for answers on exam paper have been removed to reduce the number of pages. Exam is longer than given to accommodate a wide range of questions.

WRITE IN PEN ONLY
CONCISELY ANSWER ALL QUESTIONS IN SPACE PROVIDED ON EXAM PAPER.
ANSWERS ACCEPTABLE IN POINT FORM.

1 1. Explain the principle of color development in the BIOLOG™ plate.

1 2. State all settings on phase contrast microscope and slide setup on microscope stage to determine bacterial motility. Do not include procedure steps.

1 3. (i) Determine bacterium titre (bacteria/ml) using the following experimental data. Include calculations.

Dilution Plated	Plate count[a]	
	plate 1	plate 2
10^{-3}	TNTC	TNTC
10^{-4}	244	235
10^{-5}	23	27
10^{-6}	2	1

[a] 0.1 ml bacterial dilution plated
[b] TNTC = too numerous to count

(ii) If plate counts for plate 2 at 10^{-6} dilution was 30 instead of 1 would you include this count in the titre calculation? Justify your answer.

1 4. In your answer describe statistical analysis used to obtain P-value. What information is obtained from P-value of 0.042 with respect to microbial diversity as investigated in your lab?

1 5. a) Explain why the *Rhizobium* you isolated is a pure culture.

1 b) Would *Rhizobium* grow on *Azotobacter* mannitol medium. Explain you answer.

3 6. a) It is relatively easy to select *Azotobacter* from a soil sample that contains a great variety of other microorganisms. State the selective experimental steps and explain selective nature for each.

1 b) What identification steps allowed you to identify *Azotobacter* to the species level.

1 7. The majority of bacteria in this lab require specific media for growth. For *Erythromicrobium ramosum* state nutritional category and carbon/energy source (may be separate) and nitrogen source.

1.5 8. Microbial Diversity can be examined numerous ways. List 4 methods use to investigate diversity in your lab. State diversity information obtained by each method.

1.5 9. a) State the function of the following experimental equipment or solution used to isolate bacteriochlorophyls from photosynthetic bacteria.
(i) filter apparatus
(ii) acetone (two functions)
(iii) sonicator

1 b) After extraction of Winogradsky sample a student obtained a scan with two major peaks, 663 nm and 771 nm. Explain what information is obtained from this scan.

1 10. State the function of each of the following medium components, K_2HPO_4, $MgSO_4$, yeast extract, distilled water. pH to 7.2. This is a general question, not related to any specific bacterial group isolated in your lab.

0.5 11. a) Given the following carotenoid peak maximums (live cells), 482 nm, 510 nm and 538 nm, what is the color of the aerobic anoxygenic phototrophic bacteria?

1 b) Each of aerobic anoxygenic phototrophic bacteria investigated in your lab contain BCl *a*. However, the peak maximums of the *in vivo* scan differ. Explain why.

1.5 c) Determine the biomass specific pigment production (nmoles/g cells) of an isolated carotenoid in an aerobic anoxygenic phototrophic bacteria given the following information. Protein concentration of a 250 ml culture is 0.14 mg/ml. Final volume of sample is 0.25 ml. Entire sample applied to TLC. Absorbance maximum is 1.5 at 515 nm. State the name of the bacteria and carotenoid.

2.5 d) Explain the function of each of the following components used to investigate pigment production in aerobic anoxygenic phototrophic bacteria.

(i) acetone:methanol (7:2, v/v)
(ii) nitrogen gas
(iii)10 mM Tris buffer, pH 7.8
(iv) 50% sucrose
(v) petroleum ether:absolute ether:acetone:methanol 40:10:15:3 (v/v/v/v)

1 e) Explain why the carotenoids separate on the TLC plate.

- END -